การตรวจทางอณูวิทยาของเชื้อ human papillomavirus กลุ่ม high-risk ที่ติดเชื้อในกลุ่มสตรีไทยและคุณลักษณะยีนของเชื้อ human papillomavirus 16 และ 18 ในตัวอย่างเซลล์วิทยาของปากมดลูก

นางสาว วรดี ลือชาชัยวงศ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเวชศาสตร์ (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2552

MOLECULAR DETECTION OF HIGH-RISK HUMAN PAPILLOMAVIRUS (HPV) AMONG THAI WOMEN AND GENOME CHARACTERIZATION OF HPV16 AND HPV18 IN CYTOLOGICAL SAMPLES OF UTERINE CERVIX

Miss Woradee Lurchachaiwong

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biomedical Sciences
(Interdisciplinary Program)
Graduate School
Chulalongkorn University
Academic Year 2009

Thesis Title	Molecular detection of high-risk human papillomavirus (HPV) among Thai women and genome characterization of HPV16 and HPV18 in cytological samples of uterine cervix
By	Miss Woradee Lurchachaiwong
Field of Study	Biomedical Sciences
Thesis Advisor	Professor Yong Poovorawan, M.D.
Thesis Co-Advisor	Professor Pichet Sampathanukul, M.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

..... Dean of the Graduate School
(Associate Professor Pornpote Piumsomboon, Ph.D.)

THESIS COMMITTEE

..... Chairman
(Associate Professor Kanisak Oraveerakul, D.V.M., Ph.D.)

..... Thesis Advisor
(Professor Yong Poovorawan, M.D.)

..... Thesis Co-Advisor
(Professor Pichet Sampathanukul, M.D.)

..... Examiner
(Sunchai Payungporn, Ph.D.)

..... External Examiner
(Pairoj Junyangdikul, M.D.)

วรดี ลือชาชัยวงศ์ : การตรวจทางอณูวิทยาของเชื้อ human papillomavirus กลุ่ม high-risk ที่ติดเชื้อในกลุ่มสตรีไทยและคุณลักษณะยีนของเชื้อ human papillomavirus 16 และ 18 ในตัวอย่างเซลล์วิทยาของปากมดลูก (MOLECULAR DETECTION OF HIGH-RISK HUMAN PAPILLOMAVIRUS (HPV) AMONG THAI WOMEN AND GENOME CHARACTERIZATION OF HPV16 AND HPV18 IN CYTOLOGICAL SAMPLES OF UTERINE CERVIX) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.นพ.ยง ภู่วรวรรณ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ.นพ. พิเชษฐ์ สัมปทานุกูล, 153 หน้า.

ผู้นิพนธ์ได้พัฒนาวิธีการตรวจลำดับเบสของเชื้อ human papillomavirus กลุ่ม high-risk (HPV) โดยออกแบบ primer สำหรับใช้ศึกษาคุณลักษณะทางพันธุกรรมทั้งหมดของเชื้อ HPV16 และ 18 และออกแบบ primer จากส่วนของยีน E1, E6 และ L1 สำหรับใช้ตรวจคัดกรองตัวอย่างโดยวิธี PCR ว่ามีการติดเชื้อ HPV หรือไม่ นอกจากนี้ ได้สร้างและพัฒนา primer และ probe สำหรับใช้กับการตรวจคัดกรองโดยวิธี real time PCR ให้สามารถระบุเชื้อ HPV สายพันธุ์ที่แพร่ระบาดมากสุดในตัวอย่างที่ศึกษา

การทดสอบประสิทธิผลของ primer ต่างๆ ที่สร้างขึ้น ในการตรวจสอบลำดับเบสทั้งหมดของเชื้อ HPV16 และ 18 ที่ได้จากตัวอย่างเซลล์ป้ายของปากมดลูกที่มีผลวินิจฉัยต่างๆ กัน จำนวน 7 และ 9 ตัวอย่างตามลำดับ สามารถพบ point mutation ของเชื้อ HPV16 แต่ไม่พบในเชื้อ HPV 18 กรดอะมิโน 2 ตำแหน่งที่พบการเปลี่ยนแปลงคือ พบที่ยีน E2 ตำแหน่ง 219 (proline to threonine) และ L2 ตำแหน่ง 269 (serine to aspartic acid) เกิดในตัวอย่างที่ผลวินิจฉัยเป็น squamous carcinoma และ CIN III ตามลำดับ ในการทดสอบตรวจคัดกรอง 243 ตัวอย่างที่มีผลทางเซลล์วิทยาต่างๆ กัน พบว่า primer ที่ยีน E1 มีความไวมากที่สุดคือ 79.7% และสามารถจำแนกกลุ่มที่วิธี Hybrid Capture II ให้ผลคลุมเครือได้ ส่วนการออกแบบ probe และ primer สำหรับใช้กับวิธี multiplex real time PCR ผู้นิพนธ์ได้ศึกษาสายพันธุ์ HPV ที่พบมากที่สุดจาก 515 ตัวอย่างพบว่า HPV 16 และ 31 เป็นสองสายพันธุ์ที่พบมากที่สุดตามลำดับ จึงได้พัฒนา probe ที่สามารถระบุสองสายพันธุ์นี้และเพิ่มเติม HPV18 ซึ่งเป็นสายพันธุ์ที่สำคัญ รวมทั้งหมดเป็น 3 สายพันธุ์

โดยสรุป ผู้นิพนธ์ได้ออกแบบ primer ที่เหมาะสมกับการใช้งานประเภทต่างๆ ในการศึกษากลไกการกลายเป็นมะเร็งโดยเชื้อ HPV การคัดกรอง และการใช้กับ multiplex real time PCR และได้พิสูจน์ให้เห็นว่าสามารถใช้งานได้จริงในสถานการณ์จำลอง ซึ่งพัฒนาสำหรับการสาธารณสุขของประเทศได้

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต .....วรดี ลือชาชัยวงศ์.....

ปีการศึกษา 2552 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

#488 96700 20 : MAJOR BIOMEDICAL SCIENCES

KEYWORDS: HPV / PCR / REAL-TIME PCR / GENOME / CHARACTERIZATION

WORADEE LURCHACHAIWONG: MOLECULAR DETECTION OF HIGH-RISK HUMAN PAPILLOMAVIRUS (HPV) AMONG THAI WOMEN AND GENOME CHARACTERIZATION OF HPV16 AND HPV18 IN CYTOLOGICAL SAMPLES OF UTERINE CERVIX. THESIS ADVISOR: PROF. YONG POOVORAWAN, M.D., THESIS CO-ADVISOR: PROF. PICHET SAMPATHANUKUL, M.D. 153 pp.

In this study, author established primers for high-risk HPV detection by using nucleotide sequencing method. HPV16 and HPV18 are the major cause of cervical cancer worldwide therefore, the author established HPV16 and HPV18 primer for whole genome characterization. In addition, the author established primers for HPV screening by specific at E1, E6 and L1 gene. Based on screening data of HPV distribution in this study, multiplex real-time PCR probes and primers were established for frequently detected genotypes.

Focusing on the objective, seven and nine samples which derived from HPV16 and HPV18 infected women who have different cytological data were characterized whole genome, respectively. Analysis data discovered two critical changes in HPV16, converting the E2-219P prototype to E2-219T in cervical cancer and the L2-269S prototype to L2-269D in CIN III; however, there were no critical changing in HPV18. Next, 243 different cytological samples were analyzed by using E1, E6 and L1 primers. Author found that E1 primer was high percentage of detection (79.7%). Moreover, detection by E1 primer can be clarified samples which in HCII grey zone. Furthermore, author set up probe and primer for the highest prevalence genotypes HPV16 and HPV31 which confirmed result by testing with 515 samples by using E1 primer. Then multiplex real time PCR method was developed for HPV16, HPV31 and some additional HPV18 which is more important genotype today.

In conclusion, primer designations were established for HPV screening by using PCR and multiplex real time PCR for nucleotide sequencing study which may be concern with mechanism and progressive to cancer. The author hopes that primer and probe in this study can be used for HPV studying and useful for public health study in the future.

Field of Study: Biomedical sciences Student's Signature: .....

Academic Year: 2009 Advisor's Signature: .....

Co-Advisor's Signature: .....

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and appreciation to my advisor, Professor Yong Poovorawan, for his competent supervision, guidance, encouragement and criticism which have inspired me to accomplish my study.

I am extremely grateful to my thesis co-advisor, Associate Professor Pichet Sampathanukul for his valuable suggestion, support and encouragement for the completeness of this thesis. I am very grateful to my supervisory committee, Associate Professor Dr. Kanisak Oraveerakul, Pairoj Junyangdikul and Dr. Sunchai Payungporn and Associate Professor Damrong Tresukosol Special grateful to Professor Sompop Limpongsanurak, Associate Professor Wichai Termrungruanglert, Associate Professor Somchai Niruthisard, Associate Professor Prasert Trivijitsilp, Anant Karalak and Sukumarn Swangvaree for their valuable suggestions and criticisms.

My sincere appreciation is also expressed to Professor Margaret Stanley for her valuable advice, excellent ideas and kindness support during nine months of my research in the Division of virology, Department of Pathology, University of Cambridge, Cambridge, UK. Special thanks must also be extended to Dr. David Winder, Dr. Jane Sterling and Dr. Peter Goon for their advices and suggestions.

I would like to thank staff of the Department of Pathology, Samitivej Srinakharin hospital, Thailand and Department of Obstetrics & Gynaecology, Faculty of Medicine, Chulalongkorn University for their assistances and generous providing samples.

I am particularly indebted to the Royal Golden Jubilee (RGJ) Ph.D. program, Thailand Research Fund (TRF) and the Commission on Higher Education, Ministry of Education. Special appreciation must also be extended to the Centre of Excellence in Clinical Virology, Department of Paediatrics, Faculty of Medicine, Chulalongkorn University and Division of virology, Department of Pathology, University of Cambridge, Cambridge, UK.

Finally, I would like take this opportunity to express the profound gratitude from the bottom of my heart to my beloved parents, family and friends for their love, encouragements and moral supports throughout this study.

CONTENTS

Page

ABSTRACT (THAI)..... iv

ABSTRACT (ENGLISH)..... v

ACKNOWLEDGEMENTS..... vi

CONTENTS..... vii

LIST OF TABLES..... ix

LIST OF FIGURES..... x

LIST OF ABBREVIATIONS..... xii

CHAPTER

I INTRODUCTION..... 1

- BACKGROUND AND RATIONALE..... 1

- OBJECTIVE..... 4

- CONCEPTUAL FRAMEWORK..... 5

II REVIEW OF RELATED LITERATURE..... 8

- TAXONOMY AND CLASSIFICATION OF HUMAN PAPILLOMAVIRUS (HPV)..... 8

- REPLICATION CYCLE OF HUMAN PAPILLOMAVIRUS (HPV)..... 15

- EVAUATE OF ABNORMAL CERVICAL..... 17

- EPIDEMIOLOGY AND TRANSMISSION..... 22

- IMMUNOLOGY..... 26

- VACCINE..... 27

- LABORATORY DIAGNOSIS..... 28

- ESTABLISHMENT HUMANA PILLOMAVIRUS (HPV) IN CELL CULTURE METHOD..... 31

III MATERIALS AND METHODS 32

- MATERIAL..... 32

- METHODS..... 35

IV RESULTS..... 46

- MOLECULAR CHARCTERIZATION OF CARCINOGENESIS GENOTYPES (HPV16 AND HPV18)..... 46
- DEVELOPMENTAL HPV DETECTION METHOD AND HPV SCREENING..... 60
V CONCLUSION AND DISCUSSION..... 84
- MOLECULAR CHARCTERIZATION OF CARCINOGENESIS GENOTYPES (HPV16 AND HPV18)..... 84
- DEVELOPMENTAL HPV DETECTION METHOD AND HPV SCREENING..... 90
REFERENCES..... 97
APPENDICES..... 116
APPENDIX A..... 117
APPENDIX B..... 133
BIOGRAPHY..... 153

LIST OF TABLES

Table		Page
1	Conserved primers for whole genome amplification and sequencing of HPV16……	48
2	Conserved primers for whole genome amplification and sequencing of HPV18……	49
3	General data and clinical characteristics of HPV16 patients……	51
4	General data and clinical characteristics of HPV18 patients……	58
5	Conserved primers specific for E6, E1 and L1 for amplification and sequencing of HR-HPV and some additional PHR-HPV and LR-HPV..	62
6	HPV detection based on HCII ratio (RLU/positive cut off) and PCR using our primers (E1, L1 and E6 gene primers) and primers previously published (MY09/MY11 and GP5+/GP6+)……..	66
7	Amount of HPV genotype distribution in each category cervical cytopathology (CA, HSIL, LSIL, ASC-US and Normal)……	71
8	Multiplex real-time PCR primer and probe at L1 region that are specific genotype HPV16, HPV18 and HPV31……..	76
9	Multiplex fluorescence reporters dyes suitable for each instrument….	124
10	Spectrophotometric conversions for nucleic acid templates……..	127
11	Commonly used fluorescence reporters & quenchers in real-time PCR……..	130
12	pGEM-T Easy Vector sequence reference points……	131
13	Conclusion of HPV complete genome and partial E1 gene of this study that submitted in GenBank database……..	132

LIST OF FIGURES

Figure		Page
1	Phylogenetic of L1 gene of papillomavirus……	9
2	Organization of HPV 16 genome ……	10
3	Illustrate of HPV life cycle on epithelial cell……...	12
4	Genome structure and transcription map of HPV16……	13
5	Diagram picture represent cervix structure and cell type……..	18
6	Diagram pictures of Pap smear results……..	19
7	Show how squamous cells look under the microscope as they progress toward cancer……..	20
8	Progressive of cervical cancer from normal cell to tumor cell and classify categories of dysplasia abnormality…….	22
9	The HPV type distribution in 25,368 women from Asia, stratified by grade of cervical lesion…….	25
10	Strategy for whole genome amplification of HPV…….	37
11	Phylogenetic tree based on whole genome analyses of HPV16……	52
12	Nucleotide variations of LCR among HPV16 from our study and GenBank database……	53
13	Nucleotide and amino acid variations among HPV16……	55
14	Phylogenetic tree of HPV16 with special emphasis on the E2, E6, L1 and L2 genes……..	56
15	Phylogenetic tree based on whole genome analyses of HPV18……	59
16	Schematic diagrams represent the construction of positive controls for HPV E1 gene, HPV L1 gene and HPV E6 gene……..	63
17	Human papillomavirus genotype 16 was used as positive control for sensitivity detection……	65
18	Relationship between HPV cytology of samples and Hybrid capture II ratio (RLU/CO)…….	68
19	Proportion between positive and negative samples detected by using E1 primers at an RLU/CO……..	69

LIST OF FIGURES (CONT.)

Figure		Page
20	Schematic maps of primers and probes used in multiplex real-time PCR for HPV16, HPV18 and HPV31 detection……..	77
21	Schematic diagrams represent the construction of positive controls for L1 genes of HPV16, HPV18 and HPV31……..	78
22	Representative results obtained from multiplex real-time PCR……..	80
23	Amplification plots of HPV16 L1 gene obtained from the amplification of 10-fold serially diluted of DNA standards……..	82
24	Standard curves of HPV16 detection…….	82
25	Schematic diagram of the PCR process……	118
26	Schematic representative of amplification plot and the important terminology associated with real-time PCR analysis……	120
27	Detection chemistries commonly used in real-time PCR. Detection mechanism of TaqMan probe chemistry……	123
28	Representative of absolute quantitation by real-time PCR…….	126
29	Circle map of pGEM-T Easy vector (Promega)…….	131

LIST OF ABBREVIATIONS

μg	=	microgram
μl	=	microlitre
ADC	=	adeno- and adenosquamous-carcinoma
ASCUS	=	atypical squamous cells of undetermined significance
ASC-H	=	atypical squamous cells cannot exclude HSIL
bp	=	base pair
CA	=	cervical cancer
CIN I	=	cervical intraepithelial neoplasias grade1
CIN II	=	cervical intraepithelial neoplasias grade2
CIN III	=	cervical intraepithelial neoplasias grade3
DNA	=	Deoxyribonucleic acid
E	=	early gene
EDTA	=	Ethylene Diamine Tetraacetic Acid
HC II	=	Hybrid capture II
HPV	=	Human papillomavirus
HR	=	high-risk
HSIL	=	high-grade squamous intraepithelial lesions
L	=	late gene
LBC	=	liquid base cytology
LCR	=	long control region
LR	=	low-risk
LSIL	=	low-grade squamous intraepithelial lesions
mg	=	milligram
ml	=	millitre
NCR	=	non-coding region
nm	=	nanometre
PCR	=	Polymerase Chain Reaction
pg	=	picogram

LIST OF ABBREVIATIONS (CONT.)

PHR	=	probably high-risk
SCC	=	squamous cell carcinoma
RLU/CO	=	relative light unit/ positive cut off

CHAPTER I

INTRODUCTION

BACKGROUND AND RATIONALE

Persistent infection with a carcinogenic human papillomavirus (HPV) is found in virtually all cases of cervical cancer, which is the second leading cause of death from cancer in worldwide women [1]. The prevalence of genital HPV infection varies among countries (1 - 40% in women) [2]. Although most infection resolves within two years, a substantial number persist and some of them turn into cervical cancer [2]. HPV has been identified as a causal agent for cervical squamous neoplasia and associated with neoplasia at several other mucosal sites. Genital HPVs are classified into high risk (HR-HPV), potentially high risk (probably HR-HPV) and low-risk (LR-HPV) types, according to their potentials to induce invasive cancer [3]. Infection with high-risk HPV genotypes is found to be present as high as 99.7% of cervical cancer cases [4]. Therefore, detections of the virus in screening and diagnostic setting are essential and standard methods are still in developing. On the other hand, molecular characterization of cervical carcinogenesis is on the way of development and research as well.

Human papillomavirus (HPV) infection

HPV is the cause of cervical cancer that ranks second most common cancer related death in women worldwide and is the most prevalent female cancers in Thailand. At present, HPVs have more than 100 different genotypes but only some genotypes actually contribute to development of human malignancies. HPVs are broadly grouped into cutaneous types and mucosotropic types, based on their preferred tissue tropism. Cutaneous types are found in the general population and cause common warts. Mucosotropic HPVs are classified into high-risk and low-risk type, referring to their associations with malignancy and benign lesion, respectively [5]. The most well known are types HPV16 and HPV18 which are responsible for approximately 80% of the worldwide cases of cervical cancer. In the north-east of Thailand, HPV genotypes 16 and 18 have been reported to cause two out of three cervical cancer cases [6].

However, Clifford *et al* studied the global distribution of HPV including Thailand at Lampang (northern province) and Songkla (southern province), and found that heterogeneity was a significant finding in Asia [7]. HPV 16 is the most common cause of cervical cancer and numerous variants of HPV16 have been identified in different geographic locations and ethnic groups [8]. In Thailand, data regarding genome characterization of HPV16 and HPV18 are no data available in Thailand.

Human papillomavirus (HPV) detection

Millions of women are diagnosed every year with cervical abnormalities. An aggressive management approach cannot be justified because almost all abnormalities clear without treatment [9]. However, these abnormalities cannot be ignored because most precancers and cancers are diagnosed in women with previously found mild abnormal cytological findings [10]. Cytological screening of cervix or "Pap test" has long been proven a successful means to prevent mortality and lessen cases from invasive cervical carcinoma. Up to present, annual checkup is compromised sensitivity. Nowadays, HPV detection test is promising. Several molecular methods have been developed in order to identify HPV in liquid-base cytology (LBC) samples and tissue samples [11, 12]. HPV in liquid-base cytology samples is convenient for detection and it is easy to manipulate samples collection than tissue samples. Although, HPV in liquid-base cytology samples are not good quality to relate between progressive of cancer and HPV genotype, HPV in liquid-base cytology samples has an advantage for epidemiology study and self sample collection than tissue samples collection. Molecular techniques applied for HPV DNA detection [13] include direct probe methods using Southern blotting and in situ hybridization, signal amplification methods such as hybrid capture II (HCII) assay [14], and target amplification methods by PCR [15]. They are time-consuming and require relatively large amounts of highly purified DNA. The HCII test cannot identify specific HPV genotypes. For HPV genotyping, target products amplified by PCR are subjected to sequence analysis [16], RFLP analysis [17], or hybridization with type-specific probes [18, 19, 20]. The commercially available HCII assay is widely used for HPV detection and routine screening but, due to the limited number of genotypes included in the hybridization probe mixtures, it does not allow for

comprehensive typing of viruses. Furthermore, a previous study has reported that a crucial issue with HCII is a grey zone between borderline negative and positive results, which are difficult to interpret [21]. The authors have also suggested that another method or combination of methods such as PCR should be used in parallel with HCII. Consensus PCR detection covers a wider range than HCII. The most widely publicized HPV-testing studies have been performed with L1 specific MY09/MY11 and GP5+/GP6+ primer sets. However, the L1 region is subject to frequent multiple nucleotide variations, which may affect the detection of specific HPV types. In contrast, the E1 gene contains a nucleotide region sufficiently conserved for primer design and a recently developed test kit focuses on E1 for HPV genotyping (Papillocheck, Greiner Bio-One, Frickenhausen, Germany) that gives comparable results of detecting HPV in cervical specimens with to the Linear Array test [22]. Another popular HPV detection method is real-time PCR, high sensitivity and specificity HPV multiplex PCR assays to be developed [23, 24, 25]. Furthermore, real-time methods have been used for quantitative HPV [26], biomarker for cancer progression or to assess physical status (episomal and/or integrated) of HPV16 [27]. Real-time PCR is a reliable, accurate, efficient, cost- and time-effective method adequate tool to detect pathogens [28]. Because of HPV genome variation, the developments of HPV detection are still required from further study.

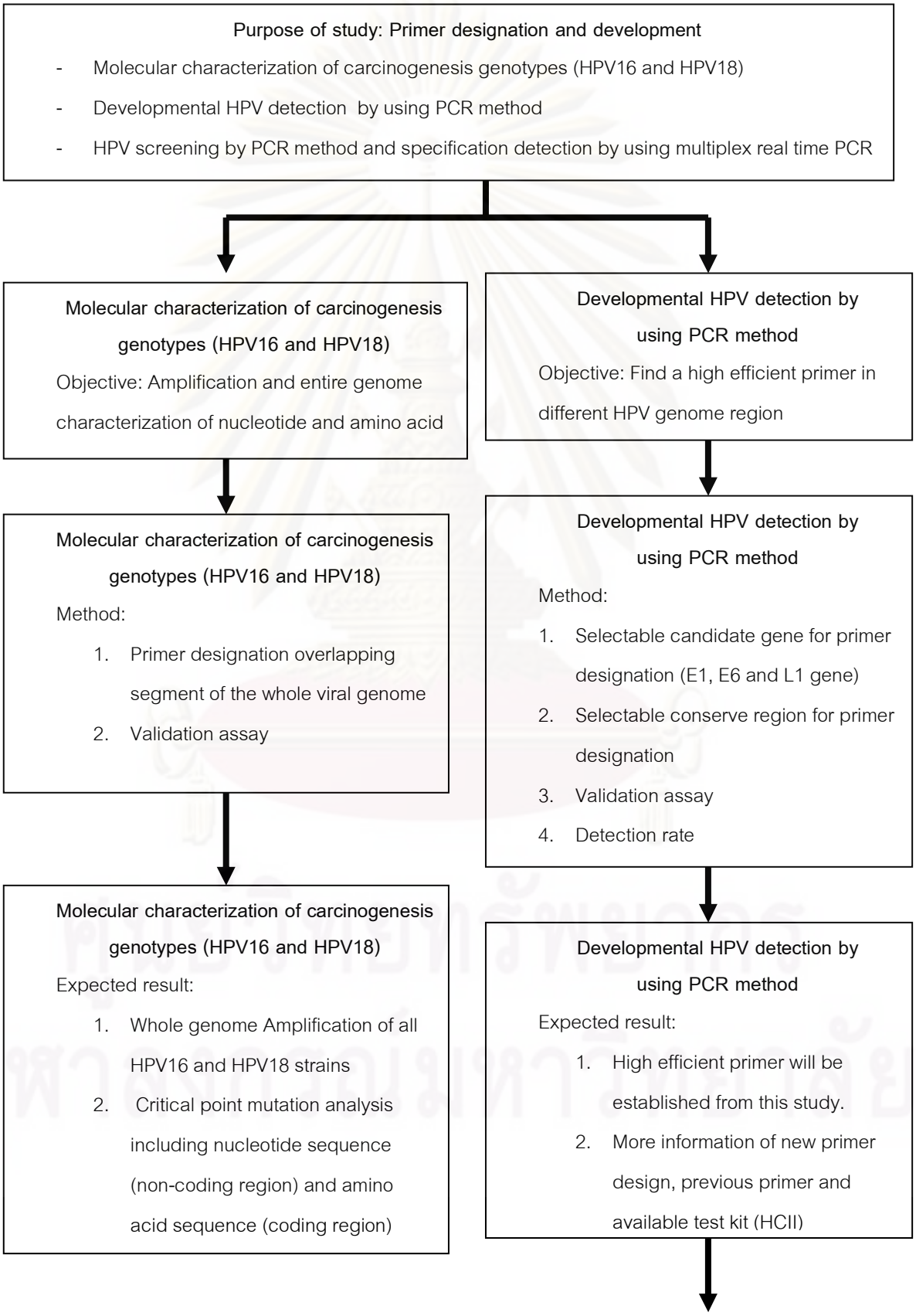
In this study, molecular diagnostic assays based on PCR were developed which examined the correlation between HCII ratio (RLU/CO) and results obtained by PCR amplification using previous primer sets (MY09/MY11 and GP5+/GP6+) and by PCR amplification of the E1, E6 and L1 genes from samples with different cytological findings. HPV prevalence detection focused on HR-HPV and additional genotype of probable HR-HPV and LR-HPV especially the genotypes 6 and 11 and identifying the genome sequence of HPV types present in Thailand. Then, a rapid, accurate and sensitive real-time PCR method was establish for the detection of high prevalence HPV genotype in Thai women. Finally, genome sequences of HPV16 and HPV18 have been which identified as major causes of cervical cancer in the women worldwide were characterized.

Objectives

1. To establish whole genome primer for HPV16 and HPV18 studying
2. To characterize and analyze the genomic sequences of HPV16 and HPV18 from specimens with different cytological findings in Thai women.
3. To compare the genome of HPV16 from Thai women and the HPV16 vaccine strain.
4. To characterize the genome between HPV18 in Thai women and HPV18 vaccine strain.
5. To compare among our PCR method based on E1 E6 and L1, with a commercial available kit (hybrid capture II) and established PCR methods (MY09/MY11 and GP5+/GP6+ primer sets) in samples that have different cytological data.
6. To develop an effective method, based on PCR, for HPV genotyping in Thai women.
7. To find the viral gene that provides the most sensitive for HPV genotyping.
8. To find the prevalence of HPV genotypes [(HR-HPV, probable HR-HPV and LR-HPV (6, 11)] in Thai women which have different cytological data.
9. To develop a rapid and cost-effective assay, based on multiplex real-time PCR, for the high detection of prevalent HPV genotypes in Thai women.

Conceptual Framework

Primer designation and development for HPV molecular detection and characterization



HPV screening by PCR method and specification detection by using multiplex real time PCR method

Method:

1. HPV screening and detection by using PCR method (high efficient primer from developmental step)
2. Probe and primer designation by selecting L1 region
3. Validation assay

↓

HPV screening by PCR method and specification detection by using multiplex real time PCR method

Objective:

1. Samples different cytological categories were genotyping with high efficient primer
2. Development primer and probe for specific detection high prevalence and important genotype by using multiplex real time PCR method

↓

HPV screening by PCR method and specification detection by using multiplex real time PCR method

Expected result:

1. More information and correlation data of HPV genotype and cytological data
2. High efficient probe and primer will be established from this study.
3. Fast and accurate specific HPV detection

Key Words

Human papillomavirus, HPV, PCR, multiplex real-time PCR, genotyping, detection, genome characterization, cytological finding

Expected Benefits

1. Molecular diagnosis techniques based on PCR and multiplex real time PCR can be applied for rapid, specific and sensitive detection of the viruses from clinical and normal specimens for detection and HPV screening.
2. Molecular techniques based on PCR detection and sequencing result will provide information on HPV genotype and HPV sequences in samples Thai women and serve as preliminary data of HPV in Thailand.
3. To develop economical and easy methods for HPV detection PCR methods are easy to access in many hospitals in Thailand whereas available kits are only found in main or large hospital.
4. Analysis of the data on HPV accurate available test kits and comparison with PCR will provide useful information with regard to future HPV screening and detection.
5. Genomic sequences of the HPV16 and HPV18 will be submitted to the GenBank database for further epidemiological and phylogenetic analysis potentially represent genetic evolution and potential relationship between clinical stage data and genome variation.
6. Determination and analysis of the most prevalent HPV genotypes will provide information essential for the development of an HPV vaccine suitable for Thai women.
7. It will provide useful information for potentially identifying the nucleotides associated with viral function, viral persistence and pathogenicity.

CHAPTER II

REVIEW OF RELATED LITERATURE

Taxonomy of Human papillomavirus (HPV)

According to the International Committee on Taxonomy of Viruses (ICTV), Papillomaviruses are classified in *Papillomaviridae* family which shared many features with Polyomaviruse including an icosahedral capsid composed of 72 pentamers, a nonenveloped virion, a double-stranded, circular DNA genome, and the site of viral replication and virion assembly as nucleus. However, major different is Papillomavirus genomes and capsids are larger than polyomavirus. In addition, papillomavirus transcription is unidirectional and no homologous region with polyomaviruses [29].

Classification of Human papillomavirus (HPV)

Over 100 different HPV genotypes have been identified based on DNA sequencing analysis [30]. They can be classified according to the species they infect and the sites or disease with which they may be associated [31]. The major viral protein structure, L1 gene that use for phylogenetic tree reveals that papillomaviruses can be generated to 12 genera, designate by the first 12 letters of Greek alphabet (figure1). Five of the 12 genera, alpha, beta, gamma, mu and nu are infected in Human whereas others infected in animal. The homology of L1 major viral protein is use for classifies HPV to genera, species, type, subtype, and variants.

- The HPV with shares at least 60% homology is classified as genera.
- The same genera with shares 60-70% homology are classified as species.
- In the same species with shares 70-90% homology are classified as type
- In the same type with shares 90-98% homology are classified as subtype.
- In the same type with shares more than 98% homology are classified as variant.

The HPVs can be broadly grouped into cutaneous types and mucosotropic types based on their tissue preferred tissue tropism. The cutaneous types are found in the general

population and cause common warts. The mucosotropic HPVs are classified into high-risk and low-risk type, referring to their association with cervical cancer [5]. Alpha genuses that are medical importance are associated with genital and mucosal cancers. The high-risk HPV type was found in species 5, 6, 7, 9 and 11 [2, 32]. HPV16, which associated with cervical cancer, is member of species 9. HPV18 is member of species 7 and HPV 6, which causes of genital warts, is member of species 10.

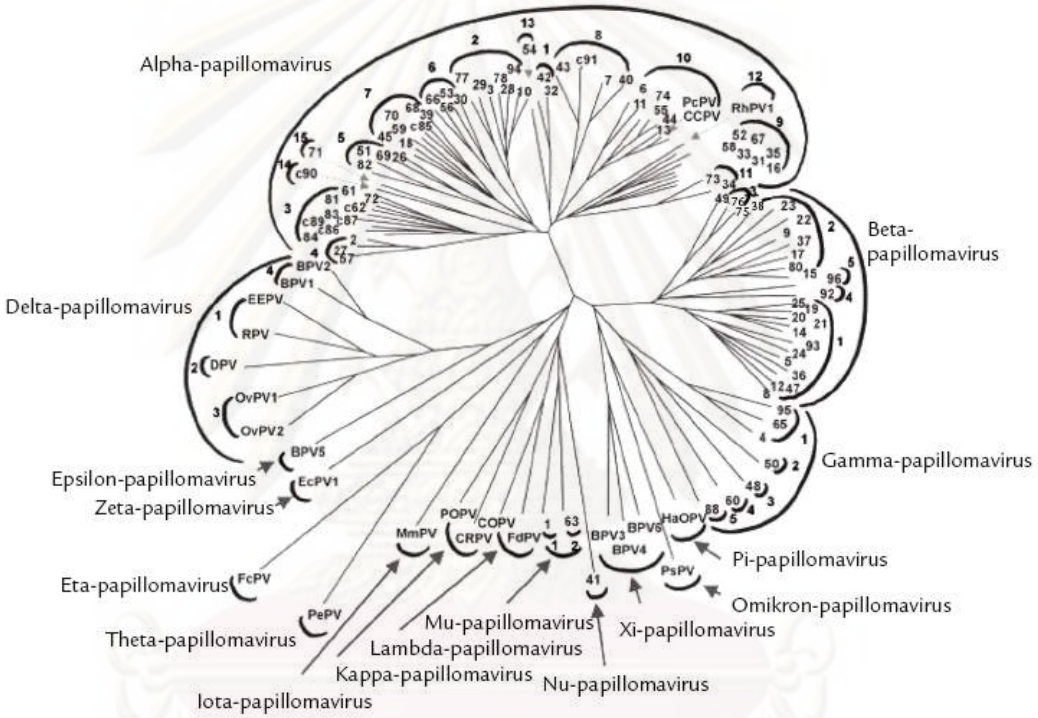


Figure 1 Phylogenetic of L1 gene of papillomavirus. At the end of branch is the papillomavirus type, at the end of the first set of semicircular grouping is the species and the end of the second set of larger semicircular groupings is the papillomavirus genus. Most HPV types are in the alpha-papillomavirus genus, as well as beta, gamma and mu genera [29].

Genital HPV have been classified into high- and low-risk HPV types, according to their potential to induce invasive cancer. Based on previous research, HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 have been considered high risk

(HR-HPV) and types 26, 53, and 66 potentially high risk (probably HR-HPV), whereas types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108 have been regarded as low risk [2].

Structure and genome organization of Human papillomavirus (HPV)

HPV is a highly variable member of small, non-enveloped, icosahedral DNA viruses that replicate in the nucleus of squamous epithelial cells. The virion particles consist of a single molecule of double-stranded circular DNA about 8,000 bp in size. DNA genome encases in a naked iscosahedral capsid about 55 nm in diameter. The virus particles have a density in cesium chloride of 1.34 g/mL [33]. The genome encodes about 10 designated translational open reading frames (ORFs) that are classified as either early (E) or late (L) ORFs, based on their location in the genome (figure 2). The viral genes, expressed from several promoters via splicing of polycistronic mRNAs, are termed either early (E) or late (L) depending on when they are expressed during infection [4]. It's infected the keratinocytes in the basal layers of squamous epithelium. Viral gene expression and replication were controlled by keratinocytes differentiation. Non-structural viral protein, early genes are expressed in undifferentiated or intermediately differentiated keratinocytes and two structural viral proteins; late genes are expressed in the terminal keratinocytes differentiation (figure 3).

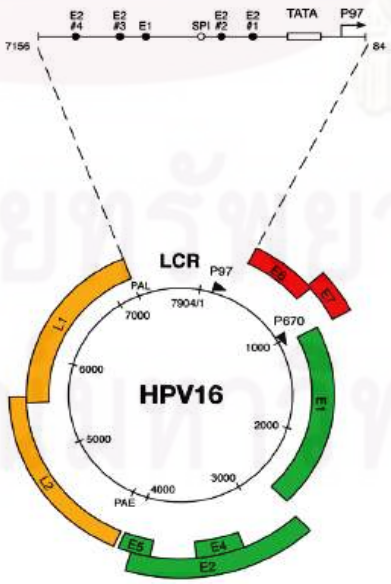


Figure 2 Organization of HPV 16 genome (7,904 bp) is shown as a black circle with the early with the early (p97) and late (p670) promoters marked by arrows. The early genes (E1, E2, E4, E5, E6 and E7) are expressed from either p97 or p670 at different stage during epithelial cell differentiation. The late genes (L1 and L2) are expressed from p670. All viral genes are encoded on one strand of the double-stranded circular DNA genome. The long control region (LCR) is extended for E2 binding site and TATA region of p97 [34].

Some literature divided papillomaviruses to major three regions: early, late, and long control region (LCR or noncoding region [NCR]) [35]. The three regions of papillomaviruses are separated by two polyadenylation (pA) sites: early pA (A_E) and late pA (A_L) sites (figure 4). The early region in papillomaviruses genomes occupies over 50% of the virus genome from its' 5' half and encodes six common open reading frame (E1, E2, E4, E5, E6 and E7) [36]. The late region covers almost 40% of the virus genome, encodes Major capsid protein (L1) and minor capsid protein (L2). The LCR region, covers about 10% of the virus genome, have a segment approximately 850 bp. This region is no protein-coding function but its carry the origin of replication as well as multiple transcription factor binding sites that are important in regulation of RNA polymerase II-initiated transcription from viral early as well as late promoters. The followings are the characteristics and functions of each genes of HPV [37].

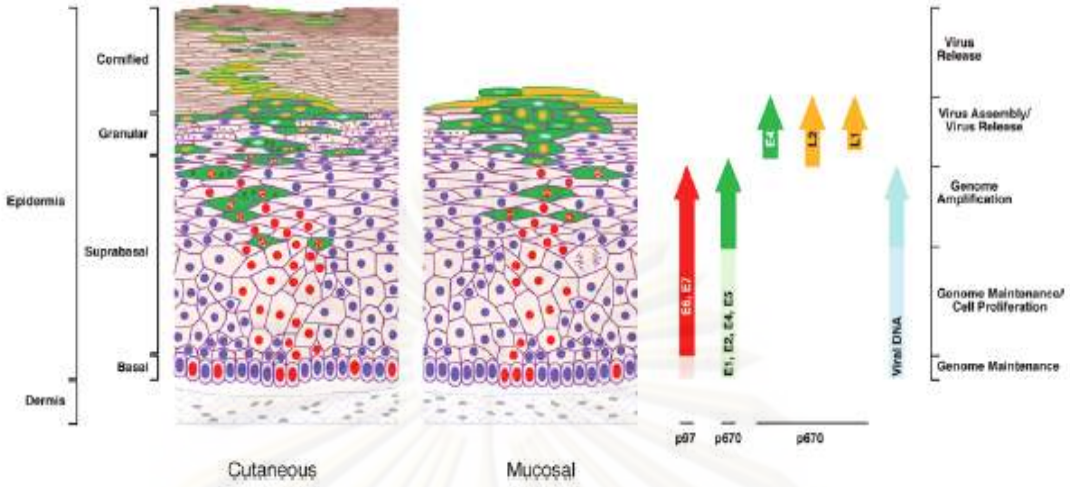


Figure 3 Illustrate of HPV life cycle on epithelial cell. The different cell layers present in the epithelium are indicated on the left. Cells in epidermis expressing cell cycle markers are shown with red nuclei. The appearance of such cells above the basal layer is a consequence of virus infection. The expression of viral proteins necessary for genome replication occurs in cells expressing E6 and E7 following activation of p670 in the upper epithelial layers (cells shown in green with red nuclei). The L1 and L2 gene (yellow) are expressed in a subset of the cells that contain amplified viral DNA in the upper epithelial layers. Cells containing infectious particles are eventually shed from the epithelial surface (cells shown in green with yellow nuclei). The timing and extent of expression of various viral proteins are summarized using arrows at the right levels of E1, E2, E4 and E5 allows maintenance of viral genome (genome maintenance/ cell proliferation). The first appearance of L2 allows genome packing to begin, with the expression of L1 allowing the formation of infectious virions (virus assembly). The accumulation of E4 close to the epithelial surface may improve the efficiency of virus release [34].

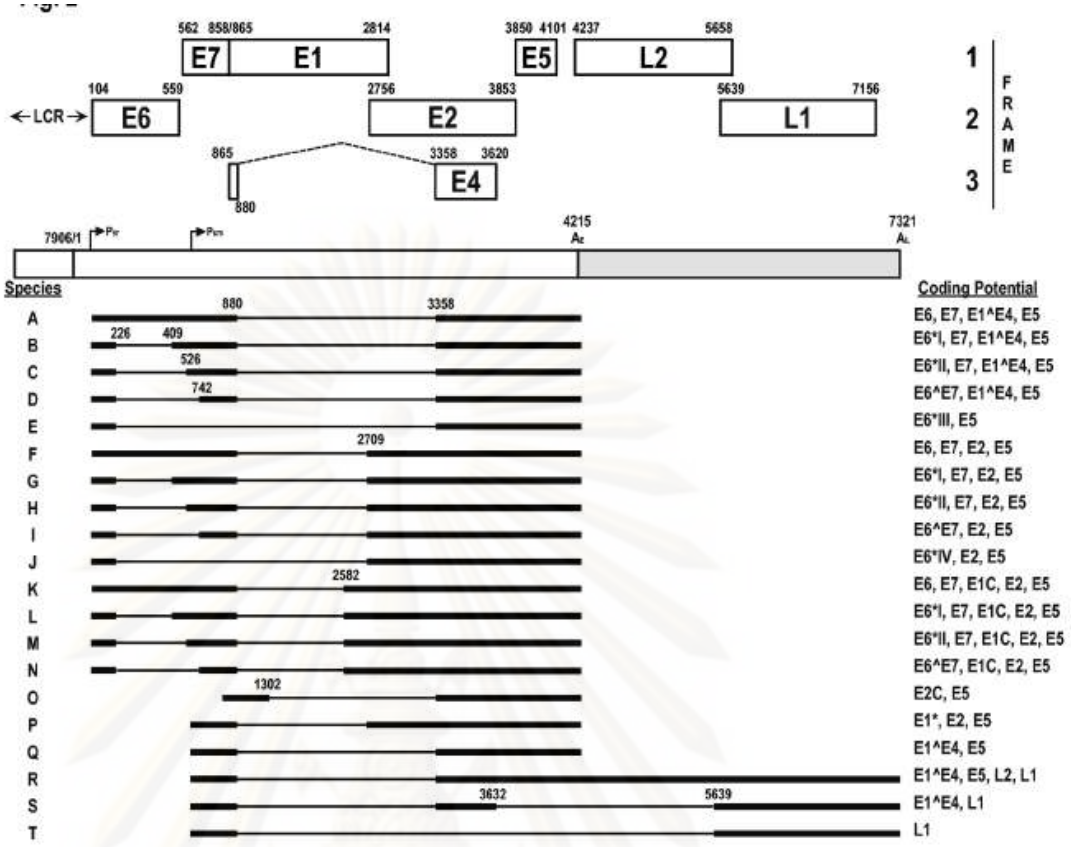


Figure 4 Genome structure and transcription map of HPV16. The bracket line in the middle of the panel represents a linear form of the virus genome and the numbers above each ORF are nucleotide positions of the first nucleotide of start codon and last nucleotide of the stop codons in HPV16 genome. The ORF4 had two exons and formation of the intact E4 ORF requires RNA splicing (dashed line). All early transcription expressed from promoter p97 are illustrated with a 5'end form nucleotide 97 whereas, all late transcription expressed form promoter p670 [35].

The term early (E) can be classified to E1, E2, E4, E5, E6 and E7 genes and late gene can be classified to L1 and L2 genes.

- HPV E1 ORF encodes proteins
  - 68 kDa
  - Requires for the maintenance of the viral genome and its replication.
  - From complex with E2 protein

- HPV E2 ORF encodes proteins
    - 50 kDa
    - The major transcriptional regulatory proteins that interact with specific binding sites in the non-coding region. The E2 encodes proteins have both positive and negative effect on transcription
    - Down-regulate the expression of E6 and E7
    - Loss of the replicative environment for viral DNA synthesis
    - Assembly of infectious virions in the upper epithelial layers
    - Improve the efficiency of genome encapsidation during natural infection

- HPV E4 ORF encoded proteins
    - E4 function not yet fully establish
    - Arrest in G2 and associate with E2 protein
    - Antagonize E7 mediate cell proliferation
    - Disruption of the cytokeratin network, resulting in the characteritic koilocytotic apperance of HPV infected cells
    - Possible regulation of mRNA stability
    - Affect the integrity of the cornified envelope

- HPV E5 ORF encoded proteins
    - This protein localizes at endosomal membranes and the Golgi. Sometime find in cellular membrane.
    - Hydrophobic membrane associate protein (activate the epidermal growth factor receptor) resulting in stimulation cell growth
    - Maintenance of replication competent environment in the upper epithelial layers
    - Inhibit expression of the p21 tumor suppressor gene, thus, affecting cell cycle control

- HPV E6 ORF encoded protein
    - 150 amino acid

- Target to p53 tumor suppressor protein for degradation

- HPV E7 ORF encoded protein
  - 100 amino acid
  - Target to pRb, control cell cycle provides biochemistry evidence of their role in oncogenesis

- HPV L1 ORF encode protein
  - Major capsid protein
  - Depend on change in mRNA splicing and transcripts that terminate at the late polyadenylation site
  - Assmbly of virion in the upper epithelial layers

- HPV L2 ORF encode protein
  - Minor capsid protein
  - Associates with L1 through a hydrophobic region near C-terminus of the protein that is though to insert into the central hole in the pentavalent L1 capsomer

Replication cycle of HPV

1. **Attachment of the virus with a host cell**

Infection by papillomaviruses requires that virus particles gain access to the epithelial basal layer and enter the dividing basal cells. Receptors for these viruses infection are still not clearly understood [38]. However, heparan sulphate proteoglycans may play role in initial binding and /or virus uptake [38-40]. In addition, HPV required secondary receptor, the α6 integrin for infection [41-43]. Papillomavirus particles disassemble in late endosomes and/or lysosomes, with the transfer of viral DNA to the nucleus begin facilitated by minor capsid protein L2 [44, 45]. Some previous study reported that viral transcripts could be detected as earl as 12 h post-infection, with mRNA level increasing over the course of several days [45]. HPV entry to cell like episome, without integration into the host cell genome, go into basal layers and require

expression of viral replication protein and begin to their life cycle (as described in figure 3 and 4).

2. **Entry of the virus into a host cell**

The life cycle of HPV is related with differentiation program of the infected host cell, the keratinocyte, with production of mature virion particles restricted to differentiated suprabasal cells. Papillomavirus infects though microwounds of the epithelium that expose cells in the basal layer to viral entry.

3. **Synthesis of viral DNA and viral proteins**

The first viral proteins to be expressed are the replication factors, E1 and E2. The E1 protein exhibits helicase activity, allowing for the separation of viral DNA strands ahead of the replication complex [46]. E1 functions have both ATPase and 3'-5' helicase activities [46, 47]. E1 and E2 replication are expressed from early promoter; the ability of E2 to activate and repress expression contributes to the control of viral copy number in undifferentiated cells. On differentiation, there is switch to late promoter which increased E1 and E2 expression leading to viral DNA amplification [48]. The HPV E4 and E5 ORFs are expressed during the early phases of the viral life cycle. The E4 ORF is translated from spliced transcripts as a fusion with the first 5 amino acids of E1 to generate E1^E4 fusion protein. The E4 ORF is no start codon and it used the E1 sequence to expression. E5 protein acted on epidermal growth factor (EGF) receptor and resulted in increased proliferation [49]. The E6 and E7 expressed late of the viral infection phase. E6 protein interacted with p53, tumor suppressor that regulated the expression of proteins involved cell cycle control [50]. Furthermore, E6 protein function had an effect with telomerase enzyme, which insufficient of this enzyme may be leading to abnormal cell chromosome. The E7 protein target was pRb, pocket protein included Rb, p107, and p130, and these proteins are differentially expressed through the cell cycle [51, 52].

4. **Assembly of the progeny viral particle**

This stage requires the replicated genome are packed into infectious particles. Capsid proteins (L1 and L2) are expressed after expression of genome amplification, with L2 expression preceding the expression of L1 [53, 54]. During epithelial cell

differentiation, the timing of capsid synthesis is regulated both at the level of RNA processing and at the level of protein synthesis [55, 56]. The assembly of infectious virions in the upper epithelial layers id though to require E2 in addition to the capsid proteins L1 and L2 [56, 57].

5. **Release of the infectious progeny viruses**

Finally step, virus release may facilitated by the E4 protein. E4 is from complex with E1, E1^E4 proteins from high-risk HPV associate with keratin network in cells and over-expression of this complex can induce cell collapse [49] and can affect the integrity of the cornfield envelope [59, 60].

Evaluate of abnormal cervical

The cytological method for HPV screening is Papanicolaou (Pap) smear which established 50 year ago. The American Cancer Society (ACS) and the National Cancer Institute (NCI) have recommended that a Pap smear should be performed annually after onset of sexual activity. Furthermore, the American College of Obstetricians and Gynecologists recommend that women who had a history of human immunoefficiency virus (HIV), human papillomavirus (HPV) infection, cervical dysplasia and multiple sexual partners should tested by Pap smear screening.

Cervical smear

A doctor will be used a spatula takes a scraping of cells from the cervix (neck of the womb) and "smears" the cells in a thin layer on a glass slide. After staining with a Papanicolaou stain, the cells are examined by a cytologist in a laboratory who will check for signs of pre-cancerous change. Each slide contains between 300,000 to 400,000 cells and takes about 10 minutes to scrutinize. The doctor will try to obtain cells from the cervix where the skin (squamous) cells on the outside meet the gland (columnar) cells on the inside of the canal. This junction is called the Transformation Zone (T.Z.) and it is here that cancers most often start. A good quality smear will therefore contain both sorts of cells to prove that the T.Z. has been sampled correctly (figure 5).

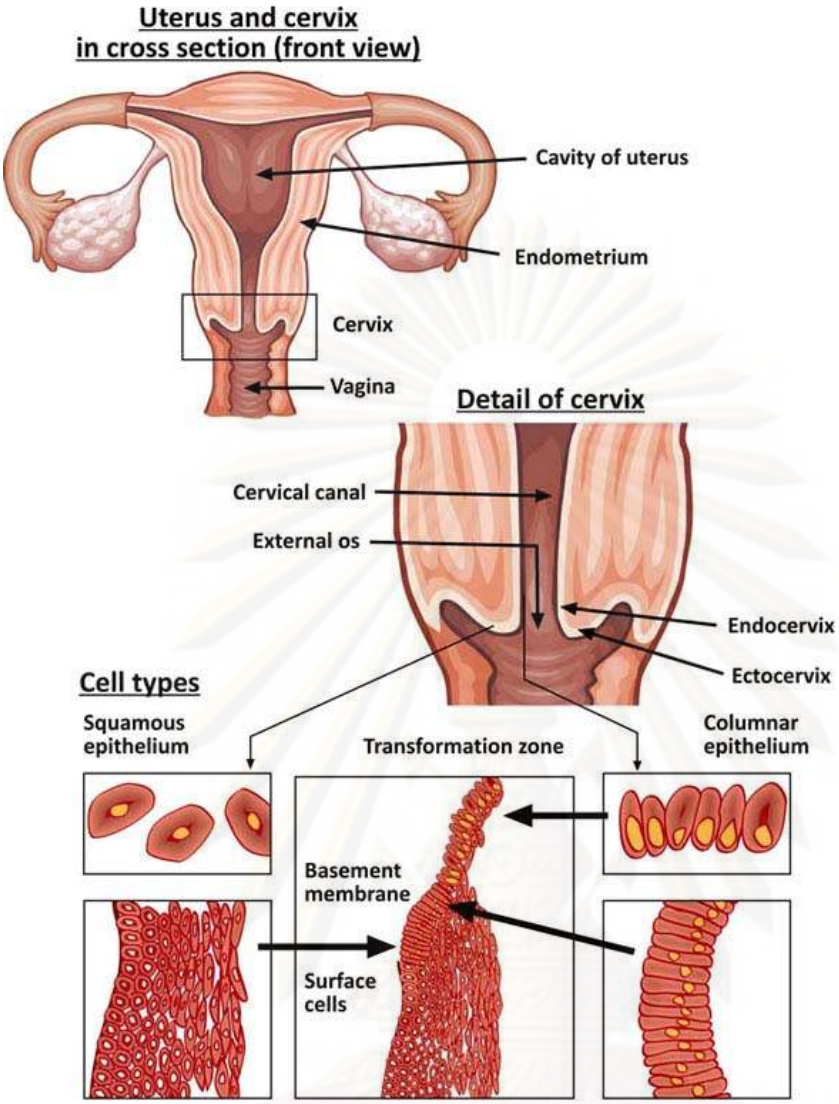


Figure 5 Diagram picture represent cervix structure and cell type. (http://www.colposcopy.org.uk/cervicalsmears.htm)

Pap smear classification

The traditional Pap smear classification designation as following these [61]:

- Class I (normal)
- Class II (atypical cells)
- Class III (cervical dysplasia)
- Class IV (carcinamo in situ)
- Class V (invasive cancer)

Traditional system was lacked of standardize cytological criteria for Pap smear

Diagnosis, therefore, many observers can make variation result. In 1972, the cervical intraepithelial neoplasia (CIN) system was proposed as following these: (figure 6)

- CIN I or mild dysplasia
- CIN II or moderate dysplasia
- CIN III or serve dysplasia ad carcinoma in situ

In 1988, the new Bethesda system (TBS) introduced in term “squamous intraepithelial lesion” (SIL) to designate a pre-cancer lesion. It subdivided into high- and low-grade SIL (figure 6)

- Low-grade SIL (LGSIL) are mild dysplasia (CIN I) and lesion suggested of HPV infection
- High-grade SIL (HGSIL) are moderate and serve dysplasia (CIN II-III) and carcinoma in situ (CIS)

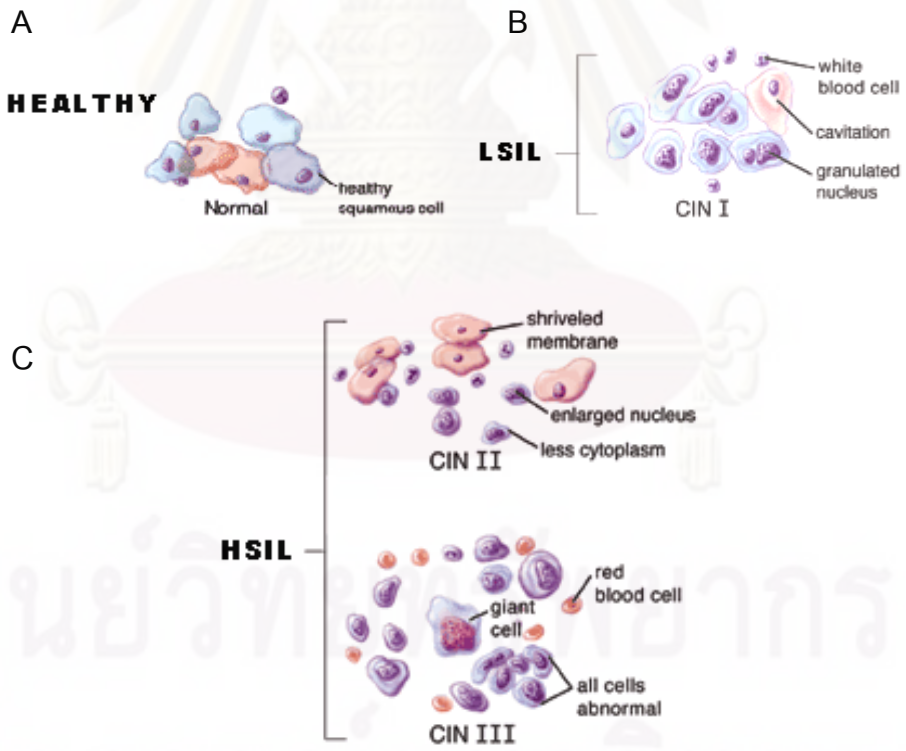


Figure 6 Diagram pictures of Pap smear results. (A) Healthy Pap result showed squamous cells from the surface of the cervix. There are no signs of infection and no

abnormal cells. (B) Low-grade squamous intraepithelial lesion result showed graduate nucleus. (C) High-grade squamous intraepithelial lesion result showed giant cell and all cell abnormal (http://www.hopkinsmedicine.org/cervicaldysplasia/diagnosis_1.htm).

The two categories that appear on Pap test results are "squamous" and "glandular". Squamous cells are found in the lower cervix, and are susceptible to becoming squamous cell carcinoma (figure 7). The term "glandular" refers to mucus-producing glands of the upper cervix. These glandular cells are susceptible to becoming adenocarcinoma.

Figure 7 This figure shows how squamous cells look under the microscope as they progress toward cancer. Compared to a normal cell (left), slightly abnormal cells (ASCUS, atypical squamous cells of undetermined significance) have larger, darker nuclei. A cell with some precancerous changes (LSIL, low-grade squamous intraepithelial lesions) appears misshapen. Cells with more severe changes (HSIL, high-grade squamous intraepithelial lesions) have large black nuclei where DNA rapidly reproduces. Cancerous cells are the most misshapen, and are very dark (http://www.thinprep.com/info/understanding_results/define_pap_terms.html).

In addition, other words that cytologist will use for classify Pap smears result is “Dysplasia/Dyskaryosis” which refer to pre-cancerous changes in the cells (figure 8). Dysplasia/Dyskaryosis can be classified into 3 types following these:

- **Dysplasia/Dyskaryosis**

  In borderline smears there is a slight abnormality of the cells and their nuclei. A

repeat smear after an interval of around 6 months is usually requested. In the United States borderline changes are known as 'Atypical Squamous Cells of Uncertain Significance' (ASCUS). Essentially, the nucleus in the centre of the cell gets bigger and the jelly around the nucleus (cytoplasm) gets smaller. These changes are sometimes caused by certain strains of the Human Papilloma Virus (HPV) or "wart" virus

- **Mild Dysplasia/Dyskaryosis**

  About 50% of women with mild dysplasia will have their changes revert to normal if you wait 6 months. So, women with a smear showing mild changes will be asked to have a repeat smear 6 months later. If mild changes persist on the second smear, those women will undergo further assessment by colposcopy. Mild dyskaryosis in the United States is known as a 'Low-grade Squamous Intraepithelial Lesion' (LSIL).

- **Moderate or Severe Dysplasia/Dyskaryosis**

  Women with smears showing moderate or severe pre-cancerous changes will be referred for colposcopy as they have a significant risk of proceeding to cervical cancer if left untreated. Moderate or severe dyskaryosis is known in the United States as a 'High-grade Squamous Intraepithelial Lesion' (HSIL).

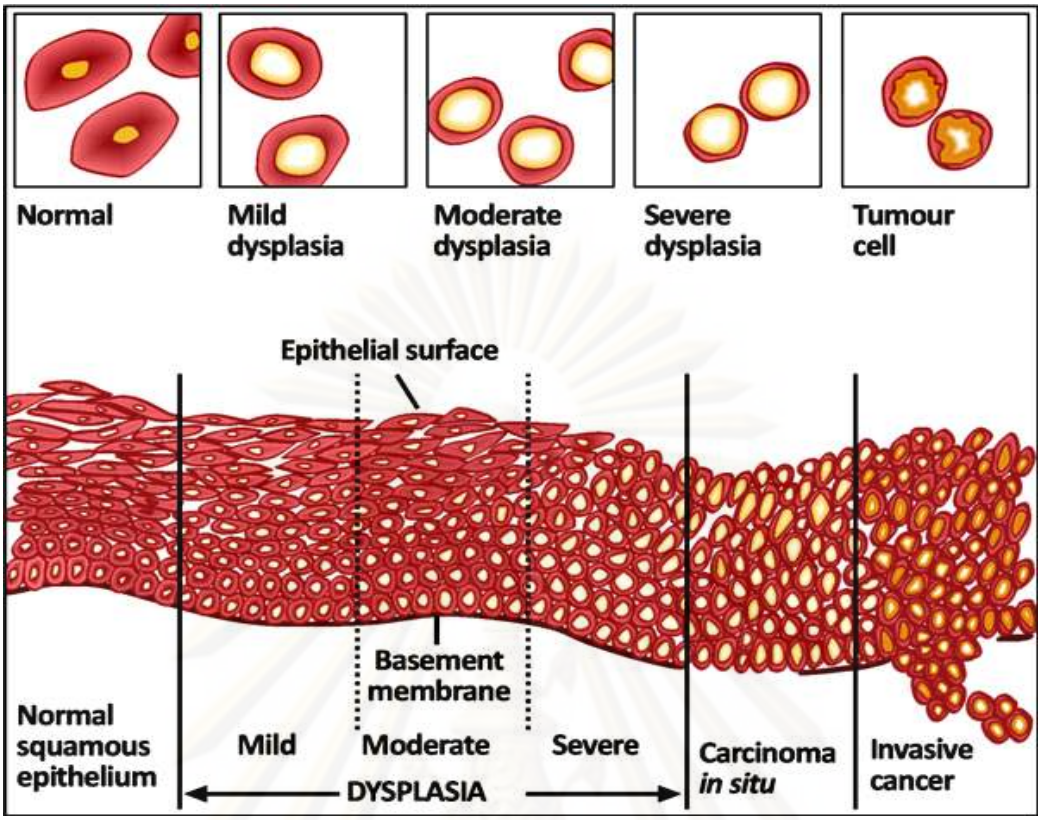


Figure 8 Progressive of cervical cancer from normal cell to tumor cell and classify categories of dysplasia abnormality. Cells showing pre-cancerous changes show no special features to the naked eye. A cervix which contains these cells will look normal. Pre-cancerous cells do not cause any symptoms, and may remain dormant for several years before proceeding to cancerous change i.e. start nibbling into the tissue beneath (http://www.colposcopy.org.uk/cervicalsmears.htm).

Epidemiology and transmission

- **Incidence rate transmission and risk factor of HPV infection**

  Genital HPV infection is the most commonly diagnosed sexually transmitted infection (STI) in young sexually active population in developed countries [62]. An estimated 30 million new cases of genital HPV are diagnosed every year worldwide [63]. Infections with oncogenic types of HPV represent 50-70% of all HPV infections [64]. The prevalence and incidence estimates of genital HPV infection vary with the characteristics of the study population, the study design, the specimen sampling, and the HPV detection method. Higher incident rates

are observed in young women, black Hispanic, and those reporting higher numbers of recent and life time sexual partners [65, 66, 67, 68]. Moreover, women who use oral contraceptive (OCs) and tobacco, had a history of herpes simplex virus and vulvar warts, and history of anal intercourse and higher frequency of vaginal intercourse are high risk for HPV infection [65, 66, 67, 68].

Age and number of sexual partners, both lives time and recent, have associated with HPV infection. The high infection is occurred in women age between 15-25 years and steady with women increase age about 40 years. Nevertheless, some previous study reported that second high infection rate found in postmenopausal women [69, 70, 71].

- **HPV genotyping and prevalence**

  There are many studies try to establish which HPV genotypes are prevalence in without with and cervical neoplasia such as LSIL (low grade squamous intraepithelial lesion), HSIL (high grade squamous intraepithelial lesion), SCC (squamous cell carcinoma) and ADC (adeno- and adenosquamous-carcinoma). First, worldwide distribution of HPV in cytological normal women is showed that the most relevant different between regions was in the prevalence of HPV16 in relation to other HPV types. HPV35, HPV45, HPV52, HPV56 and HPV58 were all common in HPV positive women in African (sub-Saharan) than in Europe. However, the prevalence of HPV16 in African (sub-Saharan) was five times less than other regions. By contrast, the proportion of HPV18 positive women was similar to other regions [72]. HPV35, HPV45 and HPV58 were more common genotype in Africa than in Europe in women with high-grade squamous intraepithelial lesion [73] and squamous cell carcinoma [74]. These patterns suggested that the prevalence of HPV16 is most popular than in Africa. The proportion of high-risk HPV infections preventable by vaccine for HPV16 or HPV18 might vary by region, being highest in Europe and lowest in Africa (sub-Saharan). However, regional heterogeneity decreases with increasing severity of lesion as HPV16 becomes increasingly dominant [75]. Second, genotyping distribution in LSIL is revealed that HPV16 was the most common genotype followed HPV31, HPV51 and HPV53 [76]. HPV positive LSIL from Africa were

significantly less likely to be infected with HPV16 than LSIL from Europe, with LSIL from North America and South/Central America showing intermediate risk [76]. Furthermore, HPV31, HPV33, HPV45, HPV52, HPV58 were identified the next common genotypes in SCC (squamous cell carcinoma). About 40% of HPV positive LSIL were positive for these five genotypes, which are responsible for approximately 15% of cervical cancer worldwide [77]. Other high-risk HPV genotype HPV35, HPV39, HPV51, HPV56 and HPV59 are found up to 40% of HPV positive LSIL but are responsible for only 3% of cervical cancer worldwide [74]. Moreover, probably high-risk HPV (HPV66 and HPV53) were found relatively common in LSIL but are rarely detected in cancer and very low risk for progression to cancer. Third, HPV genotype distribution in HSIL is performed that worldwide HSIL infected with HPV16, HPV18 and HPV45 are progressive to SCC than others high-risk genotypes. Some previous study concluded that these types are greater potential to induce fully malignant transformation especially HPV 18 increased oncogenic potential in cell culture [77, 78], and /or these infections somehow preferentially evade the host immune system. Due to HPV18 are increased oncogenic potential to cervical cancer; therefore, this strategies is true for HPV45 because both of them are genetic similarity [79]. Moreover, HPV16 which persistence and infection in HSIL are related with variants [74] and may be related to its ability to escape immune response of host cell when comparing with others HPV genotypes [74]. Finally, HPV genotype distribution in cervical cancer is separated between squamous cell carcinoma (SCC) and adeno- adenosquamous carcinoma (ADC). In SCC, HPV16 was the most predominate type followed by HPV18, HPV45, HPV31, HPV33 in all regions except Asia, whereas HPV58 and HPV52 were more frequently identified. In ADC, HPV prevalence was significantly lower than SCC, and HPV18 was the predominant type in every region followed by HPV16 and HPV45 [74]. HPV distribution in Asia revealed that type specific prevalence of HPV 6, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 70, 73 and 82 were found in abnormal cytological data. Overall HPV much more prevalence was cervical cancer, HSIL, LSIL and normal cytology/histology. HPV16 are found mostly in cervical cancer

cytology whereas HPV18 are found mostly in HSIL (figure 9). HPV58 and HPV52 were the most common types in cervical cancer group in eastern and southeastern Asian but not in south central Asian [80]. After HPV16 and HPV18, the next most common HPV types were HPV58, 33, 52, 45, 31 and 35; therefore, these types should be considered for second-generation HPV prophylatic vaccines [80].

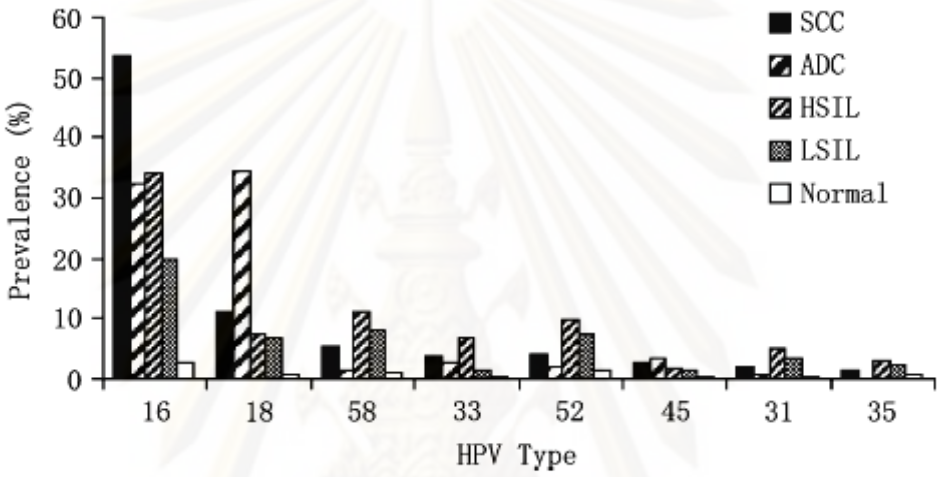


Figure 9 The HPV type distribution in 25,368 women from Asia, stratified by grade of cervical lesion [80]

However, the meta-analysis of previously studies is limited in methodology that uses primer to amplify broad-spectrum HPV genotype. But the primer cannot amplify all genotypes with exactly the same sensitivity [81]. Variations in overall HPV positive studies may be had an effect from (a) quality of cytologic/histologic assessments, (b) experience of pathologist to classify cytology data (c) frequency of cervical screening [76]. Geographic differences in the relative prevalence of HPV genotype may be related to the complex interplay between different HPV genotypes and/or variants with host immunogenetic factor (HLA polymorphisms) [82]. A recent study showed that HPV16 appears less influenced by immune status than other HPV genotypes [83].

Immunology

Host immunity is a complex mechanism that included innate and adaptive immunity. Innate system is responsible for triggering or activating adaptive immunity, that specific memory. Antibody-mediated humoral immunity clears free virus particles and can prevent re-infection. Cell-mediated immune responses are important for clearance of viral infected cells and generation of immune memory.

- **Innate immunity and HPV**

  Women with transient HPV infections are less develop antibody responses or cell mediated responses than women with persistent infections [84]. Innate immune response can eliminate antigens before there is chance to develop memory responses. During natural replication, the antigenic capsid proteins are not expressed until the cell has differentiated to the superficial layers of the epithelium where dendritic cells can not recognize them. Trancription of E6 and E7 against the innate immune system by downregulating functions related to interferon (IFN).

- **Humoral immunity and HPV**

  Some women most clear through innate mechanisms before there is adequate antigen exposure to develop adaptive immune responses. Humoral immunity against HPV capsid protein can take several months to occur, antibody level has been shown to be stable for over 10-15 years of follow-up. Antibody production is important in preventing the spread of infection and reinfection; cell-mediated response is responsible for viral clearance [85]. This response involves interactions with professional antigen presenting cells (APC), T helper cells, cytolytic T cells, and cytokines

- **Adaptive immunity and HPV**

  Cell mediated immune responses are critical in viral clearance after infection is established. Oncogenes (E6 and E7) and E2 are targeted of cell mediated immune response of HPV infection.

Vaccine

There are three points in the natural history of HPV infection which the body's immune system can be boosted to fight against the virus have following these 1) a prophylactic vaccine which injects to women before infects with HPV can prevent virus entry to mucosa 2) a therapeutic vaccine which inject during viral replication can eradicate cells expressing late genes that produce of the new virion 3) a therapeutic vaccine after viral integration can control or stop the growth of oncogenic protein E6 and /or E7 viral gene. In addition, production of killed virus vaccines is not possible because there are difficult to culture HPV in laboratory. The major viral capsid protein L1, alone or conjunction with minor capsid protein, L2 can self assemble into virus-like particle (VLPs) without the viral genome [86-90]. This type of vaccine can be imitated the natural infection [91, 92]. And animal vaccinated with VLP vaccine do not develop disease [93-96]. However, VLP vaccine are type-specific protection, therefore, the development of this type of vaccine focus on HPV16 which high prevalence in worldwide.

Currently vaccine of HPV is L1 virus like particle (VLP) vaccines which have 2 commercial products from GSK, bivalent vaccine (HPV16/18) and Merck's HPV vaccine Gardasil®, quadrivalent vaccine (HPV 6, 11, 16, 18). Nevertheless, VLP vaccine is expensive and two oncogenic types delivered via intramuscular and required cold chain. The third phase of these vaccines study is designed to compare the immunogenicity of bivalent and quadrivalent vaccine in healthy adult females 18-45 years old.

New generation of vaccine should have advantage following these: 1) broaden coverage to include protection against not only HPV16/18 but most others genital oncogenic HPV type; 2) induce long term protection at mucosal surface; 3) be cheap, theremostable and, hopefully, delivered via non-injectable method; and 4) provide therapeutic as well as prophylactic efficiency [97]. Furthermore, Current available prophylactic vaccine haves no therapeutic effect for established infection or for disease. Proportion of intraepithelial HPV-associated disease undergoes immune-mediated regression, the development of immunotherapeutic strategies is an opportunity to determine proof-of-principle for therapeutic vaccine [98].

Laboratory Diagnosis

There are several diagnostic techniques can be applied for HPV detection. Many factors should be considered in deciding which tests to use. Sensitivity, specificity, turn-around-time, reproducibility, ease of performance and costs should all be taken into account. There are 3 types of nucleic acid method for HPV detection which have following these; 1) direct nucleic acid probe method 2) hybridization signal amplification method and 3) target amplification method

- **Direct probe methods**

  Southern blot is a gold standard method for HPV genomic analysis and used by some previous study [99-101]. Another direct probe method is ISH that uses immunohistochemistry and antigenic expression within the context of histopathology [101-106]. The disadvantages of direct probe method are low sensitivity, time-consuming and need large amount of purify DNA.

- **Signal amplification methods**

  Signal amplification methods are really an extension of direct probe techniques that have achieved a sensitivity boots in detection methods. Hybrids capture II (HCII) and branched DNA (bDNA) assay is included in this categories. HCII assay can be detected HPV genotypes following low-risk HPV types: HPV6, 11, 42, 43 and 44 and high/intermediate-risk HPV types: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68.

- **Target amplification methods**

  PCR is popular target nucleic acid amplification. Polymerase chain reaction is a valuable tool because it allows in vitro multiplication of unique regions of DNA; therefore, they can be detected within large background. The method is most flexible and sensitive of all DNA analysis technique. This assay can be used for detection, viral load quantification, nucleotide sequencing and mutation analysis [107-115]. However, the contamination need to awareness.

HPV analysis method

1. **Methods for HPV genotyping and detection**

- **Hybrid capture II (HCII)**

HCII which FDA approved is nucleic acid hybridization assay for the quantitative detection of DNA of 13 carcinogenic (probe A) and 5 begin HPV types (probe B) in cervical samples. However, this test is show number of disadvantage, such as the inability to identify specific HPV genotypes and the risk of cross hybridization of additional HV types with probe mix [116, 117]. They do not provide specific information regarding which HPV genotypes are present within a specimen.

- **Polymerase chain reaction (PCR)**

The most widely used PCR assay use consensus primers, such as GP5+/GP6+, MY09/My11 and SPF, which highly conserved region of the HPV L1 gene, amplifying numerous genital HPV types in one reaction [118, 119]. PGMY09/11 primers were designed to improve MY09/MY11 sensitivity across the type spectrum with increase detection of multiple infections and improved reproducibility and specificity [119]. SPF primers are analogue to PGMY09/11, they include inosine that matched with any nucleotide and allows PCR increasing to high HPV detection rates than previous primer sets [120, 121].

- **Linear Array HPV genotyping test**

The commercial linear array was developed based on PGMY09/11 PCR in combination with line blot assay. The linear array can detected 37 high-risk and low-risk HPV genotypes including 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73 (MM9), 81, 82 (MM4), 83 (MM7), 84 (MM8), IS39, and CP6108. However, this method quite expensive and contaminate with previously amplified material can lead to false positives [122].

- **INNO -LiPA HPV assay**

The commercial linear array was developed based on SPF PCR, which can be detected 25 different HPV genotypes. This method has proved to be sensitive, specific, simple and rapid for using [123, 124].

- **Restriction fragment length polymorphism (RFLP)**

It used to identify HPV genotype-specific restriction pattern derived from PCR amplification product from consensus primer sets. The restriction enzymes used for most analyzes are *Bam*HI, *Dde*I, *Hae*III, *Hin*fI and *Pst*I. Nevertheless, RFLP data are difficult to interpretation result, especially when mixed infections are occurred in specimens.

- **Direct nucleotide sequencing methods**

The previous product PCR using consensus primers are another approach that can be used to discriminate HPV genotypes. This technique can be use to identify mutation within known HPV genotypes. Direct sequencing has an advantage to give information of nucleotide sequence; however, time-consuming and large purification DNA are required by this method.

2. **Methods for HPV viral load**

Due to several previously study reported that viral load of HPV infected patients with high can be caused for developing to cervical cancer [125-130]. Real-time PCR can be detect HPV viral load by quantitative of fluorescent reporter, the signal of reporter increase in direct proportion to the amount of PCR product in a reaction. Real-time method doesn't need to use agarose gel, making it a useful tool for large scale screening samples. Quantitative of target DNA, such as viral pathogen, using real-time PCR has the advantage of reliable, reproducible and rapid methods. Furthermore, real-time reaction can be detected multiplex fluorescence reporter dye; therefore, multiple HPV infection can be detect and quantitative in the same time by using this methods [131].

3. **Methods for HPV integration**

The physical stage of the virus can be determined by the failure to amplify full-length E2 using PCR, but also by using more comprehensive Southern blot hybridization. Real time PCR assays which measure E2 and E6 copy numbers, have recently been developed to determine the integration state. However, this assay has limitation such as low viral load and length of E2 amplicon when interpretation integration studies [131]

4. **Methods for HPV-RNA detection**

- **PreTect HPV Proofer**

  This assay incorporates nucleic acid sequence-based amplification of E6/E7 mRNA transcripts before type-specific detection for HPV16, HPV18, HPV31, HPV33 and HPV45 [132]. Compared with HPV DNA detection, the presence of E6/E7 mRNA transcripts was less sensitivity, but more specific for the detection of disease and follow-up [133].

- **Real-time quantitative PCR**

  High RNA quality is required for the application of real-time reverse transcriptase PCR to evaluate E6/E7 mRNA expression level [134]. The quantification of E6/E7 transcription may be useful as a prognosis tool to identify women at increased risk of developing cervical cancer.

Establishment HPV in cell culture method

The study of HPV in cell culture has been difficult because of the difficult in recreating the three-dimensional structure of the epithelium on which the virus depends to complete its life cycle. The current method of transfecting molecularly cloned HPV genome transfected into early-passage Human foreskin keratinocytes (HFKs) or primary human keratinocytes (PHKs) extends the life span of the cell, analysis of stable transfectants become difficult. The efficiency in vitro system for HPV study is organotopic culture of PHKs or selected epithelial cell lines have been a core format for HPV investigations. Briefly, keratinocytes stratify and differentiate into squamous epithelium in 10 or more days when cultured on a dermal equivalent, consisting of collagen with embedded fibroblasts, and held at the medium: air interface. This “raft” culture system can support the viral productive program [135, 136]. However, this system has several limitations such as transfection of non-supercoiled DNA is inefficient and PHKs senesce after only a few passages [137].

CHAPTER III

MATERIALS AND METHODS

Materials

1. **Materials and reagents required for sample collection**
   - LBC (ThinPrep®, Hologic, West Sussex, UK)

2. **Reagents for nucleic acid extraction**
   - Glycogen (USB, Cat no. 16445)
   - Guanidine Thiocyanate Ultrapure (USB, Cat no. 75818)
   - Sodium chloride (BDH Laboratory Supplies, Cat no. 102415K)
   - EDTA Tetrasodium Dihydrate (USB, Cat no. 15700)
   - Tris, Ultra Pure, Molecular Biology Grade (Research Organics, Cat no. 9680T)
   - Boric acid, ACS Reagent (Research Organics, Cat no. 1748B)
   - Phenol, Saturated (PIERCE, Cat no. 17914)
   - Ethanol (BDH Laboratory Supplies, Cat no. 10107)
   - Chloroform (SIGMA, Cat no. C-2432)
   - Iso-Amyl alcohol (BDH Labolatory Supplied, Cat no. 27212)

3. **Reagents for nucleic acid amplification and quantitation**
   - Eppendorf MasterMix (2.5X) (Eppendorf, Cat no. 0032 002.250)
   - Biotools QuantiMix EASY PROBES KIT (BIOTOOLS, Cat no. 10.601)
   - TaqMan probe (Oligo company)
   - SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen, Cat no. 11732-020)

4. **Reagents for agarose gel electrophoresis and DNA staining**
   - GeneRuler 100bp DNA Ladder Plus (Fermentas, Cat no. SM0321)
   - 10 bp DNA Ladder (Invitrogen, Cat no. 10821-015)

- Agarose, low EEO, Molecular Biology Grade (Research Organics, Cat no. 1170A)
- SeaKem LE agarose (BioWhittaker Molecular Applications, Cat no. 50004)
- Ethidium Bromide (SIGMA, Cat no. E-1510)

5. **Materials and reagents for cloning and transformation in *E.coli***
   - One Shot TOP10 Chemically Competent *E.Coli* (Invitrogen, Cat no. C4040-03)
   - pGEM-T Easy Vector System for T/A cloning strategy (Promega, Cat no. A1360)
   - X-Gal (Promega, Cat no. V3941)
   - IPTG (Isopropyl-Thio-B-D-Galactopyranoside) (Eppendorf, Cat no. 0032 006.353)
   - Tryptone powder (BIO BASIC INC., Cat no. G211)
   - Yeast Extract (GIBCO, Cat no. 20047-056)
   - Agar Bacteriological (GIBCO, Cat no. 20001-020)
   - FastPlasmid Mini (Eppendorf, Cat no. 955150601)

6. **Reagents for nucleotide sequencing**
   - Perfectprep Gel Cleanup (Eppendorf, Cat no. 955152000)
   - ABI PRISM Bigdye Terminator v3.1 Cycle Sequencing Kit (Applied Bisystems, Cat no. 4336917)
   - 310 Genetic Analyzer Performance Optimized Polymer 6 (Applied Biosystems, Cat no. 402837)
   - Buffer (10X) with EDTA (Applied Biosystems, Cat no. 402824)
   - Template Suppression Reagent (TSR) (Applied Biosystems, Cat no. 401674)

7. **General materials for molecular biology research**
   - MicroAmp PCR tube (Perkin Elemer)
   - Microcentrifuge tube : 0.5 and 1.5 ml. (AxyGen® Scientific)
   - Polypropylene conical tube : 50 and 15 ml. (AxyGen® Scientific)
   - Pipette tip : 10 µl, 200 µl and 1000 µl (AxyGen® Scientific)

- Cryotube (Nunc)
- Microscope slide and cover slit (Sail brand)
- Glassware : Beaker, Flask , Cylinder and reagent bottles (Pyrex)

Equipments

- Centrifuge (Beckman GS-6R)
- Refrigerated microcentrifuge (Universal 16R Hettich)
- – 70 °C freezer (Forma Scientific)
- – 20 °C freezer (Philco)
- DNA Thermal Cycler 9600 (Perkin Elmer)
- Mastercycler personal (Eppendrof)
- Rotor-Gene RG-3000 (Corbett Research)
- Gel Doc 1000 UV transilluminator (Biorad)
- Bio Photometer (Eppendorf)
- PCR Cabinet (Augusta)
- Perkin-Elmer 310 Sequencer (PE Applied Biosystems)
- Autoclave (Hydroclave MC10 Harvey)
- Hot air oven (Memmert)
- Multi-block heater (Lab-line)
- Balance (PB1502 Mettler Toledo)
- Microwave oven (Sanyo)
- Forceps

Software for bioinformatic and data analysis

- CLUSTAL X program (version 1.8)
- OLIGOS primer design software (version 9.1)
- BioEdit Sequence Alignment Editor (version 7.0.4.1)
- Chromas Lite (version 2.01)
- TreeView (version 1.5.2)

- Molecular Evolutionary Genetics Analysis (MEGA) (version 3.1)
- DNASTAR package software
- Rotor-Gene 3000 (version 6.0; Corbett Research)

Methods

Collection of specimens

The Ethics Committee of the hospital and faculty of Medicine, Chulalongkorn University, approved all study protocols. The HPV positive samples were chosen from among the specimens obtained during the patients' routine check up or investigation, treatment. All the studied specimens were anonymous with a coding number for analysis and permission was granted by the director of the hospital. In addition, all specimens were exclusively used for academic research and the patients were not remunerated. The HPV positive samples were randomly chosen from positive specimens' representative for each cytological category. The HPV genotype 16 and 18 positive samples were randomly chosen from positive specimens' representative for each cytological category.

Sample collection

HPV samples of Thai women representing patients with different cytological data from Bangkok province were obtained from Samitivej Srinakharin hospital and King Chulalongkorn Memorial hospital, Thailand. The specimens originated from the patients' routine check up or investigation and treatment. All specimens were collected for cytology by LBC (ThinPrep®, Hologic, West Sussex, UK). The specimens were sent as anonymous with a coding number. All HPV samples were stored at -70 °C until used.
Seven and nine HPV positive genotype 16 and HPV18 samples, respectively were collected for cytology by LBC (ThinPrep®, Hologic, West Sussex, UK) and tested for HPV DNA by using Hybrid capture II (Digene). Subsequent to BLAST analysis of the whole genome amplified by PCR, the respective genotypes were determined by direct sequencing.

Cytological diagnosis

All 515 samples were initially screened by a cytotechnician and once more by a cytopathologist. Also, all 515 cases were confirmed by a gynecologist with clinical experience. Our cytopathologists have applied the following standards:

- Cytological Report System: The Bethesda System 2001
- Recommendation for management: ASCCP guideline
- TQM-CQI in gynaecology and non-gynaecology: Mandated by Clinical laboratory improvement Amendments of 1988 (CLIA 88) applicable guide.

Primer design

The following complete nucleotide sequences of HR-HPV, probably HR-HPV and LR-HPV genotypes were downloaded from the GenBank database: HPV6 (NC001355), HPV11 (M14119), HPV16 (HPV16R, EU118173), HPV18 (X05015, EF202155), HPV26 (NC001583), HPV31 (J04353), HPV33 (M12732), HPV35 (M74117), HPV39 (M62849), HPV45 (EF202159, EF202167), HPV51 (M62877), HPV52 (X74481), HPV53 (NC001593), HPV56 (EF177181, EF177178), HPV58 (D90400), HPV59 (X77858), HPV66 (EF177191, EF177185), HPV68 (DQ080079), HPV70 (HPU212941, HPU22461), HPV73 (X94165) and HPV82 (AB027021). All of HPV sequences were used for establish primer for conventional PCR. Subsequently, alignments were performed using CLUSTAL X (Version 1.81 from ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX) and BioEdit sequence alignment Software Version 5.0.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Assay target regions were first identified by visual inspection of the sequence alignment. Primers were chosen from constant regions of all specific sequences. Primers were analyzed using the primer design software OLIGOS Version 9.1 and FastPCR Version 3.8.20 (Ruslan Kalendar, Institute of Biotechnology, University of Helsinki, Finland) to predict the percentage of G+C content, potential for dimerization, cross-linking and secondary structure.

The following complete nucleotide sequences of HPV16 genotypes were downloaded from the GenBank database: AF534061, AF536179, U89348, NC001526, K02718, AY686581, AF125673, AY686580, FJ006723, EU118173, AY686579, AF402678, AF536180, and AF472508. All of HPV16 sequence were collected and designed primer for entire genome characterization (figure 9). The following complete

nucleotide sequences of HPV18 genotypes were downloaded from the GenBank database: AY262282, X05015, EF202143, EF202144, EF202155, EF202146, EF202147, EF202148, EF202149, EF202150, EF202151, EF202152, EF202153, EF202154 and EF202155. All of HPV18 sequence were collected and designed primer for entire genome characterization (data not shown).

Subsequently, the program that use for primer designation is the same as described previously. The whole genome HPV16 PCR amplification product diagram showed on figure 10 and HPV18 PCR amplification product use the same criteria as HPV16.

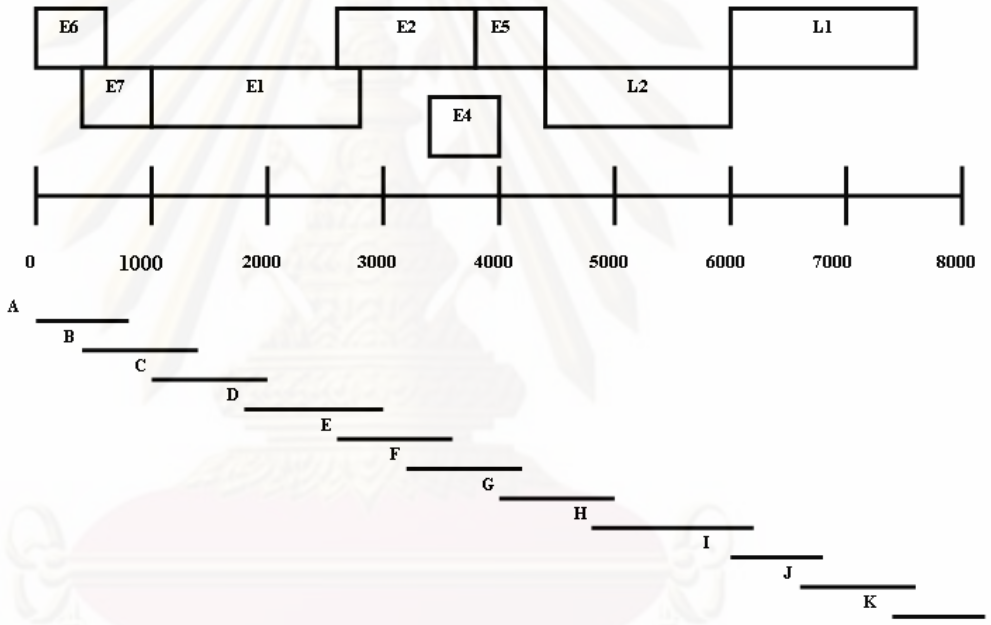


Figure 10 Strategy for whole genome amplification of HPV. The black bars (A-K) represent the 11 overlapping fragments that were amplified to cover the complete genome.

DNA extraction

The DNA is isolated from the interphase and phenol phase separated from the initial homogenate as described in the RNA isolation protocol. Remove the remaining

aqueous phase overlying the interphase and precipitate DNA from the interphase and organic phase with 0.3 ml of 100% ethanol and mix samples by inversion. Next, store the samples at room temperature for 2-3 minutes and sediment DNA by centrifugation at 2,000 g for 5 minutes at 4°C. Remove the phenol-ethanol supernatant and save it at 4°C for the protein isolation. Wash the DNA pellet twice with 1 ml of 0.1 M sodium citrate in 10% ethanol. At each wash, store the DNA pellet in the washing solution for 30 minutes at room temperature with periodic mixing and centrifuge at 2,000 g for 5 minutes at 4°C. Following these two washes, suspend the DNA pellet in 1.5 ml of 75% ethanol, store for 10 - 20 minutes at room temperature with periodic mixing and centrifuge at 2,000 g for 5 minutes at 4°C. Remove the ethanol wash and briefly air-dry the DNA pellet by keeping tubes open for 3 - 5 minutes at room temperature. Dissolve the DNA pellet in 50 µl of 8 mM NaOH by slowly passing through a pipette. Remove insoluble material by centrifugation at 12,000 g for 10 minutes and transfer the resulting supernatant containing DNA to a new tube and then store in -20°C.

Housekeeping gene detection

The house keeping gene β-globin was selected to serve as an internal control for DNA extraction, using conventional PCR as a detection method. Primer sequences for the β-globin gene have been previously described [138]. Briefly, the reaction mixture comprised 2 µl DNA, 0.5 µM of each primer, 10 µl 2.5X Eppendorf masterMix (Eppendorf, Hamburg, Germany), and nuclease-free water to a final volume of 25 µl. The amplification reaction was performed in a thermal cycler (Eppendorf, Hamburg, Germany) under the following conditions for the first round: initial denaturation at 94 ºC for 3 min, followed by 40 amplification cycles consisting of denaturation at 94°C for 30 sec, primer annealing at 60°C for 30 sec, and extension at 72°C for 30 sec, and concluded by a final extension at 72 °C for 7 min.

Human papillomavirus (HPV) detection (Polymerase chain reaction; PCR)

This study divided PCR detection into 3 steps which have specific detail in chapter IV.

- First phase: PCR detection of E1, L1, E6 and previously primer [139-141]

- Second phase: PCR detection for HPV genotyping distribution
- Final phase: Entire genome characterization of HPV16 and HPV18 in samples which have different cytological finding

Briefly, two microlitre of DNA sample was combined with a reaction mixture containing 10 µl of Eppendorf MasterMix (2.5X), 0.5 µM of each primer and sterile distilled water to a final volume of 25 µl. Amplification of the target gene was carried out in an automated thermocycler, Mastercycler personal (Eppendorf, Axygen, USA) under the following conditions: After an initial 3 minutes of denaturation step at 94°C, 40 cycles of amplification were performed, each including 30 seconds of denaturation at 94°C, 30 seconds of annealing at 45-60°C (depends on the Tm of the primers) and 30 -180 seconds (depends on the amplicon length, approximately 30 seconds for 500 bp in length) of extension at 72°C, followed by a final 10 minutes extension at 72°C.

Agarose gel electrophoresis

A total of 10 µl of amplified product was mixed with 5 µl of loading buffer and run on a 2% agarose gel (molecular biology grade) at 100 Volts for 40-60 minutes. After electrophoresis the DNA bands were stained with ethidium bromide for 10 minutes and visualized by UV transillumination.

Purification of amplified product

The PCR products of interest were cut and purified from agarose gel using the Perfectprep Gel Cleanup (Eppendorf). After DNA was stained with ethidium bromide and visualized by UV transillumination, the DNA band of interest was cut and combined with 400 µl of binding buffer. Then incubate the tube at 50°C for 10 minutes until the gel was completely melted. Add 150 µl of isopropanol into the DNA+binding buffer mixture and then load all of the suspension into the spin column. Centrifuge at 8,000 rpm for 1 minute and then discard the flow through. Reassemble the column and then add 750 µl of wash buffer containing ethanol into the column. Centrifuge at 8,000 rpm for 1 minute and then discard the flow through. Reassemble the column and centrifuge at 13,000 rpm for 5 minutes in order to dry the membrane of the column. Finally, the DNA was eluted by adding 40 µl of elution buffer and then centrifuge at 8,000 rpm for 1 minute.

Measurement of DNA / RNA concentrations by UV spectrophotometry

Concentration of the DNA and RNA were determined by measuring the absorption at 260 nm in a UV spectrophotometer (Bio Photometer; Eppendorf). The DNA or RNA was diluted 1:5 or 1:10 by nuclease-free water in a final volume of 50 µl to yield a reliable OD_{260} ranging from 0.2 to 0.8. The concentration of double-stranded DNA was calculated according to the formula: 1 OD_{260} = 50 µg/ml dsDNA. The concentration of single stranded RNA was calculated due to the formula: 1 OD_{260} = 50 µg/ml RNA.

Nucleotide sequencing

The sequencing reaction was performed by using ABI PRISM Bigdye Terminator v3.1 Cycle Sequencing Kit (Applied Bisystems). Briefly, 10 ng of purified DNA or 500 ng of plasmid DNA was combined with 1 µl of 10 µM of sequencing primer, 2 µl of 5X sequencing buffer, 4 µl of Bigdye Terminator v3.1 and sterile water to a final volume of 10 µl. The sequencing reaction was carried out in the Gene Amp PCR System 9600 (Perkin-Elmer) under the following condition: 25 cycles of sequencing amplification, each including 10 seconds of denaturation at 96°C, 5 seconds of annealing at 50°C and 4 minutes of extension at 60°C. After, the sequencing reactions were precipitated and purified, the sequencing products were subjected to detect by a Perkin Elmer 310 Sequencer (Perkin-Elmer) and analyzed by Chromas Lite software (version 2.01). Positive specimens were confirmed by direct sequencing serving as the gold standard and the resulting sequences were submitted to the Genbank database under accession numbers FJ610146-52 and GQ 161244-751. All nucleotide sequences of HPV16 obtained from this study were submitted to the GenBank database under designated accession numbers FJ610146-52 and HPV18 samples were submitted to the GenBank accession numbers GQ180784-92.

Cloning of PCR products with pGEM-T Easy vector

The pGEM®-T Easy Vector Systems (Promega) is a convenient system for the cloning of PCR products. The vector contains a single 3' terminal thymidine (T) at both ends. These single 3´-T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector

and providing a compatible overhang for PCR products generated by certain thermostable polymerases including *Taq, Tfl* and *Tth*. The DNA of interest was amplified based on Polymerase Chain Reaction (PCR) using *Taq* polymerase in order to insert the 3'- A overhangs at both ends of the amplified product for facilitating TA cloning strategy. After electrophoresis and gel purification of the amplified DNA, the concentration of the purified DNA was determined by measuring the absorption at 260 nm in a UV spectrophotometer (Bio Photometer; Eppendorf). Ligation reaction was set up by combining 5 µl of 2X Rapid Ligation Buffer, 50 ng of pGEM-T Easy Vector, 3 weiss units of T4 DNA Ligase, optimized concentration of purified DNA (approximately, the molar ratio of purified DNA: vector at 3:1 was used) and deionized water to a final volume of 10 µl. Mix the reactions by pipetting and then incubate overnight at 4°C. On the next day, transformation was performed by mixing 2 µl of the ligation reaction with 50 µl of thawed competent cells (One Shot TOP10 Chemically Competent *E.Coli*; Invitrogen). Incubate the suspension on ice for 20 minutes and then heat shock the cells for 45-50 seconds at exactly 42°C. After that, immediately, return the tube to ice for 2 minutes and then add 950 µl of SOC medium to recover the cells. Incubate for 1.5 hours at 37°C with shaking (~200 rpm). Plate 100 µl of transformation culture onto duplicate LB plates supplement with 100 µg/ml of amplicillin, 0.5 mM of IPTG and 80 µg/ml of X-Gal. If a higher number of colonies is desired, the cells may be pelleted by centrifugation at 1,000 xg for 10 minutes, resuspended in 200 µl of SOC medium, and 100 µl plated on each of 2 plates. Incubate the plates overnight (16–24 hours) at 37°C and then select white colonies which contain the vector with inserted DNA of interest. Culture the selected colonies in 2 ml of LB broth containing 100 µlg/ml of amplicillin by incubation overnight (16-18 hours) at 37°C. On the next day, plasmids were extracted from the bacterial culture.

Extraction and purification of plasmids DNA from bacterial culture

Plasmid was extracted and purified by using FastPlasmid Mini Kit (Eppendorf). Briefly, 2 ml of bacterial culture containing the plasmid of interest were centrifuged at 8,000xg for 1 minute and then discard the supernatant. After that, add 400 µl of lysis buffer containing enzyme into the cell pellets and mix vigorously by vortexing for 30 seconds. Incubate the tube at room temperature for 3-5 minutes until the suspension

become clear. Pipette all of the suspension into the spin column and centrifuge at 8,000xg for 30 seconds. Discard the flow through and reassemble the spin column. Add 750 µl of wash buffer containing ethanol into the spin column and then centrifuge at 8,000xg for 30 seconds. Discard the flow through, reassemble the spin column and then centrifuge at 13,000xg for 5 minutes in order to dry the membrane of the column. Finally, the plasmid DNA was eluted by adding 50 µl of elution buffer into the membrane and then centrifuge at 8,000xg for 1 minute. Concentration of the plasmid DNA was determined by measuring OD_{260} and then calculated the amount (copies/µl) of the plasmid by using the formula: the concentration of plasmid (g/µl) was divided by lengths (bp) of the recombinant plasmid (length of vector + length of insert), divided by 660 and then by multiplied by $6.02x10^{23}$. The known concentration (copies/µl) of plasmid can be used as a standard DNA for DNA quantitation based on real-time PCR.

Sequence analysis and phylogenetic tree construction

Nucleotide sequences were analyzed and assembled using the Lasergene 6 Package® (DNASTAR, Inc., Madison, WI) and BLAST analysis tool (http://www.ncbi.nlm.gov/BLAST). Complete genome sequences were prepared and aligned using Clustal W applied by the BioEdit program (version 7.0.4.1). Phylogenetic trees were constructed by neighbor-joining analysis executed by the MEGA3 © program [142]. Bootstrapping support for tree topologies was performed using neighbor-joining methods implemented with 1,000 replicates. Genetic distances were calculated applying Kimura's two-parameter method using MEGA3 ©. The rate between dN and dS substitutions was measured by SLAC (Single Likelihood Ancestor Counting) method in Datamonkey elsewhere [143].

Sensitivity and Specificity

DNA cloned as described above served as positive control for HPV detection and determination of test sensitivity. DNA concentration was determined by measuring OD_{260}. DNA samples with known concentrations were 10-fold serially diluted from $10-10^{7}$ copies μl^{-1} and then used as templates for sensitivity tests. The specificity of the PCR assay was evaluated by cross reaction tests with DNA and RNA extracted from Hepatitis

B virus (HBV), Parvovirus 4 (PARV4), Parvovirus B19, Human immunodeficiency virus (HIV) and Hepatitis C virus (HCV)

Multiplex real time PCR primer and probe designation

The following L1 nucleotide sequences of HPV genotypes 16 were downloaded from the GenBank database: NC001526, EU118173, FJ006723, FJ619146-52, GQ 161254, GQ161257, GQ161258, GQ161289, GQ161293, GQ161294, GQ161297, GQ161303, GQ161324, GQ161328, GQ161161333-35, GQ161338, GQ161359, GQ161364, GQ161372, GQ161373, GQ161387, GQ161396-98, GQ161401 and GQ161404. Furthermore, HPV18 and HPV31 nucleotide sequence data were shown following these. L1 nucleotide sequences of HPV genotypes 18 were downloaded from the GenBank database: NC001357, EF202155, GQ161244-47, GQ161249, GQ161296 and GQ161304. L1 nucleotide sequences of HPV genotypes 31 were downloaded from the GenBank database: J04353, GQ161256, GQ161263, GQ161269, GQ161276, GQ161292, GQ161313, GQ161329, GQ161353, GQ161354, GQ161379 and GQ161390. Subsequently, alignments were performed using CLUSTAL X (Version 1.81 from ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX) and BioEdit sequence alignment Software Version 5.0.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Assay target regions were first identified by visual inspection of the sequence alignment. Primers were chosen from constant regions of all specific sequences. Primers and probes were analyzed using the primer design software OLIGOS Version 9.1 and FastPCR Version 3.8.20 (Ruslan Kalendar, Institute of Biotechnology, University of Helsinki, Finland) to predict the percentage of G+C content, potential for dimerization, cross-linking and secondary structure.

Multiplex real-time PCR detection using TaqMan probes

A multiplex real-time PCR was performed using the TaqMan which have sets of primers and TaqMan probes specific for L1 gene of each specific genotype (HPV16, 18 and 31) were used in multiplex format. Each primer and probe for HPV16, 18 and 31 were shown in table 4. Each probe was used at a final concentration of 0.25 μM. HPV16 primer was used at final concentration of 0.5 μM whereas HPV18 and HPV31 were used

at final concentration of 0.75 µM, respectively. A combination of 1 µl of RNA sample with a reaction mixture containing 7.5 µl of 2X TaqMan PCR Master Mix, 4 mM $MgCl_2$, additional 0.3 µl of superscript and DNase-free water was used in a final volume of 15 µl. Multiplex real-time PCR was performed on Rotor-Gene RG-3000 (Corbett Research). Cycling conditions amplification was performed during 40 cycles including denaturation (94°C for 30 seconds), annealing (58 °C for 45 seconds) and extension (72 °C for 1 minute). Multiple fluorescent signals were obtained once per cycle at the end of the extension step with detectors corresponding to the fluorescent dyes labeled in each probe. Data acquisition and analysis of the real-time PCR assay were performed using the Rotor-Gene RG-3000 version 6.0 (Corbett Research).

Quantiation of viral loads or gene expression by real-time PCR

The concentration of DNA was determined by measuring OD_{260}. After the amount of DNA (copies/ µl) was calculated, the DNA was 10-fold serially diluted from 10^8-10 copies/ µl and served as DNA standards to construct a standard curve for quantitation based on real-time PCR. Quantitation of the gene of interest by real-time PCR was carried out in a Rotor Gene RG-3000 (Corbett Research). A multiplex real-time PCR was performed using SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen). The reaction mixture comprised 1 µl of DNA template combined with 7.5 µl of 2X Reaction Mix, 0.3 µl of SuperScript III RT/Platinum® Taq Mix, HPV16 primer at a final concentration of 0.5 µM whereas HPV18 and HPV31 were used at final concentration of 0.75 µM, respectively. Probes were added at a final concentration of 0.25 µM, additional 4 mM $MgSO_4$ and DNase free water in a final volume of 15 µl. Multiplex real-time PCR was performed on Rotor-Gene RG-3000 (Corbett Research). Cycling conditions amplification was performed during 40 cycles including denaturation (94°C for 30 seconds), annealing (58 °C for 45 seconds) and extension (72 °C for 1 minute). Multiple fluorescent signals were obtained once per cycle at the end of the extension step with detectors corresponding to the fluorescent dyes labeled in each probe. Data acquisition and analysis of the real-time PCR assay were performed using the Rotor-Gene RG-3000 version 6.0 (Corbett Research). The threshold cycle represented the refraction cycle number at which a positive amplification reaction was

measured and set at 10 times the standard deviation of the mean baseline emission calculated for amplification cycles 3-15.

CHAPTER IV

RESULTS

Molecular characterization of carcinogenesis genotypes (HPV16 and HPV18)

The HPV samples which have different cytological finding were collected and analyzed by PCR. The entire genome HPV16 and HPV18 primer sequences and positions of each specific primer were summarized in table 5 and table 6, respectively. These primers were designed based on the nucleotide sequences of the previously strains obtained from GenBank database. After amplification, the PCR products were analyzed by agarose gel electrophoresis and then the bands of interest were cut and purified. After that, each purified DNA fragment was subjected to direct sequencing using specific primers in both directions (table 5 and table 6). Finally, the sequences obtained from each fragment were assembled in order to generate full-length sequences for genetic characterization and phylogenetic analysis.

- **Optimization of the PCR conditions**

Polymerase chain reaction was performed to amplify the HPV genome. The reaction mixture comprised 2 µl DNA, 0.5 µM of each primer shown in table 5 and table 6, 10 µl 2.5X Eppendorf masterMix (Eppendorf, Hamburg, Germany), and nuclease-free water to a final volume of 25 µl. The amplification reaction was performed in a thermal cycler (Eppendorf, Hamburg, Germany) under the following conditions: initial denaturation at 94 ºC for 3 min, followed by 40 amplification cycles consisting of denaturation at 94°C for 30 sec, primer annealing at 55°C for 45 sec, and extension at 72°C for 1 min 30 sec, and concluded by a final extension at 72 °C for 7 min. HPV primer positions and PCR products are depicted in table 1 and table 2.

The PCR products were obtained as described above and used for directed sequencing. Determination of the nucleotide sequences was performed in duplicate and analyzed in both directions using forward and reverse primers to ensure that variations of nucleotide sequences were not due to sequencing errors. When a difference was observed, triplicate sequences were determined in order to confirm the consistency of

the sequencing result. Nucleotide sequences were analyzed and assembled using the Lasergene 6 Package® (DNASTAR, Inc., Madison, WI) and BLAST analysis tool (http://www.ncbi.nlm.gov/BLAST). Complete genome sequences were prepared and aligned using Clustal W applied by the BioEdit program (version 7.0.4.1). Phylogenetic trees were constructed by neighbor-joining analysis with the Tamura-Nei model executed by the MEGA3 © program [142]. The rate between dN and dS substitutions was measured by SLAC (Single Likelihood Ancestor Counting) method in Data monkey elsewhere [143]. Rate of non-synonymous/synonymous substitution (dN/dS) refer to selective pressure acting on a protein-coding gene. dN/dS ratio > 1 indicates that this gene evolving under positive selection. All nucleotide sequences of HPV16 and HPV18 obtained in the course of this project were submitted to the GenBank database under designated accession numbers FJ610146-610152 and GQ180784-92, respectively (Addendix B table 13).

Table 1 Conserved primers for whole genome amplification and sequencing of HPV16

Set	Primer name	Sequence (5'-3')	position[a]	Product (bp)
	HPV-E6F1_27	AAA ACT AAG GGC GTV ACC GAA A	27-49	
A	HPV-E6/E7R2_729	CAT CCT CMT CNT CTG AGC TGT	708-729	702, 759
	HPV-E6/E7R2_786	TGG TTC GGC YCG TCK GGC T	767-786	
	F412E6_HPV16	GTA TTA ACT GTC AAA AGC CAC TG	412-435	
B	R1312E1_HPV16	ACA TGG TGT TTC AGT CTC ATG GC	1289-1312	900
	HPV-E1F1_1219	AGT ACA GGT TCT AAA ACG AAA GT	1219-1242	
C	HPV-E1R1_2119	CAT TAT CAA ATG CCC AYT GYA CCA T	2094-2119	900
	F1832E1_HPV16	CAA TGT GTA TGA TGA TAG AGC C	1832-1854	
D	R2915E1_HPV16	AAT AGT CTA TAT GGT CAC GTA GG	2892-2915	1083
	F2529E1_HPV16	CAA TTT AAG AAA TGC ATT GGA TGG	2529-2553	
E	R3551E4_HPV16	GTC TGG CTC TGA TCT TGG TC	3531-3551	1022
	F3387E4_HPV16	GTC AGG TAA TAT TAT GTC CTA CA	387-3410	
F	R4321L2_HPV16	TGC AGA ACG TTT GTG TCG CAT T	4299-4321	934
	F4006E5_HPV16	CTG CTT TTG TCT GTG TCT ACA TA	4006-4029	
G	R5024L2_HPV16	AAG CAG GGT CTA CAA CTT TAA C	5002-5024	1018
	F4930L2_HPV16	AAC TAG TAG CAC ACC CAT ACC A	4930-4952	
H	R6382L1_HPV16	GAT GTA CAA ATA TCC AGT GGA AC	6359-6382	1452
	F6201L_HPV16	GAA CAC TGG GGC AAA GGA TC	6201-6221	
I	R6890L_HPV16	GAA TTC ATA GAA TGT ATG TAT GTC	6866-6890	689
	F6835L_HPV16	CTG TGC AAA ATA ACC TTA ACT GC	6835-6858	
J	R7764L_HPV16	GCC AAA AAT ATG TGC CTA ACA G	7742-7764	929
	F7641L_HPV16	CTG ACC TGC ACT GCT TGC CA	7641-7661	
K	R162E6_HPV16	GCA GCT CTG TGC ATA ACT GTG	141-162	521

[a] Position based on reference sequence NC_001526

Table 2 Conserved primers for whole genome amplification and sequencing of HPV18

Set	Primer name	Sequence (5'-3')	position[a]	Product (bp)
	F15E6_HPV18	CAATTGTAGTATATAAAAAAGGGAGT	15	
A	R901E6_ HPV18	GGATGCACACCACGGACACAC	901	886
	F828E7_ HPV18	CAGACGACCTTCGAGCATTCCA	828	
B	R1683E1_ HPV18	TTTACAGTCTAGACATTGAATATGG	1631	803
	F1612E1_HPV18	AAAGTGATAAAACCACGTGTACAG	1612	
C	R2508E1_HPV18	GTTGCATCATCTAACATGGCCAC	2508	896
	F2458E1_HPV18	TTGGTTGGAACCGTTAACAGATAC	2458	
D	R3393E2_HPV18	AATGTACTTCCCACGTACCTGTG	3393	935
	F3160E2_HPV18	AAAGGTGGCCAAACAGTACAAGTA	3160	
E	R4129E4_HPV18	TGCAATAGTAACATGGGCAAT	4129	969
	F4016E4_HPV18	TATGTGTGCGTATGCATGGGTAT	4016	
F	R4993L2_HPV18	TGTAATTAAAGAGGATGGACGTGT	4993	977
	F4871L2_HPV18	CATTAGTAGTACCCCATTGCCTA	4870	
G	R5824L2_HPV18	CTATATTGGTATGCAGAAACCTTAG	5824	954
	F5757L2_HPV18	AGATTATTAACTGTTGGTAATCCATA	5757	
H	R6668L1_HPV18	TACAGGAGACTGTGTAGAAGCAC	6668	911
	F6538L1_HPV18	GGCTCTATTGTTACCTCTGACTC	6538	
I	R7432L1_HPV18	GGATAAAATGGATGCTGTAAGGTG	7432	894
	F7296L1_HPV18	GTTCTGTGTGTTATGTGGTTGCG	7296	
J	R173E6_HPV18	AGTGTTCAGTTCCGTGCACAGAT	173	734

[a] Position based on nucleotide sequence of HPV18 (X05015)

1. Genome characterization of HPV16

HPV16 are the major cause of cervical cancer, so the entire genome characterizations of samples which have different cytological finding are interesting. This study has been performed on women between 26 and 83 years of age. All seven patients were positive by hybrid capture II and general information of all patients is shown in table 3. All 7 samples had shown positive results on application of a commercially available test kit (hybrid capture II) except for CU3 and CU6 which had yielded abnormal Pap smear detected by a cytopathologist. Whole genome sequences of HPV found in Thai samples (FJ610146-52) were aligned with HPV16R [144], K02718, EU118173, AF125673, U89348, FJ006723, AF536180 (African type1), AF534061 (East Asian), AF536179 (European German type), AF472508 (African type 1), AF472509 (African type 2), AY686580 (European), AY686581 (European), AF402678 (Asian-American) and AY686579 (Asia-American). The reference sequences AF402678 and AY686579 can be classified as Asian-American type which originated from Costa Rica [145]. Analysis of HPV nucleotide sequences showed 97.9-99.8 % similarity among the Thai and all reference sequences (data not shown). Phylogenetic analysis revealed that HPV16 in Thailand were closely related to the reference strain of HPV16 (Figure 20). The European-German type (AF536179) is related to CU1 (normal) and CU7 (cervical cancer; CA) samples, whereas the reference sequence AF125673 isolated from North America is closely associated with CU2 (atypical squamous cells of undetermined significance; ASC-US) and CU3 (atypical squamous cells cannot exclude HSIL; ASC-H) samples. The CU4 (cervical intraepithelial neoplasias grade1; CIN I) sample is related to the reference sequence (HPV16R) and European strain (AY686581), while CU5 (cervical intraepithelial neoplasias grade2; CIN II) is closely related to the East-Asian type (AF534061). Furthermore, CU6 (cervical intraepithelial neoplasias grade3; CIN III) is separate from all reference strains, while the African type-1,-2 and Asian-American type are not related to any samples (Figure 11).

Table 3 General data and clinical characteristics of HPV16 patients

Sample code	Age	Cervical Cytology	Hybrid capture II			Accession number	Length (bp)
			Relative light unit	Positive cut off	ratio		
CU1	26	NORMAL	3,415	552.67	6.18	FJ610146	7,906
CU2	27	ASC-US	43,670	843.33	51.78	FJ610147	7,906
CU3	31	ASC-H	ND	ND	ND	FJ610148	7,906
CU4	35	CIN I	590,822	1234	478.79	FJ610149	7,906
CU5	26	CIN II	32,019	507	63.15	FJ610150	7,905
CU6	37	CIN III	ND	ND	ND	FJ610151	7,906
CU7	83	CA	624,377	895	697.63	FJ610152	7,905

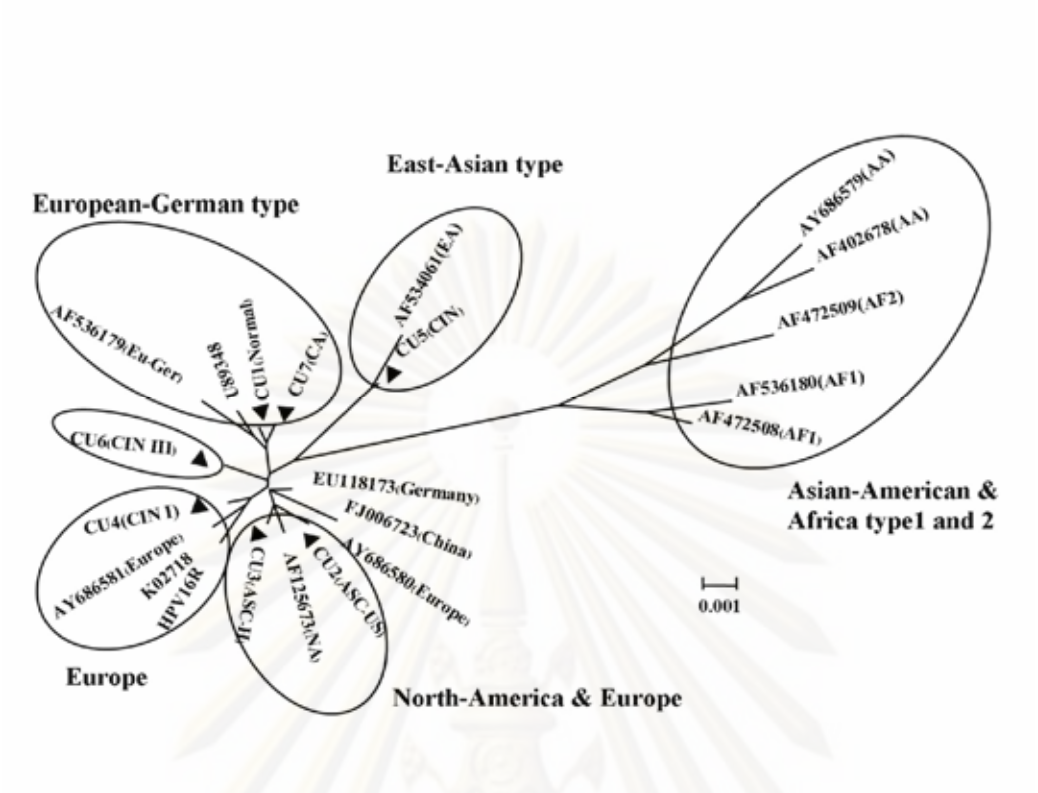


Figure 11 Phylogenetic tree based on whole genome analyses of seven samples and reference sequences from GenBank database. Phylogenetic tree construction by neighbor-joining analysis with the Kimura model implemented in the MEGA© program (version 3.1).

- **Characterization of the non-coding and coding genes of HPV16**

1. Nucleotide sequence variation in the non-coding region

Nucleotide variations in the HPV 16 genome were observed in the long control region (LCR) at positions 1-83 and 7155-7905 and upstream regulatory region of the L2 gene, positions 4102 to 4235 (position based on reference sequence; HPV16R). Most nucleotide variations of HPV16 variants can be found at up-stream regulatory L2. With each variant, we observed both insertion and deletion between the early and late gene (data not shown), including the samples from this study. The complete genome of CU5 and CU7 comprised 7,905 bp, whereas other samples consisted of 7,906 bp indicating variable genome lengths of HPV16 isolated from Thai women. As for LCR variation, a previous study had focused on the LCR segment spanning from 7480 to 7843 and

reported that nucleotide variations in this segment were usually found in Asian-American variants [146, 147]. The results of our study showed that this segment was conserved in all samples, except for position 7,842 in CU5 which is related to the EA strain. Whereas, nucleotide variations at positions 7,193 and 7,521 most pronounced in HPV16R (figure 12) [148]. Most of our samples were variation at these positions. Nucleotide alterations at position 7,521 lead to mutations of YY1 which in turn contribute to progression of cervical cancer [148, 149]. Based on the pattern of nucleotide variation, all samples displayed more similarity to the European, European-German and East-Asian type than to the African type-1,-2 and Asian-American type (South America).

			LCR													
			7	7	7	7	7	7	7	7	7	7	7	7	7	7
		HPV16	1	3	3	4	4	4	5	6	6	6	7	7	8	8
		Class/	9	9	9	6	8	8	2	0	2	8	8	9	2	4
	CYTOLOGY	Isolate	3	4	5	9	5	9	1	7	1	9	6	2	6	2
HPV16R			G	C	C	T	A	G	G	T	A	C	C	C	G	G
CU1	Normal		T	T	-	-	-	-	A	-	-	-	-	-	-	-
CU2	ASC-US		T	-	-	-	-	-	A	-	-	-	-	-	-	-
CU3	ASC-H		T	-	-	-	-	-	A	-	-	-	-	-	-	-
CU4	CIN I		-	-	-	-	-	-	A	-	-	-	-	-	-	-
CU5	CIN II		T	-	-	-	-	-	A	-	-	-	-	-	-	A
CU6	CIN III		T	-	-	-	-	-	A	-	-	-	-	-	-	-
CU7	CA		T	-	-	-	-	-	A	-	-	-	-	-	-	-
AY686580		E	T	-	-	-	-	-	A	-	-	-	-	-	-	-
AY686581		E	-	-	-	-	-	-	-	-	-	-	-	-	-	-
K02718		E	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AF125673		NA	T	-	-	-	-	-	A	-	-	-	-	-	-	-
EU118173		E	T	-	-	-	-	-	A	-	-	-	-	-	-	-
AF536179		EG	T	-	-	-	-	-	A	-	-	-	-	-	-	-
FJ006723		China	T	-	-	-	-	-	A	-	-	-	-	-	-	-
AF534061		EA	T	-	-	-	-	-	A	-	-	-	-	-	-	A
AY686579		AA	T	T	T	-	C	A	A	-	-	A	T	-	-	-
AF402678		AA	T	T	T	-	C	A	A	-	-	A	T	-	-	-
AF472508		AF1	T	-	-	-	-	A	A	-	-	A	T	-	-	-
AF472509		AF2	T	-	-	-	C	A	A	-	-	-	T	-	A	-
Binding site						GRE-1	Oct-1 TEF-1 YY1	YY1 GRE-2				YY1 YY1 YY1 TEF-1	TEF-1	YY1 YY1 YY1	TEF-1	OCT-1 YY1

Figure 12 Nucleotide variations of LCR among HPV16 from our study and GenBank database. Nucleotide positions are written vertically across the top. The binding sites for transcription factors affected by substitutions appear at the bottom. GRE, Oct, TEF and YY represent glucocorticoid response element, octamer binding factor, transcriptional enhancer and Yin-Yang factor, respectively.

2. Amino acid variations in the coding region

Amino acid analysis showed that the E4 gene was more conserved than any other gene. In contrast, we observed pronounced amino acid variations in E2, E1, E6, E5 and E7, respectively (data not shown). The nucleotide variations at E6, E7, E2, L1 and L2 detected in our samples showed in figure 13A whereas nucleotide variation which resulted in non-synonymous amino acids showed in figure 3B. The protein displaying most nucleotide variations was L2 (figure 13A) while most non-synonymous amino acids were detected in E2 (figure 13B). As shown in figure 20B, CU5 (CIN II), CU6 (CIN III) and CU7 (CA) have experienced more pronounced amino acid variations than CU1 (normal), CU2 (ASC-US), CU3 (ASC-H) and CU4 (CIN I). Based on alignment of the amino acid sequences encoded by E2, E6, E7, L1 and L2, we highlight only essential positions as described elsewhere [150]. In the E6 region, positions 83 of CU2, CU3 and CU6 changed from E6-83L to E6-83V (L83V; prototype to variant) and in the E2 region, position 219 of CU1, CU2, CU3, CU5 and CU6 changed from E2-219P to E2-219S (P219S) while position 219 of CU7 was translated to “T” (P219T). Based on variation, the rate of non-synonymous/ synonymous (dN/dS) were found only in E2 (dN/dS = 1.26). Furthermore, in the L2 region positions 243 of CU2 and CU3 changed from L2-243V to L2-243I (V243I). In the same region, positions 269 of CU1, CU5 and CU7 changed from L2-269S to L2-269P (S269P), while position 269 of CU6 translated to “D” (S269D). In the L1 region at position 266 of all samples changed from L1-266T to L1-266A (T266A) (figure 13B). Subsequently, we performed phylogenetic tree analysis based on the E2, E6, L1 and L2 genes (Figure 14). The results thus obtained indicated that the European German type is closely related with CU1 and CU7 whereas CU4 and CU5 are more closely associated with the European and East Asian type, respectively. Moreover, similarity of CU2, CU3 and CU6 depended on the gene examined for comparison. Still, they are related to the North-American and European type. Finally, figure 14 shows that E2, E6, L1 and L2 of all samples are distinct from the Asian-American type and the African type-1 and -2. Alignment of the vaccine strain’s (US Patent 6613557) polypeptide with all our samples has demonstrated 99.99-100% homology and phylogenetic tree has performed like figure 14C.

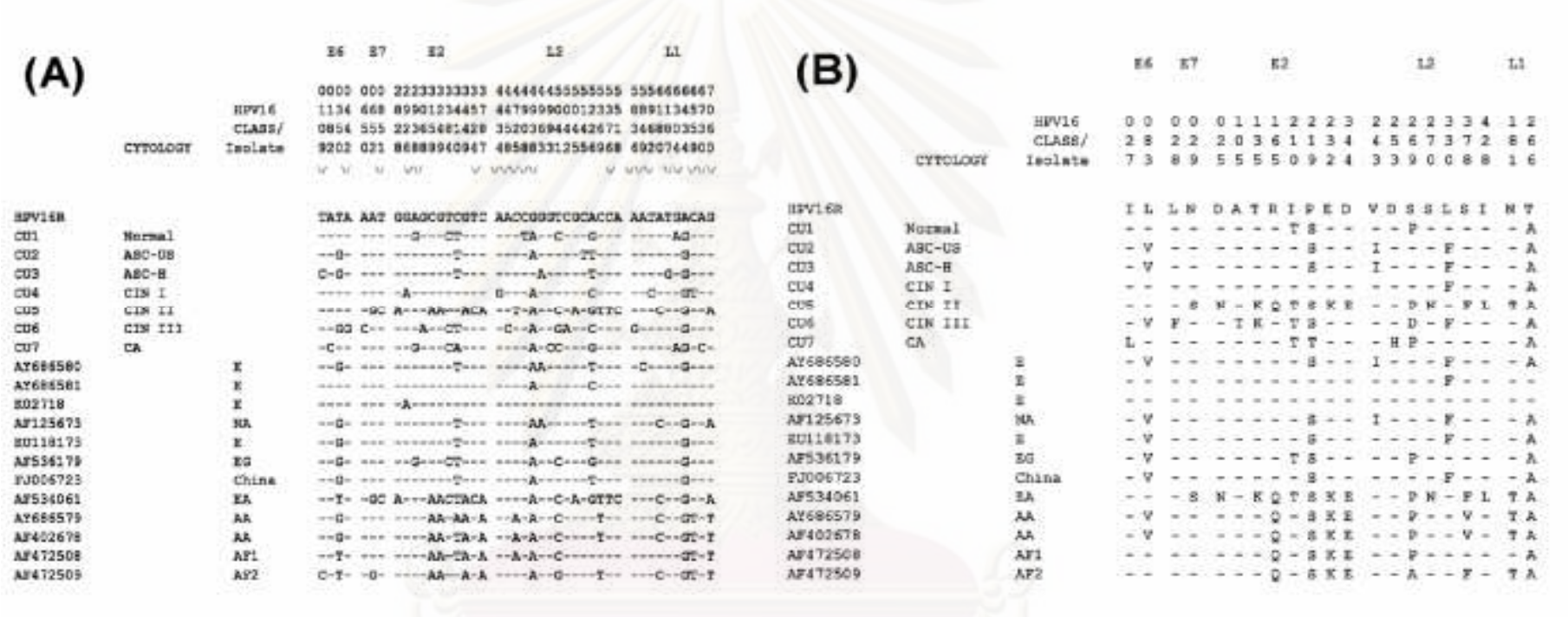


Figure 13 Nucleotide and amino acid variations among HPV16 from our study and GenBank database with special emphasis on E6, E7, E2, L2 and L1. Nucleotide and amino acid positions of E6, E7, E2, L2 and L1 are written vertically across the top. Panel (A) shows all nucleotide variation of E6, E7, E2, L2 and L1 detected in this study. V indicated that nucleotide change resulting in amino acid not change (synonymous) whereas Nucleotide change resulting in amino acid change (non-synonymous) from panel (A) were performed in the panel (B).

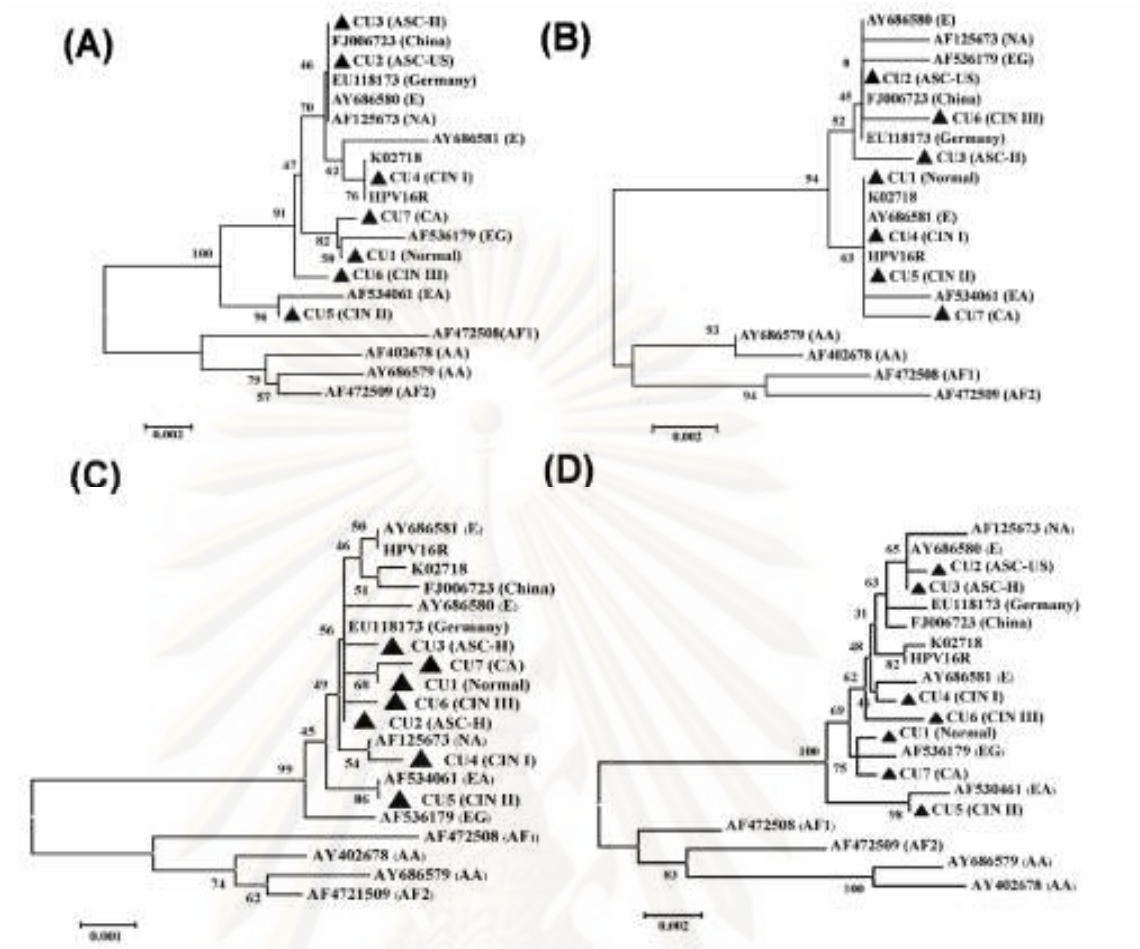


Figure 14 Phylogenetic tree construction by neighbor-joining analysis with the Kimura model implemented in the MEGA© program (version 3.1). Bootstrapping support for tree topologies was accomplished using neighbor-joining methods implemented with 1,000 replicates. Genetic distances based on neighbor-joining phylogenetic trees were calculated applying Kimura's two-parameter method using MEGA3 ©.(A), (B), (C) and (D) Phylogenetic tree of HPV16 with special emphasis on the E2, E6, L1 and L2 genes, respectively.

Conclusion, seven samples of HPV16 in infected women ranging from normal to cervical cancer are discovered two critical non-synonymous changes within the coding region converting the E2-219P prototype to E2-219T in cervical cancer and the L2-269S prototype to L2-269D in CIN III, respectively. Phylogenetic analysis based on the whole genome with special emphasis on the genes E2, E6, L1 and L2 showed the Thai

samples to be more closely related to the European than the non-European strains. The vaccine strain's L1 polypeptides showed close relationship to our samples.

2. Genome characterization of HPV18

- **Characterization of the non-coding and coding genes of HPV18**

1. Nucleotide sequence variation in the non-coding region

Nine HPV18 positive samples (CU8-CU16) displayed different cytology results such as normal (CU8-CU9), LSIL (CU10-CU11), HSIL (CU12-CU13), squamous cell carcinoma (CU14-CU15) and adenocarcinoma (CU16). HSIL and cervical cancer (CU15-CU16) samples have histological outcome to confirm the cytological result where as normal and LSIL samples lacked of histological outcome. This study has been performed on women between 19 and 70 years of age (table 4). All 9 samples had shown positive results on application of a commercially available test kit (hybrid capture II) except for CU14 and CU16 which had yielded abnormal Pap smear detected by a cytopathologist. Whole genome sequences of HPV18 found in Thai samples (accession numbers GQ180784-92) were aligned with X05015 [144], NC001357, Y262282, EF202152-EF202155 (African type), EF202147-EF202151 (European type) and EF202143-EF202146 (Asian-American type). Analysis of HPV nucleotide sequences showed 97.9-99.9 % similarity among the Thai and all reference sequences (data not shown). Phylogenetic analysis revealed that HPV18 in Thailand were closely related with European and Asian-American types (Figure 15).

The complete genome of CU11 comprised 7,844 bp, whereas other samples consisted of 7,857 bp indicating variable genome lengths of HPV18 isolated from Thai women. We observed deletion in the LCR (7245-7256) and E4 gene (position 3630-3635) of CU11 samples (data not shown). These deletions occurred in the African type and phylogenetic tree analysis showed CU11 were closely with the African type whereas other samples were related with the European and Asian-American type (Figure 15).

Table 4 General data and clinical characteristics of HPV18 patients

Sample code	Age	Cervical Cytology	Hybrid capture II			Accession number	Length (bp)
			Relative light unit	Positive cut off	ratio		
CU8	29	NORMAL	8,665	507	17.09	GQ180784	7,857
CU9	32	NORMAL	966	427	2.05	GQ180785	7,857
CU10	19	LSIL	767,331	770	96.53	GQ180786	7,857
CU11	26	LSIL	7,696	477.33	16.12	GQ180787	7,844
CU12	29	HSIL	46,212	477.33	96.81	GQ180788	7,857
CU13	45	HSIL	477,446	843.33	566.14	GQ180789	7,857
CU14	70	SQUAMOUS CELL CARCINOMA	ND	ND	ND	GQ180790	7,857
CU15	ND	SQUAMOUS CELL CARCINOMA	11,651	321	36.30	GQ180791	7,857
CU16	55	ADENOCARCINOMA	ND	ND	ND	GQ180792	7,857

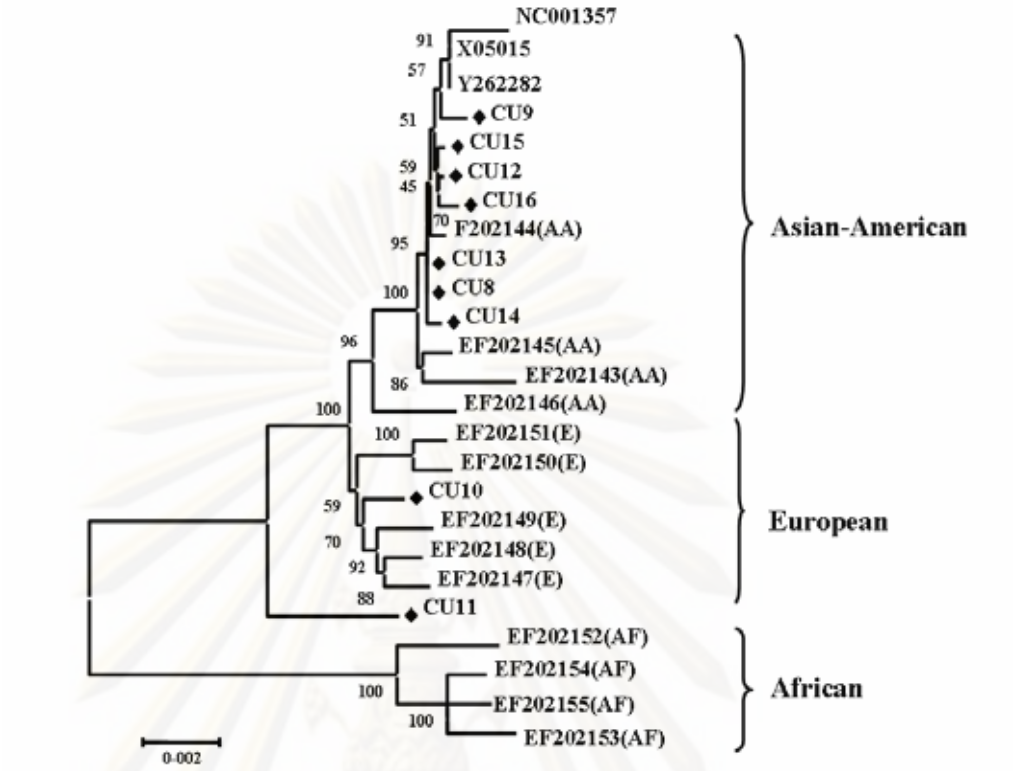


Figure 15 Phylogenetic tree based on whole genome analyses of nine samples and reference sequences from GenBank database. Phylogenetic tree construction by neighbor-joining analysis with the Kimura model implemented in the MEGA© program (version 3.1).

Based on nucleotide variation, we focused on the LCR segment at positions 41, 104 and 7,726. A previous study had reported that nucleotide variations in this segment should affect transcription factor binding sites such as Sp1 (selective promoter factor 1), YY1 (Yin-Yang factor 1) and Oct-1 (octamer binding factor), respectively [151]. Based on our results nucleotide variations at these critical positions occurred with 2 samples (CU10 and CU11). Nucleotide variation of CU10 changed both positions 41(A to G) and 104 (T to C), whereas the nucleotide sequence of CU11 changed at positions 104 (T to C) and 7726 (C to T). Variation of nucleotides 41 and 104 was associated with a higher activity of the E6/E7 promoter by modulating Sp1 and YY1 activities [152].

2. Amino acid variations in the coding region

Based on amino acid analysis, the E6 region, position N129K had not undergone amino acid variation in our samples whereas in CU10 and CU11, the L1 region, position T149N was identical to the African and European types. The amino acid at position 129 is highly conserved within HPV18. Amino acid alteration at position 149 of L1 is commonly found in many HPV genotypes and is located near the surface of the capsid protein [153]. Alignment of the vaccine strain's (US Patent 5820870) polypeptide with all our samples has shown that the L1 vaccine strain is closely related to the African type, whereas most of our samples' sequences are related to the Asian-American and European type.

Conclusion, Phylogenetic analysis based on the whole genome of HPV18 samples are more closely related to the European and Asian-American type than the African type. The vaccine strain's L1 nucleotide (US Patent 5820870) showed close relationship to the African type. However, our data can not indicate the correlation between cytological data and nucleotide or amino acid variation.

Developmental HPV detection method and HPV screening

In this study, molecular diagnostic assays were divided into 3 studies. First, molecular detection based on conventional PCR was developed, examining the correlation between HCII ratio (RLU/CO) and results obtained by PCR amplification using previous primer sets (MY09/MY11 and GP5+/GP6+) and by PCR amplification of the E1, E6 and L1 genes from samples with different cytological findings. Second, HPV prevalence detection focused on HR-HPV and additional genotype of probable HR-HPV and LR-HPV (especially the genotypes 6 and 11), identifying the genome sequence of HPV types present in Thailand. Finally, a multiplex real-time PCR method was established for the detection of high prevalence HPV genotypes in Thai women.

1. Correlation between Hybrid Capture II ratio and PCR amplification of the E1, E6 and L1 genes for HPV detection from samples with different cytological findings

The aim of this study was to develop a rapid, cost-saving and effective PCR-based method for HPV detection. The primers used in the PCR reaction followed the

general guidelines for primer design (Appendix A). The study focused on HPV detection using E1, L1 and E6 as the target.

Using the nucleotide sequences of high-risk HPV (HR-HPV); HPV16 HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV68, HPV70, HPV73 and HPV82. In addition, the nucleotide sequence of probably high-risk HPV (PHR-HPV); HPV26, HPV53 and HPV66 and low-risk HPV (LR-HPV); HPV6 and HPV11 were used in this study. The nucleotide sequences were downloading from GenBank database (as mentioned in chapter III). Multiple sequence alignments of all nucleotide sequences were performed using the CLUSTAL X program (version 1.8). The most conserved regions of the E1, L1 and E6 genes were selected and analyzed using the OLIGO primer design software (version 9.1). The previously primer sets MY09/MY11 and GP5+/GP6+ were used as standardize for this study (table 5). DNA extracted from specimens was used as template for amplification by conventional Polymerase Chain Reaction (PCR) using specific primers for each target gene. The sequences and positions of each specific primer were summarized in Table 5. These primers are designed based on the nucleotide sequences of the previously strains obtained from the GenBank database. After amplification, the PCR products were analyzed by agarose gel electrophoresis and then the bands of interest cut out and purified. After that, each purified DNA fragment was subjected to direct sequencing using specific primers in Table 5.

Construction of positive controls for PCR of E1, L1 and E6 regions

The E1, L1 and E6 genes of high-risk HPV, probably high-risk HPV and low-risk HPV were amplified from clinical specimens and used to construct HPV plasmids DNAs by inserting genes into the pGEM-T Easy Vector (Promega) as described in Chapter III and this chapter figure 16. The E1, L1 and E6 gene plasmids of HPV16 were used as a positive control for optimization of the PCRs (Figure 17). The concentration of DNA was calculated by measuring absorbance at 260 nm. The DNAs were then serially diluted ten-fold, ranging from 10^7-10 copies/μL to perform sensitivity tests.

Table 5 Conserved primers specific for E6, E1 and L1 for amplification and sequencing of HR-HPV and some additional PHR-HPV and LR-HPV

Gene	Round	Primer name	Sequence (5'-3')	[a]position	Product (bp)
E6	1	HPV-E6F1_27	AAA ACT AAG GGC GTV ACC GAA A	20-41	919
		HPV-E6R1_1019	CAC TAC AGC CTC HAC NDN AAA CCA	916-939	
	2	HPV-E6F1_27	AAA ACT AAG GGC GTV ACC GAA A	20-41	650, 707
		HPV-E6/E7R2_729	CAT CCT CMT CNT CTG AGC TGT	650-670	
		HPV-E6/E7R2_786	TGG TTC GGC YCG TCK GGC T	708-727	
E1	1	HPV-E1F1_1219	AGT ACA GGT TCT AAA ACG AAA GT	1110-1132	855
		HPV-E1R1_2119	CAT TAT CAA ATG CCC AYT GYA CCA T	1941-1965	
	2	HPV-E1F2_1383	GCGAAGACAGCGGNTATGGC	1249-1268	716
		HPV-E1R1_2119	CAT TAT CAA ATG CCC AYT GYA CCA T	1941-1965	
L1	1	HPV-L1F1_6153	CGT TTT CCA TAT TTT TTT HCA GAT G	5615-5639	1008
		HPV-L1R1_7162	TAG TTG GTT ACC CCA ACA AAT RCC ATT	6596-6623	
	2	HPV-L1F1_6153	CGT TTT CCA TAT TTT TTT HCA GAT G	5615-5639	630
		HPV-L1R2_6804	AGT ATC TAC CAT ATC MCC ATC TT	6223-6245	
[b] MY	1	MY11	GCM CAG GGW CTA TAA YAA TGG	6582-6601	451
		MY09	CGT CCM ARR GGA WAC TGA TC	7014-7033	
[b] GP	2	GP5+	TTT GTT ACT GTG GTA GAT ACT AC	6624-6641	136
		GP6+	GAA AAA TAA ACT GTA AAT CAT ATT C	6741-6760	

[a] Position based on reference sequence NC_001526

[b] Primer based on reference data [138-140]

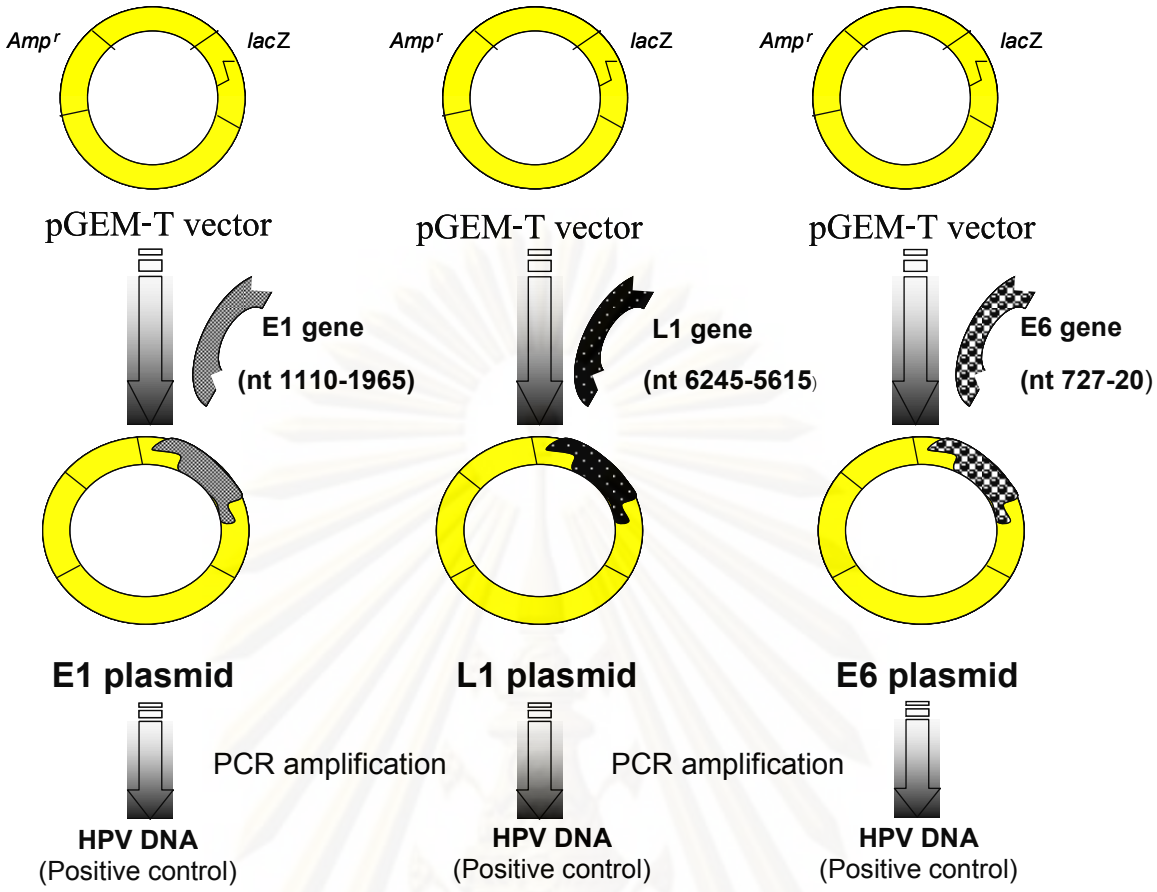


Figure 16 Schematic diagrams represent the construction of positive controls for HPV E1 gene, HPV L1 gene and HPV E6 gene (modified from previous thesis [154]).

- **Optimization of the PCR conditions**

In order to optimized the conditions for efficient PCR amplification, several Factors, including primer concentration (0.25 - 0.75μM), additional Mg^{2+}concentration (ranging from 0.5 – 3.0 μM) and primer annealing temperatures (53, 55, 58 or 60°C) were calculated optimal condition by using HPV DNA plasmids contains the E1, L1 and E6 as a positive control.

The first phase of PCR HPV detection was focused on E1, L1 and E6 genes. The presence of HPV samples were determined using the primer sets employed for large scale HPV testing (MY09/MY11), followed by a nested PCR using the primer set specific for the L1 gene described in a previous publication (GP5+/GP6+) [139-141] and our primer sets specific for E1, L1 and E6 (Table 1). The reaction mixture comprised 2 μl of DNA template, 0.5 μmol of each primer shown in table 1, 10 μl of 2.5X MasterMix

(Eppendorf, Hamburg, Germany), and nuclease-free water to a final volume of 25 µl. The amplification reactions of the first and second round were performed in a thermal cycler (Eppendorf, Hamburg, Germany) under the following conditions (except for L1 amplification using MY09/MY11 and GP5+/GP6+ which was performed as previously reported): initial denaturation at 94 ºC for 3 min, followed by 40 amplification cycles consisting of denaturation at 94°C for 30 sec, primer annealing at 55°C for 45 sec, and extension at 72°C for 1 min 20 sec, and concluded by a final extension at 72 °C for 7 min. The PCR products sizes are shown in table 1. The entire PCR product was added to loading buffer and run on a 2% agarose gel in TBE at 100 Volts for 60 minutes. After electrophoresis, the DNA bands were stained with ethidium bromide and visualized by UV transillumination. All PCR products were confirmed by direct sequencing.

- **Interpretation of the band pattern from PCR**

The oligonucleotide primer sets summarized in Table 1 were tested with HPV samples in PCR. The samples, which have PCR amplification product 716 bp is interpreted that positive E1 gene detection, 650 or 707 are interpreted as positive E6 gene detection, 630 is interpreted as positive L1 gene and MY09/MY11 and GP5+/GP6+ detection showed positive band about 136 bp (table 5).

- **Sensitivity and specificity of HPV detection**

This project has been aimed at establishing the correlation between HCII ratio (RLU/CO) and PCR amplification of the L1 gene using primer sets MY09/MY11 and GP5+/GP6+ or PCR amplification of L1, E1 and E6 using our primer sets from samples with different cytological findings. Specificity and sensitivity of PCR amplification were analyzed and the results confirmed that neither of the primer sets (Table 5) cross-reacted with HIV, Parv4, Parvovirus B19, HCV or HBV. As for HCII sensitivity, RLU/CO $\geq$ 1 (equivalent to 1 pg ml^{-1}) was interpreted as HCII positive. The sensitivity of the PCR assay was evaluated by ten-fold serial dilution of the standard HPV DNA of E1, L1 and E6. As expected, intensities of DNA bands decreased with increased dilution of the DNA standards. Sensitivity of the primers for E1 was determined at 10^2 copies μl^{-1} (figure 17),

L1 gene was determined at 10^3 copies μl^{-1} (data not shown) whereas MY09/MY11 and GP5+/GP6+ and E6 were determined at 10^4 copies μl^{-1}(data not shown).

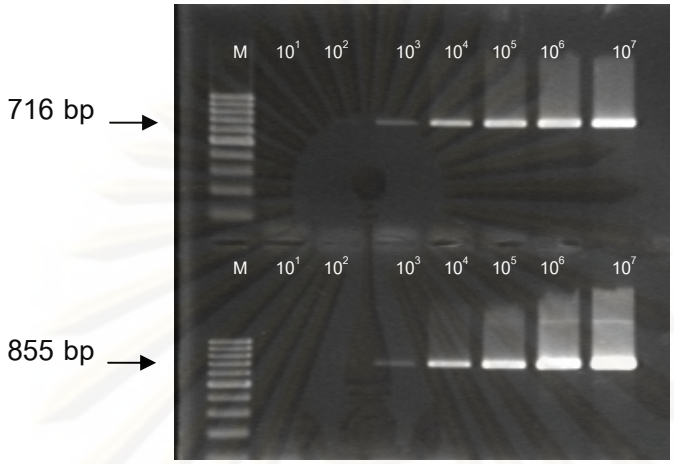


Figure 17 Human papillomavirus genotype 16 was used as positive control for sensitivity detection. The sensitivity of E1 PCR detection was evaluated by ten-fold serial dilutions ranging from 10^7 – 10^1 copies/μL of the DNA standard. DNA dilutions were as indicated on top of the lanes. Lane M: 100-bp ladder. The lower picture represented PCR1 amplification product of HPV E1 genes approximately 855 bp and the upper picture represented PCR2 (semi-nested PCR) amplification product approximately 716 bp.

- **HCII ratios and PCR detection**

All samples in this study had been tested by HCII. The ratio of HCII represented the relative light unit divided by positive cut off (RLU/CO), with a ratio <1 indicating negative HCII and a ratio $\geq$ 1 indicating positive HCII. However, a previous study has reported that HCII had a grey zone between 0.4 and 4 [155]. This grey zone refers to results between borderline negative and positive which are difficult to interpret whereas RLU/CO < 0.4 is interpreted as negative. Thus, altogether 243 samples were tested in this experiment and the samples were divided into three groups with RLU/CO ratios of <0.4 (n=21), 0.4-4 (n=64) and exceeding 4 (n=158), respectively. All 20 samples with RLU/CO <0.4 proved HCII negative and displayed normal cervical cytology, except for

1 HCII negative sample which displayed ASC-US but tested negative for E1, E6, L1 and likewise, with the previous primer sets. Of those 222 samples which RLU/CO > 0.4, 109 (49.10%), 143 (64.41%), 106 (47.73%) and 102 (45.59%) samples were amplified by MY09/MY11 and GP5+/GP6+, E1, L1 and E6 primer sets, respectively (table 6). The PCR result indicated that E1 primers yielded higher amplification efficiency than other primer sets in this study. In addition, all HPV E1 gene nucleotide sequences obtained from this study were submitted to the GenBank database under designated accession numbers FJ610146-52, GQ161244, GQ161246, GQ161248-50, GQ161253-83, GQ161285-359, GQ161361-66, GQ161368-81, GQ161384-89, GQ161396, GQ161609 and GQ161629. HCII positive samples were divided into two groups with ratios of 0.4-4 (n=64) and exceeding 4 (n=158). As the E1 primers yielded the highest percentage of detection, positive PCR results refer to the E1 product (table 6). The correlation between HCII ratio, PCR amplification of L1 according to the previous report and PCR amplification of L1, E1 and E6 using our primer sets is depicted in table 6. HCII ratios exceeding 4 can be detected by E1 primers at 79.74% whereas HCII ratios of 0.4-4 can be detected by E1 primers at 26.56%. At all HCII ratios, the percentage results obtained with our primer sets for E1 were higher than those obtained by the primers from previous studies [139-141] (table 6). Comparison of our results for L1, E1 and E6 revealed the highest percentage of amplification upon employing the E1 primer set. Furthermore, this study focused on the amplification detection upon using individual primer sets (data not shown). The result demonstrated that the percent PCR amplification is directly proportional to the HCII ratio. Irrespective of HCII ratio, some samples could not be amplified using the MY09/MY11 and GP5+/GP6+ primers whereas they could be detected with our primer sets (data not shown).

Table 6 HPV detection based on HCII ratio (RLU/positive cut off) and PCR using our primers (E1, L1 and E6 gene primers) and primers previously published (MY09/MY11 and GP5+/GP6+)

		Negative ratio (RLU/CO)	Number of samples classified by ratio (RLU/CO) of HCII (samples)		
		<0.4	0.4-4	>4	Total number
	Total	21	64	158	222
PCR categories	MY09/my11 and GP5+/GP6+	-	11 (17.18%)	98 (62.03%)	109 (49.1%)
	E1	-	17 (26.56%)	126 (79.74%)	143 (64.41%)
	L1	-	7 (10.94%)	99 (62.66%)	106 (47.73%)
	E6	-	5 (7.81%)	97 (61.39%)	102 (45.95%)
	Any primers	-	17 (26.56%)	131 (82.91%)	143 (64.41%)

- **Cytological data and grey zone analysis**

Cytology revealed that samples which HCII ratio >4 were usually found in all cytological category groups, especially in LSIL (32%), ASC-US (28%) and normal (26%) (figure 18). HPV samples with HCII ratio of 0.4-4 (known as the grey zone) displayed normal cytology at the highest percentage (77%) with ASC-US and LSIL coming second at 9% each (Figure 18B). According to the manufacturer's guidelines for HCII, the cut-off for negative samples is below 1.00. In this study 9 samples displayed HCII ratio of 0.4-0.99 and therefore classified as negative. However, HPV was detected in 2 samples by using E1 primers (2/9; 22.22%). One sample was diagnosed as ASC-H, whereas cytological data was absent for the other sample. All HCII negative specimens had normal cytology. In addition, our study focused on the proportion between positive and negative samples within the grey zone detected by E1 primers. The proportions

between positive and negative samples at ratios 1-1.99, 2-2.99 and 3-3.99 were 4/31 (12.90%), 7/18 (38.89%) and 4/6 (66.67%), respectively (figure 19A). Furthermore, samples within the grey zone can be interpreted as HCII positive yet display normal cytology data indicating that these patient groups required for HPV detection follow up in the future (figure 19B).

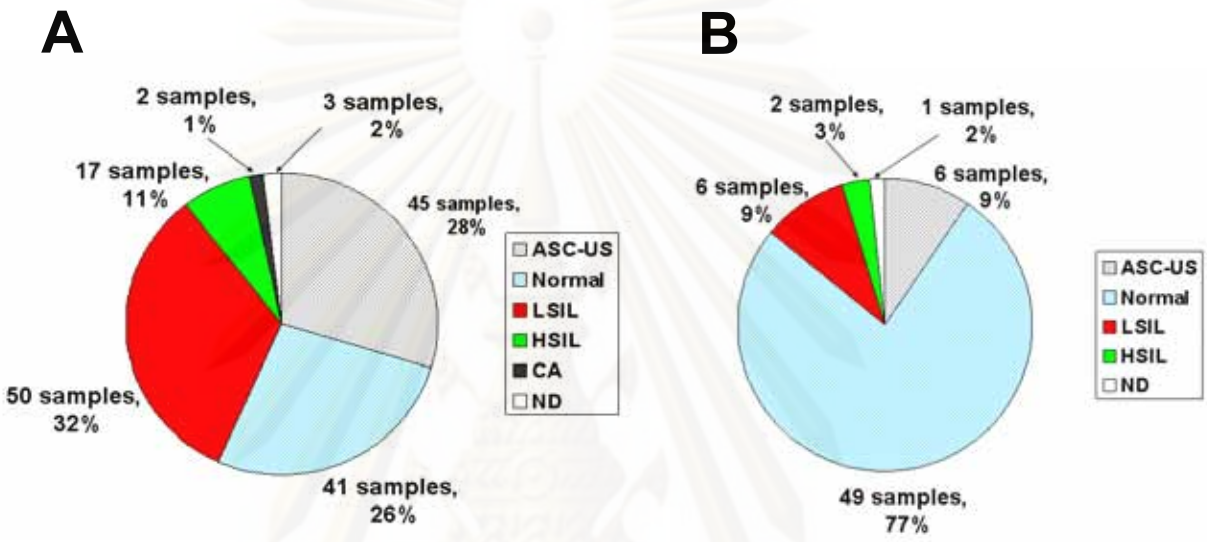


Figure 18 HPV cytology of samples with RLU/CO >4 (A) and samples with RLU/CO 0.4-4 (B). Samples were classified as follows: ASC-US (atypical squamous cells of undetermined significance), LSIL (low-grade squamous intraepithelial lesion), HSIL (high-grade squamous intraepithelial lesion), CA (cervical cancer) and ND (no data).

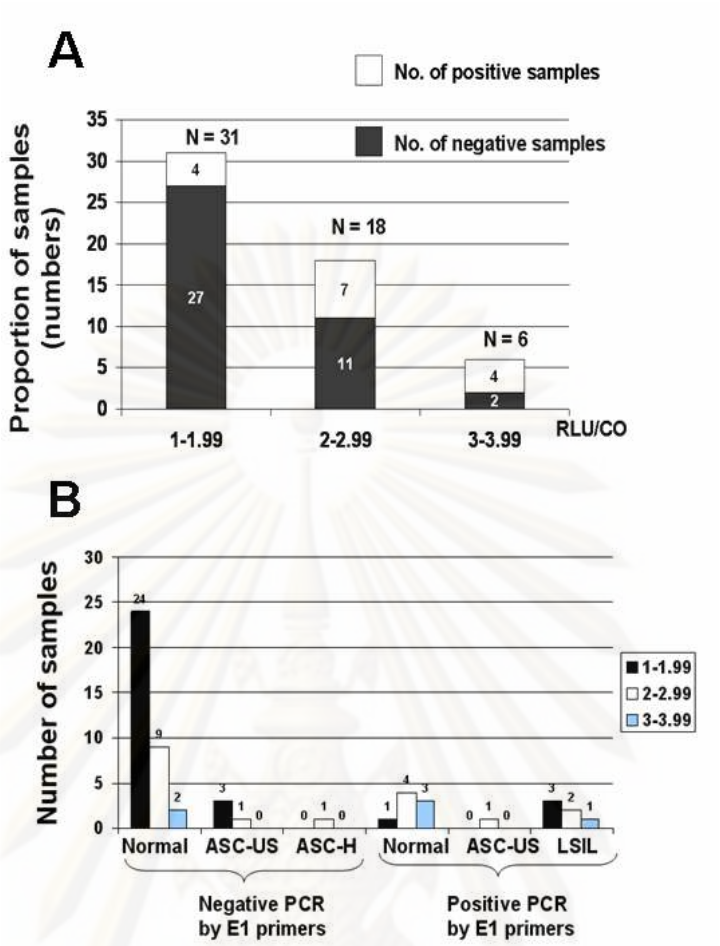


Figure 19 Proportion between positive and negative samples detected by using E1 primers at an RLU/CO range of 1-1.99, 2-2.99 and 3-3.99. Positive samples are depicted on top (A). Classification of cytological data compared with numbers of positive and negative PCR by using E1 primer at an RLU/CO range of 1-1.99, 2-2.99 and 3-3.99 (B).

Conclusion, the correlation between HCII ratio (RLU/CO) and PCR amplification using previous primer sets (MY09/MY11 and GP5+/GP6+) and the HPV E1, L1 and E6 genes primers from this study with different cytological findings samples. Altogether 243 samples were divided into three groups with RLU/CO ratios of <0.4 (n=21), 0.4-4 (n=64) and exceeding $\geq$4 (n=158), respectively. PCR amplification with the E1 primers proved more sensitive than with previously primers (MY09/MY11 and GP5+/GP6+), E6 or L1 primers. The E1 assay can be used for HPV detection with sensitivity of 10^2 copies μl^{-1}.

Samples with RLU/CO exceeding 4 and grey zone samples of 0.4-4 were amplified using E1 primers at 79.74% and 26.56%, respectively. Cytological data of grey zone samples were usually identical to normal samples (77%) whereas RLU/CO > 4 can be found in all categories' cytological data. HPV screening by HCII within the grey zone should be analyzed together with cytological data and PCR screening preferably using E1 primers.

2. Human papillomavirus genotypes among infected Thai women with different cytological findings by analysis of E1 genes

As mentioned previously, E1 primer is more sensible than L1 and E6 primers; therefore, the second HPV detection was used E1 primer for HPV genotyping distribution among infected Thai women with different cytological data. Polymerase chain reaction was performed to amplify the E1 region of HPV. The reaction mixture comprised 2 µl DNA, 0.5 µM of each primer, 10 µl 2.5X Eppendorf masterMix (Eppendorf, Hamburg, Germany), and nuclease-free water to a final volume of 25 µl. The amplification reaction was performed in a thermal cycler (Eppendorf, Hamburg, Germany) under the following conditions for the first round: initial denaturation at 94 ºC for 3 min, followed by 40 amplification cycles consisting of denaturation at 94°C for 30 sec, primer annealing at 55°C for 45 sec, and extension at 72°C for 1.00 min, and concluded by a final extension at 72 °C for 7 min. The primers employed were HPV-E1F1_1219 (nt 1160-1183); 5'-AGTACAGGTTCTAAAACGAAAGT-3' and HPV-E1R1_2119 (nt 2076-2100) 5'-CATTATCAAATGCCCAYTGYACCAT-3' (table 1). These primers yield a PCR product of 940 bp. Second round PCR was performed under the following conditions: Denaturation at 94 ºC for 3 min, followed by 40 amplification cycles consisting of denaturation at 94°C for 30 sec, primer annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, and concluded by a final extension at 72 °C for 7 min. The primers employed were HPV-E1F2_1383 (nt 1315-1335) 5'- GAAGACAGCGGNTATGGC-3') and HPV-E1R1_2119.

- **Genotype distribution in relation to cervical cytology**

All 515 samples originated from women between 19 and 83 years of age and tested positive for HPV DNA by PCR amplification of the E1 gene. Samples with PCR

negative for the house keeping gene (β-globin) were excluded from this study. More specifically, 16 samples (3.11%) could be categorized as CA (cervical cancer), 98 samples (19.03%) as HSIL (high-grade squamous intraepithelial lesion), 136 samples (26.41%) as LSIL (low-grade squamous intraepithelial lesion), 79 samples (15.34%) as ASC-US (Atypical squamous cells of undetermined significance) and 186 samples (36.12%) as normal.

- **Cervical cancer cytology**

The 16 cases positive for squamous cell carcinoma (15 samples) and adenocarcinoma (1 sample) contained HR-HPV of three different genotypes which were HPV 16 (11/16; 68.75%), 18 (3/16; 18.75%) and 52 (2/16; 12.5%) (table 7). Based on our results, HR-HPV genotypes were found in 100% of the CA group.

- **HSIL cytology data**

These 98 samples mostly contained the HR-HPV genotypes 16 (40/98; 40.82%), 52 (15/98; 15.31%), 31 (13/98; 13.26%), 33 (4/98; 4.08%) and 18 (4/98; 4.08%) and other less prevalent HPV genotypes (miscellaneous) such as HPV 6, 30, 35, 39, 42, 44, 53, 55, 56, 58, 68, 70, 71, 73, 74 and 90. All miscellaneous genotypes were found in 22 samples (22/98; 22.45%) (table 7). Based on our results, HR-HPV genotypes were found in 89.8% (88/98) of the HSIL group.

- **LSIL cytology data**

These 136 samples mostly contained genotypes16 (19/136; 13.97%), 31 (19/136; 13.97%), 66 (13/136; 9.84%), 52 (12/136; 8.82%), 39 (9/136; 6.62%) and 51 (9/136; 6.62%) as well as the less prevalent miscellaneous HPV genotypes 6, 18, 30, 32, 33, 34, 35, 40, 42, 53, 55, 56, 58, 59, 68, 70, 71, 73, 74, 81, 82 and 90. Miscellaneous genotypes were found in 55 samples (55/136; 40.44%) (table 7). HR-HPV genotypes were found in 69.12% (94/136) of the LSIL group.

Table 7 Amount of HPV genotype distribution in each category cervical cytopathology (CA, HSIL, LSIL, ASC-US and Normal)

	Cytological data (Number of samples)					
	CA	HSIL	LSIL	ASC-US	Normal	Total
HPV6	-	1	1	2	1	5
HPV11	-	-	-	-	1	1
HPV16	11	40	19	12	42	124
HPV18	3	4	1	2	2	12
HPV30	-	1	2	1	1	5
HPV31	-	13	19	12	20	64
HPV32	-	-	1	1	4	6
HPV33	-	4	1	1	-	6
HPV34	-	-	4	6	6	16
HPV35	-	1	6	1	7	15
HPV39	-	2	9	3	7	21
HPV40	-	-	1	-	-	1
HPV42	-	1	4	1	8	14
HPV44	-	1	-	-	-	1
HPV51	-	-	9	2	3	14
HPV52	2	15	12	5	10	44
HPV53	-	1	3	-	3	7
HPV55	-	1	1	1	1	4
HPV56	-	3	7	2	2	14
HPV58	-	3	1	1	-	5
HPV59	-	-	2	5	8	15
HPV66	-	-	13	7	13	33
HPV68	-	1	6	5	9	21
HPV70	-	1	2	-	1	4
HPV71	-	1	2	3	10	16
HPV73	-	2	1	1	-	4
HPV74	-	1	2	2	2	7
HPV81	-	-	1	-	-	1
HPV82	-	-	1	1	-	2
HPV85	-	-	-	-	1	1
HPV90	-	1	5	2	23	31
HPV91	-	-	-	-	1	1
Total	16	98	136	79	186	515

- **ASC-US cytology data**

In the 79 samples with ASC-US cytology, we detected mostly HPV genotypes 16 (12/79; 15.19%), 31 (12/79; 15.19%), 66 (7/79; 8.86%), 52 (5/79; 6.33%), 59 (5/79; 6.33%) and 68 (5/79; 6.33%) along with less prevalent (miscellaneous) genotypes 6, 18, 30, 32, 33, 34, 35, 39, 42, 51, 55, 56, 58, 71, 73, 74, 82 and 90. Miscellaneous genotypes were found in 33 samples (33/79; 41.77%) (table 7). HR-HPV genotypes were found in 67.09% (53/79) of the ASC-US group.

- **Normal cytology data**

The 186 samples with normal cytology mostly contained HPV genotypes 16 (42/186; 22.58%), 90 (23/186; 12.37%), 31 (20/186; 10.75%), 66 (13/186; 6.99%), 52 (10/186; 5.38%) and 71 (10/186; 5.38%) as well as less prevalent (miscellaneous) genotypes 6, 11, 18, 30, 32, 34, 35, 39, 42, 51, 53, 55, 56, 59, 68, 70, 74, 85 and 91. We detected miscellaneous genotypes in altogether 68 samples (68/186; 36.56%) (table 7). HR-HPV genotypes were found in 59.14 % (110/186) of samples with normal cytology.

- **Distribution of HPV genotypes based on E1 amplification**

Amplification with primers HPV-E1F2_1383 and HPV-E1R1_2119 yielded a 716-bp product, which upon direct sequencing facilitated HPV genotype determination. Direct sequencing is the gold standard method for confirming PCR results. Our group classified HPV based on Munoz et al into HR-HPV (high-risk HPV), probably HR-HPV, LR-HPV (low-risk HPV) and UR-HPV (undetermined risk). All 515 HPV DNA positive samples were typed and revealed 32 different genotypes: 70.09% (n=361) were determined as HR-HPV, 7.76 % (n=40) as probably HR-HPV, 5.24% (n=27) as LR-HPV and 16.89% (n=87) as UR. HPV16 was the most prevalent genotype at 24.08% (124/515), followed by HPV31 (12.43%; 64/515), HPV52 (8.54%; 44/515), HPV66 (6.41%; 33/515) and HPV90 (6.02%; 31/515). HPV genotypes 18, 32, 33, 34, 35, 39, 42, 51, 53, 56, 59, 68, 71 and 74 were less frequent (from 1.0% to 4.0%) and others were rare (below 1.0%). The percentage of frequency was arrived at based on previously published criteria [155].

HPV genotype 16 appeared to be most prevalent, irrespective of cervical cytology whereas genotype 18 was mainly found in the HSIL and CA groups. Furthermore, HPV genotypes 52 and 31 were detected in the LSIL, HSIL and CA groups whereas HPV genotype 33 was found only in HSIL. HPV genotypes 31, 33 and 52 were mostly found in abnormal cytological samples. In addition, HR-HPV genotypes 66 and 68 were usually found in LSIL to normal groups (table 7). Our results demonstrated that using the E1 primers facilitated PCR amplification not only of HR-HPV genotypes but also of probably HR-HPV, LR-HPV and others.

All of HPV specimens can be efficiently detected by the PCR were subjected to nucleotide sequencing and BLAST alignment to confirm of the HPV genotypes detected. Some sequences obtained in this study have been submitted to the GenBank database and assigned accession numbers (Appendix B, table 13).

Conclusion, cervical cytological data may not be sufficient for cervical cancer screening and prevention; therefore, additional information of HPV genome detection will be used for confirmation and provided essential data of HPV infection. In this project, we determined HPV genotypes among infected Thai women with different cytological findings by characterizing E1 genes. The 515 samples positive for HPV-DNA by PCR were typed and revealed 32 different genotypes. HR-HPV (HPV16, 18 or 52) was detected in all samples with cervical cancer cytology. HR-HPV was present in 89.8% of HSIL, 69.12% of LSIL, 67.09% of ASC-US, and 59.14% of normal samples, respectively. HPV16 was most prevalent, irrespective of cervical cytology. Moreover, HPV31 and 52 were most prevalent in the HSIL and LSIL groups whereas HPV66 was found mostly in the LSIL group. The LSIL group displayed the highest variation of HPV genotypes. Furthermore, HPV31 and 52 predominated in the HSIL and LSIL groups, especially HPV52 which was found in cancer samples.

Based on study described above, HPV16 is the most widely HPV distributed type being present in all categories of cytology, whereas HPV18 is mainly distributed in HSIL case. The finding that HPV16 and HPV18 are both highly prevalent in specimens from Thai women led to the genome characterization of the types present, which analyzed on sample with different cytological finding from normal to cervical cancer. Furthermore, next section of this study focused on developmental method for molecular

diagnosis of the HPV16 and HPV31 which has been report as described above that are high distribution in our samples and HPV18 concerned with cervical cancer.

3. Multiplex real-time PCR for HPV16, HPV18 and HPV31 detection

In this study, I selected the 2 most commonly found genotypes (HPV16 and HPV18) and HPV18 that is found in cervical cancer to establish multiplex real time PCR method. The diagnostic method was further developed for an even more rapid, specific and sensitive assay based on the multiplex real-time PCR method using primers and triple fluorescent labelled TaqMan probes corresponding to the L1 genes of HPV16, HPV18 and HPV31, as these have the highest distribution in my studies.

A TaqMan probe is an oligonucleotide with a reporter fluorescent dye attached to the 5' end and a non-fluorescent quencher attached to the 3' end. The fluorescent signal increases when the probe is cleaved by the 5' to 3' exonuclease activity of Taq DNA polymerase during the PCR reaction, thereby separating the reporter dye from the quencher. Applying the multiplex primers from the above study and TaqMan probes labeled with 3 different fluorescent reporter dyes (FAM, JOE and ROX which have emission wavelength at 518, 554 and 607 nm respectively) for specific targets corresponding to HPV16, HPV18 and HPV31. Therefore, HPV detection based on a multiplex real-time PCR using TaqMan probes should yield more efficient, specific and sensitive assay for rapid detection and quantitation of the viral load in the original specimen.

- **TaqMan probe designation**

TaqMan probes were designed following the general guideline described in Appendix A. Using the nucleotide sequences available in the GenBank database (as mentioned previously in chapter III), multiple sequence alignments of L1 genes of the HPV16, HPV18 and HPV31 were performed using the CLUSTAL X program (version 1.8). Both primers and probes were chosen and analyzed using the primer design software (OLIGOS version 9.1) to ensure that they could be combined in a multiplex format. The primers and probes used in this study are shown in Table 8 and Figure 20.

- **Construction of positive controls for multiplex real time PCR**

The L1 genes of Human papillomavirus types 16, HPV18 and HPV31 were used to construct plasmid DNAs by inserting genes into the pGEM-T Easy Vector (Promega) (see Chapter III). Then, the L1 genes plasmid of HPV16, HPV18 and HPV31 were used as a positive control for optimization of multiplex real-time PCR (Figure 21). The concentration of DNA was calculated by measuring absorbance at 260 nm. The DNAs were then serially diluted ten fold, ranging from 10^7-10 copies/μL to perform sensitivity tests.

Table 8 Multiplex real-time PCR primer and probe at L1 region that are specific genotype HPV16, HPV18 and HPV31

L1 specific type	Primer and Probe name	Sequence(5'-3')	Position	Product size (bp)	Tm (°C)
HPV16[a]	F1L_6170T16	CACAGAAAATGCTAGTGCTTATGC	6020-6043	200	68
	R1L_6378T16	ATAACTGTGTTTATTAACTCTAATGG	6195-6220		66
	ProbeT16	FAM-TGCAGGTGTGGATAATAGAGAATGTATA-BHQ1	6050-6077		76
HPV18[b]	F1L_6189T18	TGAGGACGTTAGGGACAATGTGT	6032-6054	120	68
	R1L_6322T18	TAAAGGACGCGATTTACAAGCAGT	6139-6152		68
	ProbeT18	JOE-AGTTATGTATTTTGGGCTGTGCCCCT-BHQ1	6076-6101		78
HPV31[c]	F1L_6096T31	TGGTTTAGAGGTAGGTCGCGG	5863-5883	193	66
	R1L_6287T31	GCTCTCCAATAGGTGGTTTGC	6036-6056		66
	ProbeT31	ROX-GAAAACTCTAATAGATATGCCGGTGG-BHQ2	5942-5967		74

[a] Position based on reference sequence NC_001526

[b] Position based on reference sequence NC_001357

[c] Position based on reference sequence J04353

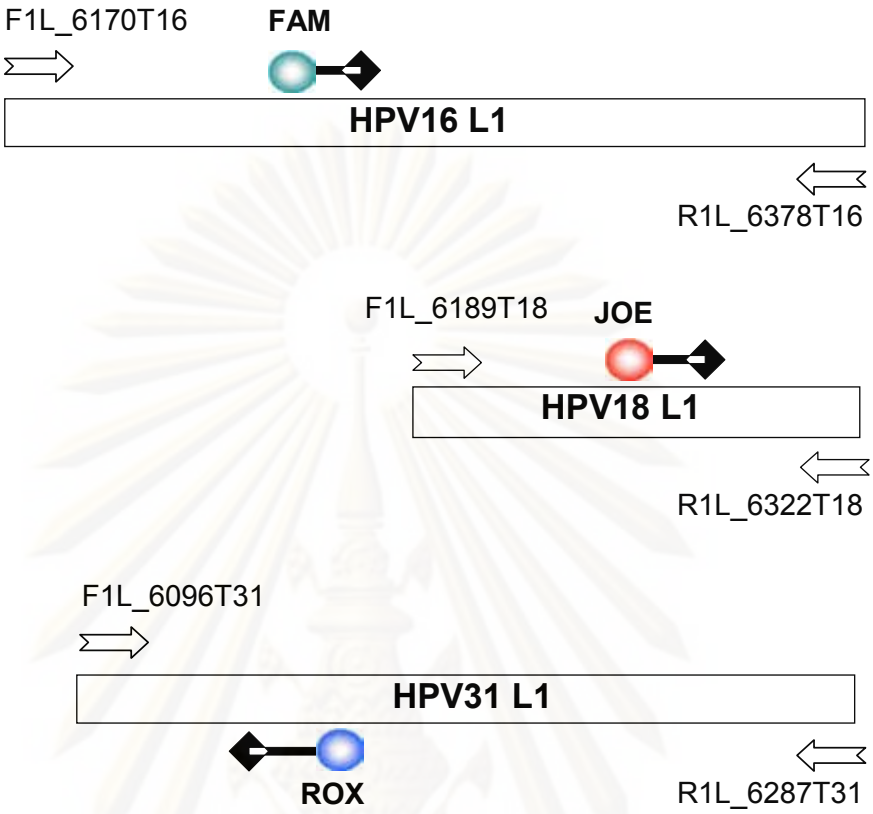


Figure 20 Schematic maps of primers and probes used in multiplex real-time PCR for HPV16, HPV18 and HPV31 detection. HPV16 probe was labeled with FAM (emission wavelength at 518 nm), HPV18 probe was labeled with JOE (emission wavelength at 554 nm) and HPV31 probe was labeled with ROX (emission wavelength at 607 nm) (modified from previous thesis [154]).

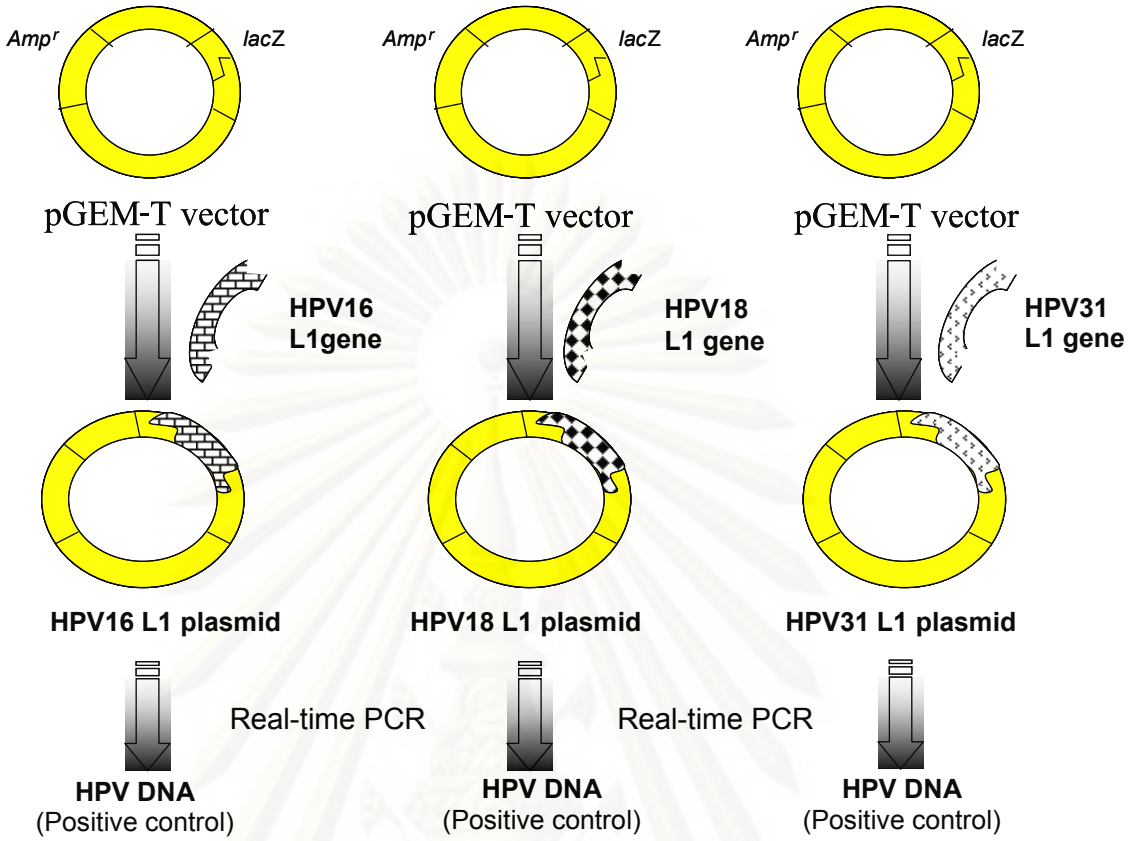


Figure 21 Schematic diagrams represent the construction of positive controls for L1 genes of HPV16, HPV18 and HPV31 (modified from previous thesis [154]).

- **Optimization of the multiplex real-time PCR conditions**

For maximum efficiency of amplification in multiplex real-time PCR, several factors, including each primer concentration (0.25 - 0.75 µM), each probe concentration (0.1 – 0.5 µM), additional Mg^{2+} concentration (ranging from 0.5 – 3.0 µM) and thermocycling condition (annealing at 55, 58 or 60°C), were subjected to be optimized by using DNA plasmid as a positive control. According to the result of optimization (data not shown), Multiplex real-time RT-PCR was performed using the Biotool QuantiMix EASY PROBES KIT (Biotools). Three sets of primers and TaqMan probes specific for L1 genes of HPV16, HPV18 and HPV31 were used in multiplex format. Each probe was used at a final concentration of 0.25 µM. The HPV16 primer was used at final concentration of 0.5 µM, whereas HPV18 and HPV31 were used at final concentration of 0.75 µM. A combination of 1 µl of DNA sample with a reaction mixture containing 7.5 µl

of 2X TaqMan PCR Master Mix, 4 mM $MgCl_2$, with 0.3 µl of superscript and DNase-free water was used to bring it up to a final volume of 15 µl.

Multiplex real-time PCR was performed on Rotor-Gene RG-3000 (Corbett Research). Cycling conditions amplification was performed during 40 cycles including denaturation (94°C for 30 seconds), annealing (58 °C for 45 seconds) and extension (72 °C for 1 minute).

- **Interpretation of multiplex real-time PCR detection**

The primers and probes summarized in Table 4 were selected for real-time PCR detection in multiplex format of the FAM, JOE and ROX fluorescent signals, corresponding to the HPV16, HPV18 and HPV31 respectively. Double or triple fluorescent signals resulting from multiplex real-time PCR can be interpreted as a multiple infection of HPV, whereas only one signal indicated a specific HPV genotype infection (figure 22).

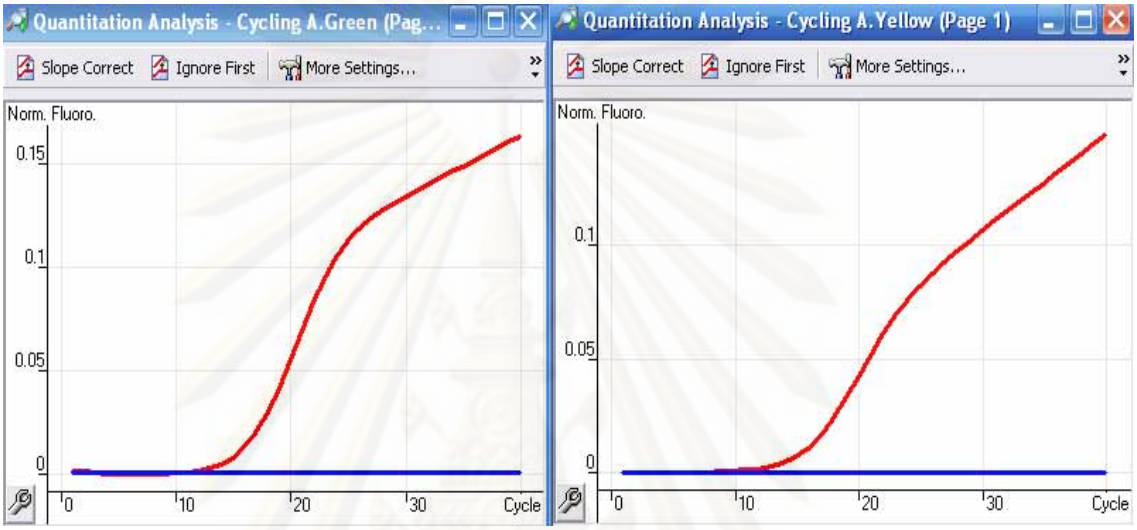
- **Specificity and Sensitivity test of multiplex real-time PCR**

The specificity of multiplex real-time PCR assay was evaluated by cross-reaction tests showing no cross-reactivity to the human DNA and RNA virus, Hepatitis B virus (HBV), Parvovirus 4 (PARV4), Parvovirus B19, Human immunodeficiency virus (HIV) and Hepatitis C virus (HCV). No significant false positive or non-specific signal was observed in any of the samples tested. Taken together, these results indicate high specificity of the primers and probes used in the multiplex real-time PCR assay.

The PCR amplification product of HPVL1F1_6153 and HPVL1R2_6804 (table 5) were amplified L1 gene and the PCR products were confirmed by nucleotide sequencing. Then, the L1 genes amplification products of HPV16, HPV18 and HPV31 were used to construct plasmid DNAs by inserting into the pGEM-T Easy Vector (Promega). The concentration of the plasmid DNA was calculated by measuring absorbance at 260 nm. The plasmid DNA concentrations were then serially diluted ten-fold, ranging from 10^7-10 copies/µl to perform sensitivity tests. As expected, the threshold cycle (Ct) increased in direct proportion to the dilution of the DNA standards. The FAM signal can be detected HPV16 DNA standard dilutions as low as 10^2copies/µl

in a multiplex real-time PCR assay (figure 23). In addition, the JOE and ROX signal can be detected HPV18 and HPV31 DNA standard dilutions at 10^3 copies/µl in a multiplex real-time PCR (data not shown).

A



B

Figure 22 Representative results obtained from multiplex real-time PCR. (A) HPV sample that infected with multiple genotypes HPV16 and HPV31 was yielded double fluorescent signals of FAM (green channel) and ROX (yellow channel) corresponding to HPV16 and HPV31 respectively. (B) HPV sample that infected with specific genotypes HPV16 was yielded only a FAM fluorescent signal (green channel) of HPV16.

- **False positive and false negative sample test with multiplex real-time PCR**

Thirty samples of standard known genotypes sample test of HPV16 (10 samples), HPV18 (10 samples) and HPV31 (10 samples) that exactly known genotype by nucleotide sequencing at L1 gene were tested with multiplex real time PCR following the condition previously described. The result showed that all of them were accurately detected by this multiplex real time PCR detection system (data not shown).

The others high-risk HPV genotypes: HPV33 (3 samples), HPV35 (3 samples), HPV39 (3 samples), HPV51 (3 samples, HPV56 (4 samples), HPV58 (3 samples), HPV59 (3 samples), HPV68 (3 samples), HPV73 (2 samples) and HPV82 (1 samples) were tested with this multiplex PCR system. The result showed that my multiplex real time PCR detection system cannot detect other high-risk genotypes; therefore, it does not give a cross genotyping result (data not shown).

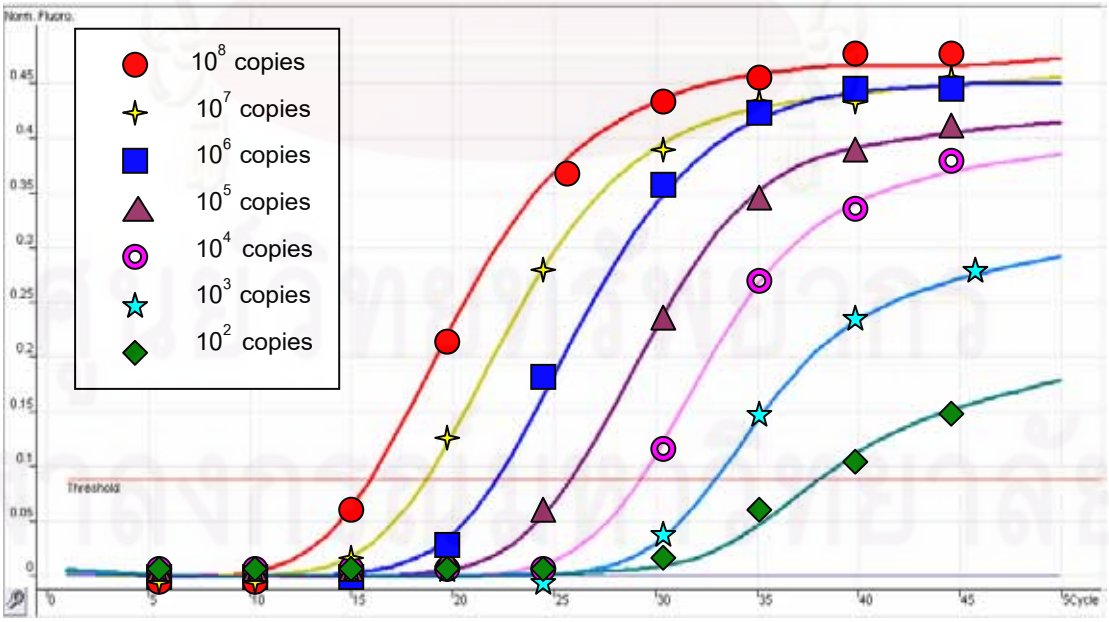


Figure 23 Amplification plots of HPV16 L1 gene obtained from the amplification of 10-fold serially diluted of DNA standards. The result revealed that the sensitivity of the real-time PCR was 10^2copies/µl.

- **Evaluation of real-time PCR performance**

The standard curve of multiplex real time assay was constructed by plotting the logarithm of initial DNA concentrations against the threshold cycle (Ct) obtained from each dilution. The standard curve of HPV16 real time assay is shown in figure 24. The slope of the standard curve is used to determine the PCR reaction efficiency, which is theoretically exponential: 100% amplification efficiency would imply doubling of amplicon concentration each cycle. The standard curves with a slope between approximately -3.1 and -3.6 are typically acceptable for most applications requiring accurate quantification (90 -110 % reaction efficiency). An R^2 value is the fit of all data to the standard curve plot. If all the data lie perfectly on the line, the R^2 will be 1.00. As the data fall further from the line, the R^2 decreases. An R^2 value ≥ 0.985 is acceptable for most assays. The HPV16 detection standard curve yielded a slope of -3.625 (efficiency = 90%) and R^2 = 0.993. These values indicate satisfactory amplification efficiency and overall performance of the real-time PCR assay.

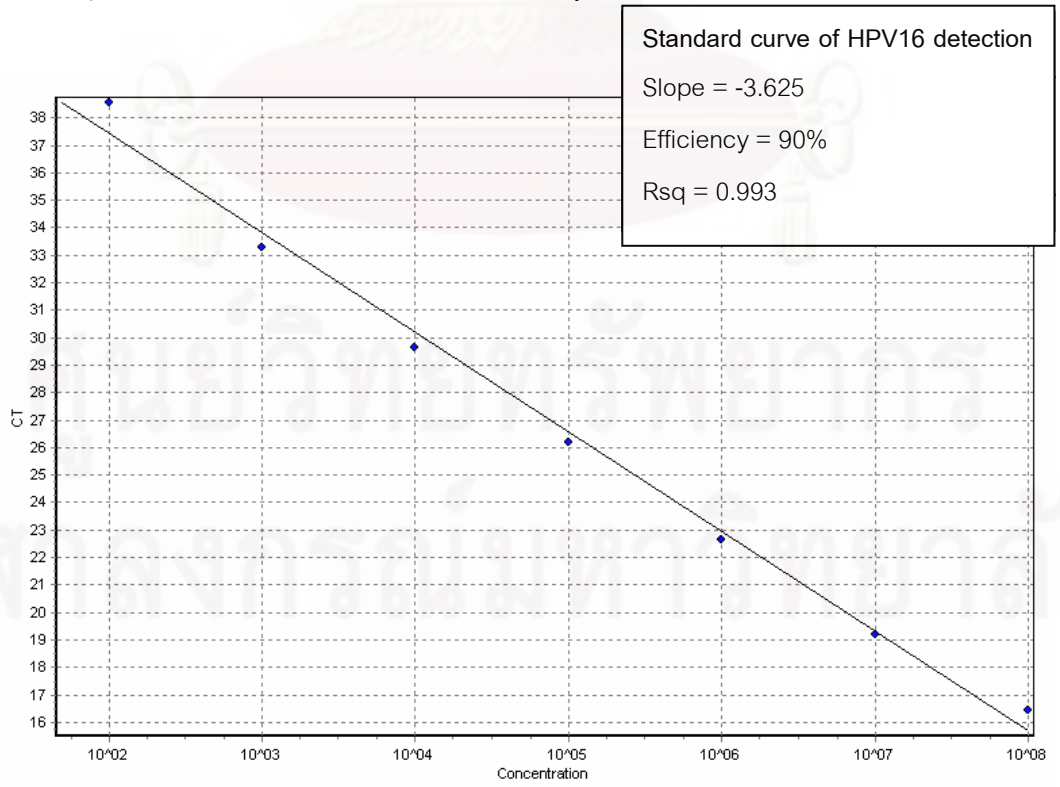


Figure 24 Standard curves of HPV16 detection was constructed by plotting the log of starting DNA concentrations (X-axis) against the threshold cycle (Ct) (Y-axis) obtained from each dilution.

- **Samples test with multiplex real-time PCR**

One hundred and five of HPV samples were tested in multiplex real-time PCR. All of the samples were a confirmed result by amplifying them with E1 primer (table 5) and positive PCR amplification results were confirmed by nucleotide sequencing. Based on this study, ninety-six samples have a correlated result with nucleotide sequencing data (96/105; 91.43%). Nine samples that missed detection can be divided in to undetected HPV16 (4 samples), HPV18 (2 samples) and HPV31 (3 samples) respectively. Moreover, a multiple infection can be found in 3 samples that showed HPV16 and HPV18 infection by multiplex real-time PCR, but the result of PCR and nucleotide sequencing indicated HPV16 only. Multiplex real-time PCR provided an additional data of multiple infection more than conventional PCR; however, undetected samples can be found in the multiplex real-time PCR.

In conclusion, the real-time PCR assay described in this study provides a rapid, sensitive and cost-effective approach for HPV16, HPV18 and HPV31 detection, which are the high distribution genotypes found in this study.

CHAPTER V

CONCLUSION AND DISCUSSION

Molecular characterization of carcinogenesis genotypes (HPV16 and HPV18)

From previous data, the most popular HPV distribution in Thailand is HPV16 that high prevalence in all cytological categories groups. While, HPV18 is emerge in HSIL or cancer categories group. Then, the HPV 16 and HPV18 are the most interesting genotype for whole genome characterization.

1. Genome characterization of HPV16

This section has been focused on analyzing the entire genome of HPV16 obtained from patients with different cervical cytology data. A pathologist determined cervical cytology and the respective samples were subjected to a commercially available test such as Hybrid capture II. We found HPV16 genome variations in Thai women in that the total length of the HPV16 genome can amount to 7,905 or 7,906 base pairs comprising the long control region (LCR), early gene, late gene and upstream regulatory region. Various nucleotide variations were discernible within the upstream regulatory region between early and late gene (data not shown). Each type of HPV16 will find insertion and deletion in this region accounting for various genome lengths displayed. For example, the LCR of most samples, except CU4 (CIN I) showed a change from G to T at position 7193 (G7193T) and all our samples exhibited a change from G to A at position 7521 (G7521A). These positions were critical for HPV infection because both positions are integral parts of transcription factor binding sites and thus, influence infection properties [148]. Various previous studies have reported that the disruption of Yin Yang 1 (YY1) binding sites within the LCR may result in the up-regulation of E6/E7 expression potentially allowing malignant conversion without integration [148, 149]. However, we could detect variations in the YY1 binding sites in CU5 at position 7,842 related to the EA strain. Xi et al suggested that nucleotide changes in the LCR variants of HPV 16 might be more closely associated with a risk for disease progression possibly resulting in pathogenesis [156, 157]. According to a

previous study reporting an E6 variation at position 350, the E6-350G variant was found mostly in Europe and America, but not in Southeast Asia [147]. Yet, in the course of this project we detected both E6-350G and E6-350T in 3 and 4 samples, respectively. Some epidemiological studies have revealed that in HPV16 a nucleotide change at nt 350 within E6 from T to G correlates with high-grade lesions and cancer [147, 158], whereas another study speculated that it might be population dependent [159]. The T350G (L83V) variation within E6 was suggested to be associated with an increased risk of persistent infection and cytological progression to cervical intra-epithelial neoplasia grade 2/3 [79]. However, in contrast with some previous studies [79, 147] our research did not show this correlation. The dissimilarity may be explained by various factors. For example, variants of HPV16 may affect the biological properties of the protein. Also, the host's immune response to specific viral epitopes encoded by variants and the high level of diversity among HPV16 E6 and E5 proteins may be a potential force driving the evolutionary selection of the E2 since HPV16 E2 variants frequently co-segregate with the E6 T350G (L83V) variant that has been associated with viral persistence [160]. This co-segregation was proposed to act as an additional risk factor for the development of cervical cancer [79, 145]. Moreover, some previous studies revealed that variations in the E2 protein might alter the affinity for cellular transcription factors or for HPV 16 DNA because variants have been suggested to be associated with the risk of cervical neoplasia [145, 161, 162]. Yet another research group has previously suggested that amino acid variation in L1 at codon 202 (H202D), would decrease the infectious potential of HPV because it can prevent viral capsid formation [163]. However, we did not detect this polymorphism (data not shown). Moreover, Lee and collaborators suggested that HPV should be analyzed at E6-83, L2-243, L1-266, L2-269 and E2-219 and concluded that five combinations were possible based on the E6-83L prototype and two combinations based on the E6-83V variant [150]. Our results correlated with Lee's except for the CA and CIN III samples. The CA patient's sample showed that E2-219 was neither the E2-219S variant nor the E2-219P prototype but instead, it was E2-219T. Furthermore, rate of non-synonymous/ synonymous (dN/dS) were found only in E2 (dN/dS = 1.26). Similarly, the CIN III patient's sample indicated that L2-269 was neither the L2-269P variant nor the L2-269S prototype but instead, it was L2-269D (figure 21).

Variations at these positions can not be seen in other reference sequences, be that the European, European-German, Asian-American, North-American, or African type 1 and 2 (south-America) Thus, these alterations deserve close observation as they may be responsible for malignant progression. In addition, a previous study from Thailand revealed that this E6 mutation coincided with a specific E7 mutation at residue 29 leading to a substitution from "N" (asparagine) to "S" (serine) [164]. This E7 variant was also more frequent in cervical cancer samples as compared to precursor lesions [165]. However, in this study we detected this change in only one CU5 sample (CIN II), whereas none of the other samples had mutated at this residue. In the E5 region, amino acid variations were focused at amino acid positions 11 to 24 as this area encodes the trans-membrane helical region and positions 46 to 50 are conserved among HPV types [166]. As for amino acid variations, the E5 gene displayed conservation at positions 46 to 50, but differed from the reference sequence (HPV16R) at amino acid position 20. According to a previous study, E5 amino acid variations may alter the protein's capacity for transformation by affecting the interactions with the EGFR, the 16 kDa subunit of the H+-ATPase or, potentially, other cellular proteins [166]. Based on complete genome analysis, HPV 16 from Thai women is more closely related to the European, European German, East Asian and North American type than to the Asian American or African type1 and 2. Lately, HPV vaccines have been commonly used worldwide and many research groups have attempted to elucidate their efficiency. Consequently, we analyzed and compared the polypeptide of the vaccine strain (US Patent 6613557), GenBank database and all seven samples. Phylogenetic tree analysis of L1 polypeptides revealed that vaccine polypeptides were closely related to the reference sequence (HPV16R) and K02718 and thus, more similar to all seven samples than the Asian American and African type1 and 2. Phylogenetic tree comparison between the polypeptide of the vaccine strain (US Patent 6613557), GenBank database and all seven samples is shown like figure 22C. Judging from the percent homology between the polypeptide of the vaccine strain and all our samples, the vaccine will probably be efficient in protecting Thai women.

In conclusion, we found numerous nucleotide and amino acid variations in the genome of HPV16 isolated from infected Thai women. Whole genome analysis of these

samples showed HPV16 from Thailand to be more closely related to the European strain than the Asian American and African type1 and 2. This study revealed that infected women with CIN III and CA displayed amino acid alterations at critical positions in the E2 and L2 region, but our group can not draw any inference between clinical stage of disease progression and amino acid alterations as there was only one sample available for each clinical trial. However, we hope that these new data on the HPV genome, which are representative for the entire genome of HPV in Southeast Asia, can serve as basic data for scientific research on cervical cancer pathogenesis.

2. Genome characterization of HPV18

Another interesting genotype is HPV18. Whole genome characterization was analyzed in all cytological categories of Thai women samples. Whole genome sequences of HPV18 found in Thai samples (accession numbers GQ180784-92) were aligned with X05015 [144], NC001357, Y262282, EF202152-EF202155 (African type), EF202147-EF202151 (European type) and EF202143-EF202146 (Asian-American type). Analysis of HPV nucleotide sequences showed 97.9-99.9 % similarity among the Thai and all reference sequences (data not shown). Phylogenetic analysis revealed that HPV18 in Thailand were closely related with European and Asian American types (Figure 23).

The complete genome of CU11 comprised 7,844 bp, whereas other samples consisted of 7,857 bp indicating variable genome lengths of HPV18 isolated from Thai women. We observed deletion in the LCR (7245-7256) and E4 gene (position 3630-3635) of CU11 samples (data not shown). These deletions occurred in the African type and phylogenetic tree analysis showed CU11 were closely with the African type whereas other samples were related with the European and Asian-American type (Figure 23). In contrast to existing data on HPV16 variants, HPV16 in Thailand was more closely related to the European than the Asian American and the African type [167]. Thus, our study showed that HPV18 variants in Thailand related two distinct groups of HPV18 variants (European and Asian-American types). Nevertheless, due to small sample size, these finding should be verified by larger studies.

Based on nucleotide variation, we focused on the LCR segment at positions 41, 104 and 7,726. A previous study had reported that nucleotide variations in this segment should affect transcription factor binding sites such as Sp1 (selective promoter factor 1), YY1 (Yin-Yang factor 1) and Oct-1 (octamer binding factor), respectively [151]. Based on our results nucleotide variations at these critical positions occurred with 2 samples (CU10 and CU11). Nucleotide variation of CU10 changed both positions 41(A to G) and 104 (T to C), whereas the nucleotide sequence of CU11 changed at positions 104 (T to C) and 7726 (C to T). Variation of nucleotides 41 and 104 was associated with a higher activity of the E6/E7 promoter by modulating Sp1 and YY1 activities [150]. A previous report indicated that nucleotide alterations at position 104 were less likely to promote tumor recurrence than virus with the reference sequence [152] and variation was usually found in the European type. Based on the pattern of nucleotide variation, all samples displayed more similarity to the European (especially CU10) and Asian-American type than the African type. We could detect these changes in CU10 and CU11 that indicated LSIL cytological data.

Based on amino acid analysis, the E6 region, position N129K had not undergone amino acid variation in our samples whereas in CU10 and CU11, the L1 region, position T149N was identical to the African and European types. The amino acid at position 129 is highly conserved within HPV18 and in vitro analysis has shown that this change of amino acid not affect the ability in promoting p53 degradation [168]. Amino acid alteration at position 149 of L1 is commonly found in many HPV genotypes and is located near the surface of the capsid protein [153]. Hence, it does not affect the function of the L1 gene. In addition, the proteins displaying most non-synonymous amino acids were detected in E2 and L2 as reported for HPV16 by a previous study [167]. López-Saavedra et al indicated that the E2 viral protein is essential for regulating LCR activity and transcription of viral genes [169]. Some researchers reported that E2 variations were insignificant between controls which no past and current history of cervical abnormalities and tumor cases [170]. Multiple nucleotide substitutions found in HPV18-E2 variants were not affected activities over homologous LCR among HPV18 variants [167]. Moreover, based on variation, the rate of non-synonymous/ synonymous (dN/dS) alterations was not found significant for all coding regions. Thus, our data didn't

performed and analyzed amino acid variation on E2 gene. However, E2 variation could affect other biological function such as viral replication or interaction with other cellular protein [169].

Alignment of the vaccine strain's (US Patent 5820870) polypeptide with all our samples has shown that the L1 vaccine strain is closely related to the African type, whereas most of our samples' sequences are related to the Asian-American and European type. Some previous study revealed that oncogenic potential and HPV18 variants demonstrated that the African type is usually found in patients with less abnormal pathology [171]. Although, bivalent and quadrivalent vaccine cross-reactivity have been reported but long-term stimulation of immune response remains to be established in direct comparison [172]. Some report revealed that protective and broadly cross-neutralization should be emphasized on L2 [173]. Thus, a second generation HPV 18 vaccines should be tested on both HPV18-L1 and HPV18-L2 for increasing potential protection. Due to our small sample size, we could not observe any significant correlation between HPV18 variants detected in this study and the results from cervical cytology. In addition, we did not see any difference in distribution of HPV18 variants when comparing squamous cell carcinoma with adenocarcinomas. Our data support the proposed association of non-European variants (especially AA variant) with adenocarcinoma as suggested elsewhere [174]. However, our project was limited by the small sample size. It had been intended as a preliminary study aimed at HPV18 genome characterization in Thailand which would represent HPV18 in Southeast Asian.

In conclusion, we found numerous nucleotide and amino acid variations in the genome of HPV18 isolated from infected Thai women. Whole genome analysis of these samples showed HPV18 from Thailand to be more closely related to the European and the Asian-American than African type. This study revealed that our group can not draw any inference between clinical stage of disease progression and amino acid alterations as there were only one or two samples available for each clinical trial. However, we hope that these new data on the HPV18 genome which are representative for the entire genome of HPV18 in Southeast Asia can serve as basic data for scientific research on cervical cancer pathogenesis.

Developmental HPV detection method and HPV screening

The classic screening method is used on the cytologic evaluation of Papanicolaou (Pap)-stained cervical smears, in which cervical cells are scraped from the transformation zone, i.e. the area adjacent to the border of the endocervix and ectocervix, and transferred to a glass slide [175, 176]. During the last 50 years, the classic screening Pap test has undoubtedly reduced cervical cancer morbidity and mortality [177-179]. In spite of its success, the Pap test is a subjective method with a limited sensitivity of 50% and high suspectibility to intraindividual and interindividual variability [178, 180, 181]. The introduction of liquid-based cytological (LBC) has contributed to mitigating the problem of efficiency in processing samples, but the diagnostic validity in terms of sensitivity and specificity still shows important shortcomings [181, 182, 183]. Therefore, several molecular methods have been developed in order to identify HPV in liquid-base cytology (LBC) samples and tissue samples [11, 12]. First of all, the HPV detection used directed probe hybridization such as dot blot and Southern blot which are labor-intensive, time-consuming, and have low sensitivity and require large amounts of DNA [13]. Signal amplification methods such as hybrid capture II (HCII) assay, which is excellent, but it cannot identify specific HPV genotypes. Linear array (AMPLICOR HPV test; Roche Molecular System) assay and microarray systems were rapid detection and simultaneous typing of multiple HPV types; however, this assay required specialized instrumentation and was expensive cost. PCR is a target amplification method which can produce one more copies from single double stranded DNA molecule after 30 cycles of amplification. Due to large amplification by PCR, it must be taken to avoid a false-positive result, which emerged from cross-contamination with PCR amplification products. Nevertheless, most studies have focused on this method that has a high sensitivity and specificity. Each of these methods has its advantages and disadvantages, and some, or all, of these factors may influence the method of choice. When selecting which assay to use, there are several factors that should be taken into consideration. These include the requirement for qualitative, semi-quantitative or quantitative data, and the nature and number of samples to be analyzed. In addition, the available time and resources of the laboratory and the skill of the staff involved must also be considered.

1. Correlation between Hybrid capture II ratio and PCR amplification of the E1, E6 and L1 genes for HPV detection from samples with different cytological findings

Human papillomavirus (HPV) has numerous those more than 100 genotypes. The genome of HPV contains LCR, early gene and late gene that depend on stage of gene expression. HPVs have a variant genome in intra- or inter- genotypes, therefore, genotyping of HPV still need for further study. Remarkable gene E6, E1 and L1 are highlight for developmental genotyping method. In this project, it focused on the most essential HPV genome regions and hence, designed PCR primers for amplification of the L1 gene which is widely used in commercially available test kits, the E6 gene which has been shown responsible for cervical cancer development, and the E1 gene which encodes a function essential for replication of the virus and has until recently served to classify HPV genotype (Papillomacheck, Greiner Bio-One, Frickenhausen, Germany). Upon amplification, those products were confirmed by direct sequencing. All samples were detected using previously published primer sets and our primers specific for the E1, L1 and E6 gene. Using either primer set, samples with a high HCII ratio could be more readily amplified than those with a low ratio (figure 13). Moreover, it became apparent that PCR amplification of E1 provided a more accurate and sensitive diagnosis than amplification of L1 and E6. All samples that could be amplified with MY09/MY11 and GP5+/GP6+ originated from the same patients as the samples amplified with the E1 primers. Nevertheless, the percentage of PCR amplification upon employing E1 was higher than with the MY09/MY11 and GP5+/GP6+ primer set. From nucleotide alignment of high-risk HPV genomes, it became apparent that they are highly diverse and hence, only contain few short stretches of conserved regions insufficient for primer design. However, we found that some region in the E1 gene was more conserved than L1 and selected this region for primer design. Also, the nucleotide sequence amplified with the E 1 primers is longer (855 bps in the first round and 716 bps in the second round) than the L1 sequence previously reported at approximately 450 bps with MY09/MY11 and 150 bps after nested PCR with GP5+/GP6+ primers. Therefore, the E1 PCR product is more suitable for direct sequencing than L1, which will be essential for accurate determination of the HPV genotype. On a note of caution, PCR amplification can produce false negatives, as well, especially with ASC-US and ASC-H samples

(figure13B). However, PCR detection of samples by E1 primers within the grey zone of HCII should be analyzed together with cytological data.

The result demonstrated that the HCII ratio is directly proportional to the percent PCR amplification. However, the HCII grey zone was critical for HPV detection. Hence, a combination of methods should be introduced for HPV detection. Furthermore, our study showed correlation between HCII ratio and cytological data. We found that HCII ratio of 0.4-4 mainly displays normal cytology, whereas a ratio exceeding 4 is mostly found in LSIL, ASC-US and normal. Results with HCII ratio of 0.4-4 are difficult to interpret; therefore, cytological data and PCR should be introduced for HPV screening. Nonetheless, due to small sample size in the grey zone, these findings should be verified by larger studies. We hope that these data, correlating HCII and PCR methods, could be used as preliminary data for the establishment of a standardized HPV detection method in Thailand.

In conclusion, comparison between HCII ratio and PCR detection based on amplification of E1, L1 and E6 showed a correlation between HCII ratio and PCR detection as PCR can detect samples with a higher HCII ratio more readily than samples with a lower ratio. Finally, due to primer specificity PCR detection by E1 amplification proved more sensitive than by amplification of MY09/MY11 and GP5+/GP6+, L1 or E6. Correlating the results obtained by HCII, cytology and PCR will provide useful information for affected women as well as assist with future HPV therapy.

2. Human papillomavirus genotypes among infected Thai women with different cytological findings by analysis of E1 genes

Based on previous section as described above, E1 gene is sensitive and more HPV detection than E6 and L1 genes. Then, the study focused on HPV genotype based on E1 gene. Our group focused on HPV genotype distribution among different categories of cervical cytology by PCR amplification of the E1 gene. Alignment of the E1 genes of most HPV types showed high variation in nucleotide sequence (data not shown). Furthermore, parts of the E1 gene are conserved and can be used for primer design. Upon amplification the resulting products were confirmed by direct sequencing. PCR amplification of the E1 gene can help detect a broad panel of genotypes and

provides sequence information on HPV. With our E1 primers, we could detect 32 different genotypes including HR-HPV, probably HR-HPV, LR-HPV and others. Distribution of HPV genotypes showed increased prevalence of HR-HPV in direct relation to the severity of cervical cytopathology (normal cytology (59.14%), ASC-US (67.09%), LSIL (69.12%), HSIL up to 89.8% and CA 100%). The results obtained in the course of this project correlate with those published in a previous report [155] in that HR-HPV genotypes are mainly detected in HSIL and to a lesser extent in LSIL or samples displaying normal cytology. Thus, persistence of HR-HPV genotypes is associated with a risk for progression to cervical cancer.

Irrespective of cervical cytology, HPV16 was the most prevalent genotype detected. This finding correlates with a previous work focusing on L1 gene detection [5, 6, 155, 184], whereas HPV18 was the second most prevalent in the CA group. However, the CA samples available for this study mostly comprised squamous cell carcinoma, a single sample diagnosed as adenocarcinoma being found to contain HPV18. In the CA group, HPV18 was found to be less prevalent than HPV16, supporting the finding that, whereas HPV16 is associated with both squamous cell carcinoma (SCC) and adenocarcinoma, HPV18 is mainly a risk factor for development of adenocarcinoma [185]. Previous reports have revealed that HPV18 is more predominant in adeno/adenosquamous carcinoma than in SCC [177]. Our group also identified HPV 52 in two CA samples as HPV 52 is the 7^{th} most common cervical HR-HPV type worldwide, but the 5^{th} most common in Asia [186]. However, due to small sample size, this finding should be verified by larger studies.

The HPV genotypes detected in the CA group were HPV16, 18 and 52, whereas the greatest diversity of HPV genotypes in the HSIL group was found HPV16, 52, 31, 18 and 33. HPV16 and 18 are the most prevalent HPV types in cervical cancer worldwide [80, 186], the next most common HPV genotypes being 33, 45, 31, 58, 52 and 35 [186]. Meta-analysis of HPV distribution in HSIL revealed that HSIL infected with HPV16, 18 and 45 were progress to SCC [73] whereas our result showed that HPV16, 52 and 31 were mostly found in HSIL (Table2). Smith *et al* suggested that, among HSIL samples, certain HPV types have a specific risk for progressive to cancer, dependent on the geographical region in question [186]. According to a recent study conducted on

Korean women [166], HPV 58 was the most prevalent genotype in HSIL and CA group after HPV16 and HPV18. In Japan HPV52 and HPV58 were more prevalent than HPV18 in HSIL but less prevalent than HPV18 in SCC [187]. In contrast, we detected HPV31, 33 and 52 more frequently among our samples than HPV58 in HSIL. Based on the result of previous study [187], the most 6 prevalent genotypes in HSIL, HPV16, 18, 31, 33, 52, 58 in Japan, was the same as in our study. Moreover, genotype 66 (probably HR-HPV) was mostly found in LSIL, ASC-US and normal samples. HPV66 and 90 were most prevalent in samples with less abnormal cytology and were absent in HSIL and CA, whereas HPV16 can be found in all categories from normal to CA, In contrast, HPV90 is mainly found in Thai women with normal cytology but it could not be detected in any other category and hence, represents a virus of undetermined risk. According to the above data [80, 184-186], a second generation HPV vaccine should focus on HPV 31, 33, 35, 45, 52 and 58 for prevention of HPV infection in all regions of the world, including Thailand.

Genotyping could be a useful tool for classification of HPV-positive women according to oncogenic potential and thus, relative risk of progression to CA as well as for evaluating the efficiency and epidemiological impact of vaccination programs. PCR and direct sequencing used for HPV genotyping in this project did not allow for detecting co-infection or multiple infections because only one major population of HPV can be detected by these methods; however, the advantage of applying these methods lies in their capacity to detect not only HR-HPV but also probably HR-HPV, LR-HPV and others up to 32 different genotypes. Furthermore, these molecular methods have disclosed the various HPV nucleotide sequences, which can be subjected to further studies in the future. In addition, our results used to validate with L1-based assays by using MY09/MY11 and GP5+/GP6+ primers [140, 141]. We have collected 98 samples on which Hybrid captureII (HCII) had been performed and cytology data were available. The result revealed that 80.61%(79 samples) correlation between E1 and L1 primer sets. However, we observe that ratio (relative light unit of sample divided by positive cut off of HCII) and cytological data have an effected with genotyping correlation between E1 and L1 detection (data not shown). These findings should be verified by larger studies, but may be used as preliminary data of HPV type distribution in Thailand. Future research of

HPV genotype distribution in Southeast Asia is required prior to the development of a new generation vaccine, to take into account the geographical differences that occur.

3. Multiplex real-time PCR for HPV16, HPV18 and HPV31 detection

This study has described a multiplex real-time PCR for HPV detection. The selected primers and 3 TaqMan probes labeled with FAM, JOE and ROX corresponding to HPV16, HPV18 and HPV31, that had a high prevalence in this study, were used in multiplex format for simultaneous detection of triple fluorescent signals. The advantages of real-time PCR lie in its rapidity, high specificity and high sensitivity.

The primers and probes used in this study target the L1 genes, which encoded to major capsid protein. The primers and probe of the HPV16, HPV18 and HPV31 were selected from the conserved region. The multiplex real-time PCR provided advantages over the conventional PCR in terms of being less time-consuming because post-PCR processing steps are not required resulting in a decrease in the risk of contamination. Moreover, utilizing the TaqMan probe made the assay more specific and sensitive. Finally, the real-time PCR assay provided accurate quantitative data, whereas the conventional PCR yielded just semi-quantitative data. Nevertheless, the multiplex real-time PCR had a limitation in that it can detect only high distribution genotypes (HPV16, HPV18 and HPV31) as found in this study. Some of samples can detect multiplex infection by multiplex real-time PCR, whereas conventional PCR cannot be detected. Due to its the limitation, the post-conventional PCR processing step of nucleotide sequencing is required to confirm the result. Then, the majority population of HPV in sample was amplified and showed only one genotype in the individual sample. Nevertheless, nucleotide sequencing was reliable as a gold-standard method because this method provided nucleotide sequence of HPV that can serve as basic information or be used for further study.

The real-time PCR method has more advantage than conventional PCR because its rapid and can be estimate the HPV viral load in samples. The HPV viral load illustrated the productivity of viral replication, whether the level of HPV viral load is able to predict the persistence of this virus. The association of a higher HPV viral load with persistence of the infection has been reported in some studies [188-193] but not in

others [194, 195]. Many cross-sectional studies reported an increase in HPV viral load with increasing disease severity; others found either no association or a higher viral load in women with LSIL than in those with HSIL [196-200]. However, longitudinal studies have also failed to find a consistent association between a baseline measurement of HPV viral load and duration of infection, clearance of disease, and subsequent risk of progression of disease [200-202]. There are several possible explanations for these inconsistencies of HPV viral load. The prevalence of integrated forms of HPV increases with increasing disease severity, integration of HPV is followed by a decrease in HPV viral load. Some previously reported that prevalence infection with a higher viral load of HPV16 or HPV18 was associated with short term persistence and many factors concerned with HPV viral load detection such as coinfection and cross-reactivity of cellular immune response [203]. Due to its limitation and many factors of HPV viral load studies, this experiment did not study the HPV viral load in samples.

REFERENCES

[1] Michels KB, zur Hausen H. HPV vaccine for all. The Lancet 374 (2009) : 268-270.

[2] Muñoz N, Bosch FX, De Sanjosé S, Herrero R, Castellsagué X, Shah KV, Snijders, PJ, Meijer CJ. Epidemiological classification of Human papillomavirus types associated with cervical cancer. N Engl J Med 348 (2003) : 518-527.

[3] Schwartz LA. Cervical cancer: disease prevention and informational support. Can Oncol Nurs J 19 (2009) :6-9.

[4] Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Sniders PJ, Peto J, Meijer CJ, Muñoz N. Human papillomavirus is a necessary cause of invasive cancer worldwide. J Pathol 189 (1999) : 9-12.

[5] Sriamporn S, Snijders PJ, Pientong C, Pisani P, Ekalaksananan T, Meijer CJ, Parkin DM. Human papillomavirus and cervical cancer from a prospective study in Khon Kaen, Northeast Thailand. Int J Gynecol Cancer 16 (2006) : 266-269.

[6] Clifford GM, Gallus S, Herrero R, Muñoz N, Snijders PJF, Vaccarella S, Anh PTH, Ferreccio C, Hieu NT, Matos E, Molano M, Rajkumar R, Ronco G, de Sanjosé S, Shin HR, Sukvirach S, Thomas JO, Tunsakul S, Meijer CJLM, Franceschi S. IARC HPV Prevalence Surveys study Group, Worldwide distribution of human papillomavirus types in cytologically normal women in the International Agency for Research on Cancer HPV prevalence surveys: a pooled analysis. Lancet 366 (2005) : 991-998.

[7] Ho L, Chan SY, Chow V, Chong T, Tay SK, Villa LL, Bernard HU. Sequence variations of human papillomavirus type 16 in clinical samples permit verification and extension of epidemiology studies and conclusion of phylogenetic tree. J Clin Microbiol 29 (1991) : 1765-1772.

[8] Icenogle JP, Sathya P, Miller DL, Tucker RA, Rawls WE. Nucleotide and amino acid sequence variation in the L1 and E7 open reading frames of human papillomavirus type6 and type 16. Virology 184 (1991) : 101-107.

[9] Moscicki AB, Shiboski S, Hills NK, Powell KJ, Jay N, Hanson EN, Miller S, Canjura-Clayton KL, Farhat S, Broering JM, Darragh TM. Regression of low-grade squamous intra-epithelial lesions in young women. Lancet 364 (2004) : 1678–1683.

[10] Kinney WK, Manos MM, Hurley LB, Ransley JE. Where's the high- grade cervical neoplasia? The importance of minimally abnormal Papanicolaou diagnoses. Obstet Gynecol 91 (1998) : 973-976.

[11] Sherman ME, Schiffman MH, Lorincz AT. Cervical specimens collected in liquid buffer are

suitable for both cytologic screening and ancillary human papillomavirus testing. Cancer 81 (1997) : 89-97.

[12] Depuydt CE, Vereecken AJ, Salembier GM, Vanbrabant AS, Boels LA, van Herck E, Arbyn M, Segers K, Bogers JJ. Thin-layer liquid -based cervical cytology and PCR for detecting and typing human papillomavirus DNA in Flemish women. Br J Cancer 88 (2003) : 560-566.

[13] Hubbard RA. Human papillomavirus testing methods. Arch Pathol Lab Med 127 (2003) : 940-945.

[14] Lorincz AT. Hybrid Capture method for detection of human papillomavirus DNA in clinical specimens: a tool for clinical management of equivocal Pap smears and for population screening. J Obstet Gynaecol Res 22 (1996) : 629-636.

[15] Burd EM. Human papillomavirus and cervical cancer. Clin Microbiol Rev 16 (2003) : 1-17.

[16] Seme K, Fujs K, Kocjan BJ, Poljak M. Resolving repeatedly borderline results of Hybrid Capture 2 HPV DNA Test using polymerase chain reaction and genotyping. J Virol Methods 134 (2006) : 252–256.

[17] Asato T, Maehama T, Nagai Y, Kanazawa K, Uezato H, Kariya K. A Large case-control study of cervical cancer risk associated with human papillomavirus infection in Japan, by nucleotide sequencing-based genotyping. J Infect Dis 189 (2004) : 1829-1832.

[18] Bernard HU, Chan SY, Manos MM, Ong CK, Villa LL, Delius H, Peyton CL, Bauer HM, Wheeler CM. Identification and assessment of known and novel human papillomavirus by polymerase chain reaction amplification, restriction fragment length polymorphisms, nucleotide sequence, and phylogenetic algorithms. J Infect Dis 170 (1994) : 1077-1085.

[19] Kleter B, van Doorn LJ, Schrauwen L, Molijin A, Sastrowijoto S, ter Schegget J, Lindeman J, ter Harmsel B, Burger M, Quint W. Development and clinical evaluation of highly sensitive PCR-reverse hybridization line probe assay for detection and identification of anogenital human papillomavirus. J Clin Microbiol 37 (1999) : 2508-2517.

[20] Han J, Swan DC, Smith SJ, Lum SH, Sefers SE, Unger ER, Tang YW. Simultaneous amplification and identification of 25 human papillomavirus types with Templex technology. J Clin Microbiol 44 (2006) : 4157-4162.

[21] Federschneider JM, Yuan L, Brodsky J, Breslin G, Betensky RA, Crum CP. The borderline or weakly positive Hybrid Capture II HPV test: a statistical and comparative (PCR) analysis. Am J Obstet Gynecol 191 (2004) : 757–761.

[22] Iftner T, Germ L, Swoyer R, Kjaer SK, Breugelmans JG, Munk C, Stubenrauch F,

Antonello J, Bryan JT, Taddeo FJ. Study comparing human papillomavirus (HPV) real-time multiplex PCR and hybrid capture II Inno-Lipa v2 HPV genotyping PCR assays. J Clin Microl 47 (2009) : 2106-2113.

[23] Jansen K, Taddeo FJ, Weili L, DiCello AC. Fluorescent multiplex HPV PCR assays using multiple fluorophores. 2003.WO patent 2003/019143 A2.

[24] Taddeo FJ, Skulsky DM, Wang XM, Jansen KU. 2006. Real-time HPV PCR assays. WO patent 2006/116276 A2.

[25] Taddeo FJ, Skulsky DM, Wang XM, Jansen KU. 2006. Fluorescent multiplex HPV PCR assay. WO patent 2006/116303 A2.

[26] Tucker RA, Unger ER, Holloway BP, Swan DC. Real-time PCR-based fluorescent assay for quantitation for human papillomavirus types 6, 11, 16 and 18. Mol Diagn 6 (2001) : 39-47.

[27] Cañadas MP, Darwich L, Sirera G, Cirigliano V, Bofill M, Clotet B, Videla S; on behalf of the HIV-HPV Study Group. New molecular method for the detection of human papillomavirus type 16 integration. Clin Microbiol Infect 2009; doi:10.1111/j.1469-0691.2009.02964.x

[28] Takács T, Jeney C, Kovács L, Mózes J, Benczik M, Sebe A. Molecular beacon-based real-time PCR method for detection of 15 high-risk and 5 low-risk HPV types. J virol Methods 149 (2008) : 153-162.

[29] Hoory T, Monie A, Gravitt P, Wu TC. Molecular epidemiology of human papillomavirus. J Formos Med Assoc 107 (2008) : 198-217.

[30] de Villiers EM. Taxonomic classification of papillomaviruses. Papillomavirus Rep 12 (2001) : 57-63.

[31] Antonsson A, Erfurt C, Hazard K, Holmgren V, Simon M, Kataoka A, Hossain S, Hakangard C, Hansson BG. Prevalence and type spectrum of human papillomaviruses in healthy skin samples collected in three continents. J Gen Virol 84 (2003) : 1881-1886.

[32] Schiffman M, Herrero R, Desalle R, Hideseim A, Wacholder S, Rodriguez AC, Bratti MC, Sherman ME, Morales J, Guillen D, Alfaro M, Hutchinson M, Wright TC, Solomon D, Chen Z,Schusseler J, Castle PE, Burk RD. The carcinogenicity of human papillomavirus types reflects viral evolution. Virology 337 (2005) : 76-84.

[33] Scheurer ME, Tortolero-Luna G, Adler-Storthz K. Human papillomavirus infection: biology, epidemiology and prevention. J Int Gynecol Cancer 15 (2005) : 727-746.

[34] Doorbar J. Molecular biology of human papillomavirus infection and cervical cancer. Clinical science 110 (2006) : 525-541.

[35] Zheng ZM, Baker CC. Papillomavirus genome structure, expression, and post-transcriptional regulation. Front Biosci 11 (2006) : 2286-2302.

[36] Danos O, Katinka M, Yaniv M. Human papillomavirus 1a complete DNA sequence; a novel type of genome organization among papovaviridae. EMBO J 1 (1982) : 231-236.

[37] Doorbar J, Elston RC, Napthine S, Raj K, Medicalf E, Jackson D, Coleman N, Griffin HM, Masterson P, Stacey S, Mengistu Y, Dunlop J. The E1E4 protein of human papillomavirus type 16 associates with a putative RNA helicase through sequences in its C terminus. J virol 74 (2000) : 10081-10095.

[38] Patterson NA, Smith JL. Ozbun MA. Human papillomavirus type31b infection of human keratinocytes does not require heparan sulfate. J Virol 79 (2005) : 6838-6847.

[39] Shafit-Keramat S, Handisurya A, Kriehuber E, Meneguzzi G, Slupetzky K, Kirnbauer R. Different heparan sulfate proteoglycans serve as cellular receptors for human papillomaviruses. J Virol 77 (2003) : 13125-13135.

[40] Loyce JG, Tung JS, Przysiecki CT, Cook JC, Lehman ED, Sands JA, Jansen KU, Keller P. The L1 major capsid protein of human papillomavirus type 11 recombinant virus-like particles interacts with heparin and cell-surface glycosaminoglycans on human keratinocytes. J Biol Chem 274 (1999) : 5810-5822.

[41] McMillan NA, Payne E, Frazer IH, Evander M. Expression of the α6 integrin confers papillomavirus binding upon receptor-negative B-cells. Virology 261 (1999) : 271-279.

[42] Evander M, Frazer IH, Payne E, Mei QY, Hengst K, McMillan NA. Identification of the α6 integrin as a candidate receptor for papillomaviruses. J Virol 71 (1997) : 2449-2456.

[43] Bossis I, Roden RB, Gambhira R, Yang R, Tagaya M, Howley PM, Meneses PI. Interaction of tSNARE syntaxin 18 with the papillomavirus minor capsid protein mediates infection. J Virol 79 (2005) : 6723-6731.

[44] Day PM, Lowy DR, Schiller JT. Human papillomavirus infect cells via a clathrin-dependent pathway. Virology 307 (2004) : 1-11.

[45] Day PM, Baker CC, Lowy DR, Schiller JT. Establishment of papillomavirus infection is enhanced by promyelocytic leukemia protein (PML) expression. Proc Natl Acad Sci USA 101 (2004) : 14252-14257.

[46] Hughes FJ, Romanos MA. E1 protein of human papillomavirus is a DNA helicase/ATPase. Nucleic Acids Res 21 (1993) : 5817-5823.

[47] Seo YS, Mulle F, Lusky M, Hurwitz J. Bovine papilloma virus (BPV)-encoded E1 protein contains multiple activities required for BPV DNA replication. Proc Natl Acad Sci USA 90 (1993) : 702–706.

[48] Doorbar J, Ely S, Sterling J, McLean C, Crawford L. Specific interaction between HPV-16 E1–E4 and cytokeratins results in collapse of the epithelial cell intermediate filament network. Nature 352 (1991) : 824–827.

[49] Crusius K, Auvinen E, Alonso A. Enhancement of EGF- and PMA-mediated MAP kinase activation in cells expressing the human papillomavirus type 16 E5 protein. Oncogene 15 (1997) : 1437–1444.

[50] Ko L, Prives C. p53: puzzle and paradigm. Genes Dev 10 (1996) : 1054–1072.

[51] Berezutskaya E, Yu B, Morozov A, Raychaudhuri P, Bagchi S. Dfferential regulation of the pocket domains of the retinoblastoma family proteins by the HPV16 E7 oncoprotein. Cell Growth Differ 8 (1997) : 1277–1286.

[52] Davy CE, Jackson DJ, Wang Q, Raj K, Masterson PJ, Fenner NF, Southern S, Cuthill S, Millar JB, Doorbar J. Identification of a G2 arrest domain in the E1 wedge E4 protein of human papillomavirus type 16. J Virol 76 (2002) : 9806–9818.

[53] Florin L, Sapp C, Streeck RE, Sapp M. Assembly and translocation of papillomavirus capsid proteins. J Virol 76 (2002) : 10009–10014.

[54] Doorbar J, Gallimore PH. Identification of proteins encoded by the L1 and L2 open reading frames of human papillomavirus type 1a. J Virol 61 (1987) : 2793–2799.

[55] Schwartz S. Regulation of human papillomavirus late gene expression. Ups J Med Sci 105 (2000) : 171–192.

[56] Zhou J, Liu WJ, Peng SW, Sun XY, Frazer I. Papillomavirus capsid protein expression level depends on the match between codon usage and tRNA availability. J Virol 73 (1999) : 4972–4982.

[57] Day PM, Roden RBS, Lowy DR, Schiller JT. The papillomavirus minor capsid protein, L2, induces localization of the major capsid protein, L1 and the viral transcription/replication protein, E2, to PML oncogenic domains. J Virol 72 (1998) : 142–150.

[58] Zhao KN, Hengst K, Liu WJ, Liu YH, Liu XS, McMillan NA, Frazer IH. BPV1 E2 protein enhances packaging of full-length plasmid DNA in BPV1 pseudovirions. Virology 272 (2000) : 382–393.

[59] Bryan JT, Brown DR. Association of the human papillomavirus type 11 E1()E4 protein with cornified cell envelopes derived from infected genital epithelium. Virology 277 (2000) : 262–269.

[60] Lehr E, Hohl D, Huber M, Brown D. Infection with Human Papillomavirus alters expression of the small proline rich proteins 2 and 3. J Med Virol 72 (2004) : 478–483.

[61] Kurman RJ, Henson DE, Herbst AL, Noller KL, Schiffman MH. Interim guidelines for

management of abnormal cervical cytology. JAMA 271 (1994) : 1866-1869.

[62] De Schryver A, Meneus A. Epidemiology of sexually transmitted disease: the global picture. Bull World Health Organ 68 (1990) : 639-654.

[63] Khanna J, Van Look PFA, Griffin PD. Reproductive health: a key to a brighter future: biennial report 1990-1991. Geneva, Switzerland: World Health Organization, 1992.

[64] Cates WJ. Estimates of the incidence and prevalence of sexually transmitted disease in the United States. American Social Health Association Panel. Sex Transm Dis 26 (1999) (Supple.):S2-7.

[65] Moscicki AB, Hills N, Shiboski S, Powell K, Jay N, Hanson E, Miller S, Clayton L, Farhat S, Broering J, Darragh T, Palefsky J. Risks for incident human papillomavirus infection and low- grade squamous intraepithelial lesion development in young females. JAMA 285 (2001) : 2995-3002.

[66] Giuliano AR, Harris R, Sedjo RL, Baldwin S, Roe D, Papenfuss MR, Abrahamsen M, Inserra P, Olvera S, Hatch K. Incidence, prevalence, and clearance of type-specific human papillomavirus infections: the Young Women's Health Study. J Infect Dis 186 (2002) : 462-469.

[67] Winer RL, Lee SK, Hughes JP, Adam DE, Kiviat NB, Koutsky LA. Genital human papillomavirus infection: incidence and risk factors in a cohort of female university students. Am J Epidemiol 157 (2003) : 218-226.

[68] Sellors JW, Karwalajtys TL, Kaczorowski J, Mahony JB, Lytwyn A, Chong S, Sparrow J, Lorincz A; Survey of HPV in Ontario Women Group. Incidence, clearance and predictors of human papillomavirus infection in women. CMAJ 168 (2003) : 421-425.

[69] Molano M, Posso H, Weiderpass E, van den Brule AJ, Ronderos M, Franceschi S, Meijer CL, Arslan A, Munoz N; HPV Study Group HPV Study. Prevalence and determinants of HPV infection among Colombia women with normal cytology. Br J Cancer 87 (2002) : 324-333.

[70] Herrero R, Hildesheim A, Bratti C, Sherman ME, Hutchinson M, Morales J, Balmaceda I, Greenberg MD, Alfaro M, Burk RD, Wacholder S, Plummer M, Sachiffman M. Population based study of human papillomavirus infection and cervical neoplsia in rural Costa Rica. J Natl Cancer Inst 92 (2000) : 464-474.

[71] Lazcano-Ponce E, Herrero R, Munoz N, Cruz A, Shah KV, Alonso P, Hemandez P, Salmeron J, Hernandez M. Epidemiology of HPV infection among Mexican Women with normal cervical cytology. Int J Cancer 91 (2001) : 412-420.

[72] Clifford GM, Gallus S, Herrero R, Muñoz N, Snijders PJF, Vaccarella S, Anh PTH, Ferreccio

C, Hieu NT, Matos E, Molano M, Rajkumar R, Ronco G, Sanjosé Sde, Shin HR, Sukvirach S, Thomas JO, Tunsakul S, Meijer, Franceschi S, the IARC HPV Prevalence Surveys Study Group CJLM. Worldwide distribution of human papillomavirus types in cytologically normal women in the international agency for research on cervical cancer HPV prevalence surveys: a pooled analysis. Lancet 366 (2005) : 991–98.

[73] Clifford GM, Smith JS, Aguado T, Franceschi S. Comparison of HPV type distribution in high-grade cervical lesions and cervical cancer: a meta-analysis. Br J Cancer 89 (2003) : 101–105.

[74] Clifford GM, Smith JS, Plummer M, Muñoz N, Franceschi S. Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. Br J Cancer 88 (2003) : 63–73.

[75] Herrero R, Castle PE, Schiffman M, et al. Epidemiologic profile of type-specific HPV infection and cervical neoplasia in Guanacaste, Costa Rica. J Infect Dis 191 (2005) : 1796–1807.

[76] Clifford GM, Rana RK, Franceschi S, Smith JS, Gough G, Pimenta JM. Human papillomavirus genotype distribution in low grade cervical lesions: comparison by geographic region and with cervical cancer. Cancer Epidemiol Biomarkers Prev 14 (2005) : 1157-1164.

[77] Schwartz SM, Daling JR, Shera KA, Madeleine MM, McKnight B, Galloway DA, Porter PL, McDougall JK. Human papillomavirus and prognosis of invasive cervical cancer: a population-based study. J Clin Oncol 19 (2001) : 1906–1915.

[78] Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Munoz N. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 189 (1999) : 12–19.

[79] Londesborough P, Ho L, Terry G, Cuzick J, Wheeler C, Singer A. Human papillomavirus genotype as a predictor of persistence and development of high-grade lesions in women with minor cervical abnormalities. Int J Cancer 69 (1996) : 364–368.

[80] Bao YP LI N, Smith JS, Qiao YL, ACCPAB members. Human papillomavirus type distribution in women from Asia: a meta-analysis. Int J Gynecol Cancer 18 (2008) : 71-79.

[81] Iftner T, Villa LL. Chapter 12: Human papillomavirus technologies. J Natl Cancer Inst Monogr 31 (2003) : 80–88.

[82] Hildesheim A, Wang SS. Host and viral genetics and risk of cervical cancer: a review. Virus Res 89 (2002) : 229–240.

[83] Strickler HD, Palefsky JM, Shah KV, Anastos K, Klein RS, Minkoff H, Duerr A, Massad LS,

Celentano DD, Hall C, Fazzari M, Cu-Uvin S, Bacon M, Schuman P, Levine AM, Durante AJ, Gange S, Melnick S, Burk RD. Human papillomavirus type 16 and immune status in human immunodeficiency virus-seropositive women. J Natl Cancer Inst 95 (2003) : 1062–1071.

[84] Konya J, Dillner J. Immunity to oncogenic human papillomaviruses. Adv Cancer Res 82 (2001) : 205-238.

[85] Stanley MA. Immunobiology of papillomavrus infections. J Reprod Immunol 52 (2001) : 45-59.

[86] Kirnbauer R, Booy F, Cheng N, Lowy DR, Schiller JT. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. Proc Natl Acad Sci USA 89 (1992) : 12180-12184.

[87] Hageness ME, Yaegashi N, Galloway DA. Self-assembly of human papillomavirus type 1 capsids by expression of the L1 protein alone or by coexpression of the L1 and L2 capsid proteins. J Virol 67 (1993) : 315-322.

[88] Rose RC, Bonnez W, Reichman RC, Garcea RL. Expression of human papillomavirus type 11 L1 protein in insect cells: in vivo and in vitro assembly of virus like particles. J Virol 67 (1993) : 1936-1944.

[89] Christensen ND, Hopfl R, DiAngelo SL, Assembled baculovirus-expressed human papillomavirus type 11 L1 capsid protein virus like particles are recognized by neutralizing monoclonal antibodies and induce high titres of neutralizing antibodies. J Gen Virol 75 (1994) : 2271-2276.

[90] Li M, Cripe TP, Estes PA, Lyon MK, Rose RC, Garcea RL. Expression of the human papillomavirus type 11 L1 capsid protein in Escherichia coli: characterization of protein domains involved in DNA binding and capsid assembly. J Virol 71 (1997) : 2988-2995.

[91] Roden RB, Greenstone HL, Kirnbauer R, Booy FP, Jessie J, Lowy DR, Schiller JT. In vitro generation and type-specific neutralization of a human papillomavirus type 16 virion pseudotype. J Virol 70 (1996) : 5875-5883.

[92] White WI, Wilson SD, Bonnez W, Rose RC, Koenig S, Suzich JA. In vitro infection and type-restricted antibody-mediated neutralization of authentic human papillomavirus type 16. J Virol 72 (1998) : 959-964.

[93] Jansen KU, Rosolowsky M, Schultz LD, Markus HZ, Cook JC, Donnelly JJ, Martinez D, Ellis RW, Shaw AR. Vaccination with yeast-expressed cottontail rabbit papillomavirus (CRPV) virus-like particles protects rabbits from CRPV-induced papilloma formation. Vaccine 69 (1995) : 3959-3963.

[94] Breitburd F, Kirnbauer R, Hubbert NL, Noooenmacher B, Trin-Dinh-Desmarquet C, Orth G, Schiller JT, Lowy DR. Immunization with virus like particles from cottontail rabbit papillomavirus (CRPV) can protect against experimental CRPV infection. J Virol 69 (1995) : 3959-3963.

[95] Kirnbauer R, Chandrachud LM, O'Neil BW, Wanger ER, Grindlay GJ, Armstrong A, McGarvie GM, Schiller JT, Lowy DR, Campo MS. Virus-like particles of bovine papillomavirus type 4 in prophylactic and therapeutic immunization. Virology 219 (1996) : 37-44.

[96] Suzich JA, Ghim SJ, Palmer-Hill FJ, White WI, Tamura JK, Bell JA, Newsome JA, Jenson AB, Schlegel R. Systemic immunization with papillomavirus L1 protein completely prevents the development of viral mucosal papillomas. Proc Natl Acad Sci USA 92 (1995) : 11553-11557.

[97] Stanley M, Gissmann L, Nardelli-Haefliger D. Immunobiology of Human Papillomavirus infection and vaccination implications for second generation vaccines. Vaccine 26 (2008) : 62-67.

[98] Trimble CL, Franzer IH. Development of therapeutic HPV vaccines. The Lancet 10 (2009) : 975-980.

[99] Southern EM. Detection of specific sequences among DNA fragments sep- arated by gel electrophoresis. J Mol Biol 98 (1975) : 503–517.

[100] Schiffman MH, Bauer HM, Lorincz AT, Manos MM, Byrne JC, Glass AG, CadellDM, Howley PM. Comparison of Southern blot hybridization and polymerase chain reaction methods for the detection of human papillomavirus DNA. J Clin Microbiol 29 (1991) : 573-577.

[101] Schneider A, Kraus H, Schuhmann R, Gissmann L. Papillomavirus infection of the lower genital tract: detection of viral DNA in gynecological swabs. Int J Cancer 35 (1985) : 443-448.

[102] Caussy D, Orr W, Daya AD, Roth P, Reeves W, Rawls W. Evaluation of methods for detecting human papillomavirus deoxyribonucleotide sequences in clinical specimens. J Clin Microl 26 (1988) : 236-243.

[103] Bekkers RL, Melchers WJ, Bulten J, Boonstra H, Quint WG, Hanselaar AG, Massuger LF. Localized distribution of human papillomavirus genotypes in the uterine cervix. J Gynaecol Oncol 23 (2002) : 203-206.

[104] Ausubel FM. Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology. 4th ed. New York, NY: Wiley, 1999.

[105] Huang CC, Qiu JT, Kashima ML, Kurman RJ, Wu TC. Generation of type- specific probes

the detection of single-copy human papillomavirus by a novel in situ hybridization method. Mod Pathol 11 (1998) : 971–977.

[106] Schwarzacher T, Heslop-Harrison J. Practical In Situ Hybridization. New York, NY: Springer Verlag; 2000.

[107] Myer T, Arndt R, Stockfleth E, Flammann HT, Wolf H, Reischl U. Strategy for typing human papillomavirus by RFLP analysis of PCR products and sub-sequent hybridization with a generic probe. Biotechniques 19 (1995) : 632–639.

[108] Gibson UE, Heid CA, Williams PM. A novel method for real time quantitative RT-PCR. Genome Res 6 (1996) : 995–1001.

[109] Rady PL, Arany I, Hughes TK, Tyring SK. Type-specific primer-mediated direct sequencing of consensus primer-generated PCR amplicons of human papillomavirus: a newapproach for the simultaneous detection of multiple viral type infections. J Virol Method 53 (1995) : 245–254.

[110] Kalantari M, Karlsen F, Johansson B, Sigurjonsson T, Warleby B, Hagmar B. Human papillomavirus findings in relation to cervical intraepithelial neoplasia grade: a study on 476 Stockholm women, study using PCR for detection and typing of HPV. Hum Pathol 28 (1997) : 899–904.

[111] Zhao S, Lu S, Fei S. Detection of human papillomavirus in Chinese laryngeal carcinoma with digoxigenin labelled probe prepared by PCR [in Chinese]. Lin Chuang Er BiYan Hou Ke Za Zhi 12 (1998) : 153-155.

[112] Jacobs MV, Walboomers JM, van Beek J, Voorhorst FJ, Verheijen RH, Meijer CJ, van den Brule AJ, Helmerhorst TJ, Snijders PJ. A quantitative polymerase chain reaction–enzyme immunoassay for accurate measurements of human papillomavirus type 16 DNA levels in cervical scrapings. Br J Cancer 81 (1999) : 114–121.

[113] Nindl I, Lotz B, Kuhne-Heid R, Endisch U, Schneider A. Distribution of 14 high risk HPV types in cervical in cervical intraepithelial neoplasia detected by a non-radio-active general primer PCR mediated enzyme immunoassay. J Clin Pathol 52 (1999) : 17–22.

[114] Gravitt PE, Peyton CL, Alessi TQ, Wheeler CM, Coutlée F, Hildesheim A, Schiffman MH, Scott DR, Apple RJ. Improved amplification of genital human papillomaviruses. J Clin Microbiol 38 (2000) : 357–361.

[115] Stephen AL, Thompson CH, Tattersall MH, Cossart YE, Rose BR. Analysis of mutations in the URR and E6/E7 oncogenes of HPV 16 cervical cancer isolates from central China. Int J Cancer 86 (2000) : 695–701.

[116] Peyton CL, Schiffman M, LÖrincz AT, Hunt WC, Mielzynska I, Bratti C, Eaton S, Hildesheim

A, Morera LA, Rodriguez AC, Herrero R, Sherman ME, Wheeler CM. Comparison of PCR- and hybrid capture-based human papillomavirus detection systems using multiple cervical specimen collection strategies. J Clin Micro biol 36 (1998) : 3248 – 54.

[117] Vernon SD, Unger ER, Williams D. Comparison of human papillomavirus detection and typing by cycle sequencing, line blotting, and hybrid capture. J Clin Microbiol 38 (2000) : 651-655.

[118] Coutlee F, Gravitt P, Kornegay J, Hankins C, Richardson H, Lapointe N, Voyer H, Franco E. Use of PGMY primers in L1 consensus PCR improves detection of human papillomavirus in genital samples. J Clin Microbiol 40 (2002) : 902-907.

[119] Clifford G, Franceschi S, Diaz M, Muñoz N, Villa LL. Chapter 3: HPV-type distribution in women with and without cervical neoplastic diseases. Vaccine 2006;23S3:S3/26 – S3/24.

[120] Kleter B, van Doorn LJ, ter Schegget J, Schrauwen L, van Krimpen K, Burger M, ter Harmsel B, Quint W. Novel short-fragment PCR assay for highly sensitive broad-spectrum detection of anogenital human papillomaviruses. Am J Pathol 153 (1998) : 1731– 1739.

[121] Perrons C, Kleter B, Jelley R, Jalal H, Quint W, Tedder R. Detection and genotyping of human papillomavirus DNA by SPF10 and MY09/11 primers in cervical cells taken form women attending a colposcopy clinic. J Med Virol 67 (2002) : 246–252.

[122] Hubbard RA. Human papillomavirus testing methods. Arch Pathol Lab Med 127 (2003) : 940-945.

[123] Van Ham MA, Bakker JM, Harbers GK, Quint WG, Massuder LF, Melchers WJ. Comparison of two commercial assays for detection of human papillomavirus (HPV) in cervical scrape specimens: validation of the Roche AMPLICOR HPV test as a means to screen for HPV genotypes associated with a higher risk of cervical disorders. J Clin Microbiol 43 (2005) : 2662–2667.

[124] Kleter B, Van Doorn L, Schrauwen L, Molijn A, Sastrowijoto S, ter Schegget J, Lindeman J, Ter Harmsel B, Burger M, Quint W. Development and clinical evaluation of a highly sensitive PCR-reverse hybridization line probe assay for detection and identification of anogenital human papillomavirus. J Clin Microbiol 37 (1999) : 2508–2517.

[125] Hart KW, Williams OM, Thelwell N, Fiander AN, Brown T, Borysiewicz LK, Gelder CM. Novel method for detection, typing, and quantification of human papillomaviruses. J Clin Microbiol 39 (2001) : 3204-3212.

[126] De Marco F, Di Carlo A, Poggiali F, Muller A, Marcante ML. Detection of HPV in genital

condylomata: correlation between viral load and clinical out come. J Exp Clin Cancer Res 20 (2001) : 377–383.

[127] Josefsson AM, Magnusson PK, Ylitalo N, Sorensen P, Qwarforth-Tubbin P, Andersen PK, Melbye M, Adami HQ, Gyllensten UB. Viral load of human papilloma virus 16 as a determinant for development of cervical carcinoma in situ: a nested case-control study. Lancet 355 (2000) : 2189–2193.

[128] Peitsaro P, Johansson B, Syrjanen S. Integrated human papillomavirus type 16 is frequently found in cervical cancer precursors as demonstrated by a novel quantitative real-time PCR technique. J Clin Microbiol 40 (2002) : 886–891.

[129] Sun CA, Liu JF, Wu DM, Nieh S, Yu CP, Chu TY. Viral load of high-risk human papillomavirus in cervical squamous intraepithelial lesions. Int J Gynaecol Obstet 76 (2002) : 41-47.

[130] Zerbini M, Venturoli S, Cricca M, Gallinella G, De Simone P, Costa S, Santini D, Musiani M. Distribution and viral load of type specific HPVs in different cervical lesions as detected by PCR-ELISA. J Clin Pathol 54 (2001) : 377–380.

[131] Arias-Pulido H, Peyton C, Joste N, Vargas H, Wheeler C. Human papillomavirus type 16 integration in cervical carcinoma in situ and in invasive cervical cancer. J Clin Microbiol 44 (2006) : 1755–1762.

[132] Molijn A, Kleter B, Quint W, van Doorn LJ. Molecular diagnosis of human papillomavirus (HPV) infections. J Clin Virol 32 (2005) : S43–51.

[133] Cuschieri KS, Whitley MJ, Cubie HA. Human papillomavirus type specific DNA and RNA persistence-implications for cervical disease progression and monitoring. J Med Virol 73 (2004) : 65–70.

[134] Wang-Johanning F, Lu DW, Wang Y, Johnson MR, Johanning GL. Quantitation of human papillomavirus 16 E6 and E7 DNA and RNA in residual material from ThinPrep Papanicolaou test using real-time polymerase chain reaction analysis. Cancer 94 (2002) : 2199–210.

[135] Dollard SC, Wilson JL, Demeter LM, Bonnez W, Reichman RC, Broker TR, Chow LT. Production of human papillomavirus and modulation of the infectious program in epithelial raft cultures. Genes & Dev 6 (1992) : 1131-1142.

[136] Meyers C, Frattini MG, Hudson JB, Laimins LA. Biosynthesis of human papillomavirus from a continuous cell line upon epithelial differentiation. Science 257 (1992) : 971-973.

[137] Wang HK, Duffy AA, Broker TR, Chow LT. Robust production and passaging of infectious HPV in squamous epithelium of primary human keratinocytes. Gene & Dev 23 (2008) : 181-194.

[138] Shadrina MI, Semenova EV, Slominsky PA. Effective quantitative real-time polymerase chain reaction analysis of the parkin gene (PARK2) exon 1-12 dosage. BMC Med Genet 8 (2007) : 6.

[139] Manos MM, Ting Y, Wright DK, Lewis AJ, Broker TR, Wolinsky SM. Use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. Cancer Cells 7 (1989) : 209–214.

[140] Evander M, Edlund K, Bodén E, Gustafsson A, Jonsson M, Karlsson R, Rylander E, Wadell G. Comparison of one-step and two step polymerase chain reaction with degenerate general primers in population-based study of Human papillomavirus infection in young Swedish women. J Clin Microbiol 30 (1992) : 987-992.

[141] Jacobs MV, de Roda Husman AM, van den Brule AJ, Snijders PJ, Meijer CJ, Walboomers JM. Group specific differentation between high- and low-risk human papillomavirus genotypes by general primer-mediated PCR and two cocktails of oligonucleotide probes. J Clin Microbiol 33 (1995) : 901-905.

[142] Kumar S, Tamura K, Nei M. MEGA3: Integrated software for molecular evolution genetics analysis and sequencing alignment. Brief Bioinform 5 (2004) : 150–163.

[143] Pond SL, Frost SD. Datamonkey: rapid detection of selective pressure on individual sites of Codon alignments. Bioinformatics 21(2005) : 2531-2533.

[144] Myers G, Delius H, Icenogle J, Bernard HU, Favre M, van Ranst M, WheelerCM. Human papillomaviruses 1997: a complication and analysis of nucleic acid and amino acid sequences. Los Alamos National Laboratory: Los Alamos, 1997.

[145] Chen Z, Terai M, Fu L, Herrero R, DeSalle R, Burk RD. Diversifying selection in human papillomavirus type 16 lineages based on complete genome analyses. J Virol 79 (2005) : 7014–7023.

[146] Ho H, Chan SY, Burk RD, Das BC, Fuginaga K, Icenogle JP, Kahn T, Kiviat N, Lancaster W, Mavromara-Nazos P, Labropoulou V, Mitrani-Rosenbaum S, Norrild B, Pillai MR, Stoerker J, Syrjaenen K, Syrjaenen S, Tay SK, Villa LL, Wheeler CM, Williamson AL, Bernard HU, The genetic drift of human papillomavirus type 16 is a means of reconstructing prehistoric viral spread and the movement of ancient human populations. J Virol 67 (1993) : 6413–6423.

[147] Yamada T, Manos MM, Peto J, Greer CE, Muñoz N, Bosch FX. Human papillomavirus type 16 sequence variation in cervical cancers: a worldwide perspective. J Virol 71 (1997) : 2463-2472.

[148] Kurvinen K, Yliskoski M, Saarikoski S, Syrjänen K, Syrjänen S. Variants of the long control region of human papillomavirus type 16. Eur J cancer 36 (2000) : 1402-1410.

[149] Dong XP, Stubenrauch F, Beyer-Finkler E, Pfister H. Prevalence of deletions of YY1-binding sites in episomal HPV16 DNA from cervical cancers. Int J Cancer 58 (1994) : 803–808.

[150] Lee K, Magalhaes I, Clavel C, Briolat J, Birembaut P, Tommasinod M, Zehbe I. Human papillomavirus 16 E6, L1, L2 and E2 gene variants in cervical lesion progression. Virus Res 131 (2008) : 106-110.

[151] Cerqueira DM, Raiol T, Véras NM, von Gal Milanezi N, Amaral FA, de Macedo Brígido M, Martin CR. New variants of Human papillomavirus type 18 identified in Central Brazil. Virus Genes 3 (2008) : 282-287.

[152] Rose BR, Thompson CH, Zhang J, Stoeter M, Stephen A, Pfister H, Tattersall MH, Cossart YE. Sequence variation in the upstream regulatory region of HPV 18 isolates from cervical cancers. Gynecol Oncol 66 (1997) : 282–289.

[153] Combita AL, Touzé A, Bousarghin L, Christensen ND, Coursaget P. Identification of two cross-neutralizing linear epitopes within the L1 major capsid protein of human papillomaviruses. J Virol 76 (2002) : 6480–6486.

[154] Payungporn S. Molecular characterization and molecular diagnosis of recently emerged influenza A viruses (H5N1 & H3N8). Doctoral Dissertation Biomedical sciences, graduate school, Chulalongkorn University, 2006.

[155] Pannier-Stockman C, Segard C, Bennamar S, Gondry J, Sevestre H, Castelain S, Duverlie G. Prevalence of HPV genotypes determined by PCR and DNA sequencing in cervical specimens from French women with or without abnormalities. J Clin Microl 42 (2008) : 353-360.

[156] Xi LF, Koutsky LA, Galloway DA, Kuypers J, Hughes JP, Wheeler CM, Holmes KK, Kiviat NB. Genome variation of human papillomavirus type 16 and risk for high grade cervical intraepithelial neoplasia. J Natl Cancer Inst 89 (1997) : 796-802.

[157] Xi LF, Critchlow CW, Wheeler CM. Risk of anal carcinoma in situ in relation to human papillomavirus type 16 variants. Cancer Res 58 (1998) : 3839-3844.

[158] Andersson S, Alemi M, Rylander E, Strand A, Larsson B, Sallstrom J, Wilander E. Uneven distribution of HPV16 E6 protoype and variant (L83V) oncoprotein in cervical neoplasia lesions. Br J Cancer 83 (2000) : 307-310.

[159] Zehbe I, Voglino G, Delius H, Wilander E, Tommasino M. Risk of cervical cancer amd geographical variants of human papillomavirus 16 E6 polymorphisms. Lancet 352 (1998) : 1441–1442.

[160] Casas L, Galvan SC, Ordonez RM, Lopez N, Guido M, Berumen J. Asian-american variants

of human papillomavirus type 16 have extensive mutations in the E2 gene and are highly amplified in cervical carcinomas. Int J Cancer 83 (1999) : 449-455.

[161] Hall S, Lörincz A, Shah F, Sherman ME, Abbas F, Paull G, Kurman RJ, Shah KV. Human papillomavirus DNA detection in cervical specimens by hybrid capture: correlation with cytologic and histologic diagnoses of squamous intraepithelial lesions of the cervix. Gynecol Oncol 62 (1996) : 353-359.

[162] Ellis JR, Keating M, Baird PJ, Hounsell J, Renouf EF, Rowe DV, Hopkins M, Duggan-Keen D, Bartholomew MF, Young JS, Stern LS. The association of an HPV16 oncogene variant with HLA-B7 has implications for vaccine design in cervical cancer. Nat Med 5 (1995) : 464-470.

[163] Yamada T, Wheeler CM, Halpern AL, Stewart AC, Hildesheim A, Jenison SA. Human papillomavirus type16 vqriants lineages in United States populations characterized by nucleotide sequence analysis of the E6, L2 and L1 coding segments. J Virol 69 (1995) : 7743-7753.

[164] Vaeteewoottacharn K, Jearanaikoo P, Ponglikitmongkol M. Co-mutation of HPV16 E6 and E7 genes in Thai squamous cervical carcinomas. Anticancer Res 23 (2003) : 1927-1931.

[165] Song YS, Kee SH, Kim JW, Park NH, Kang SB, Chang WH, Lee HP. Major sequence variants in E7 gene of human papillomavirus type 16 from cervical cancerous and noncancerous lesion of Korean women. Gynecol Oncol 66 (1997) : 275-281.

[166] Eriksson A, Herron JR, Yamada T, Wheeler CM. Human papillomavirus type 16 variant lineages characterized by nucleotide sequence analysis of the E5 coding segment and the E2 hinge region. J Gen Virol 80 (1999) : 595-600.

[167] Lurchachaiwong W, Junyangdikul P, Payungporn S, Chansaenroj J, Sampathanukul P, Tresukosol D, Termrungruanglert W, Theamboonlers A, Poovorawan Y. Entire genome characterization of human papillomavirus type 16 (HPV16) from infected Thai women with different cytological findings. Virus Genes 39 (2009) : 30-38.

[168] de la Cruz-Hernández E, García-Carrancá A, Mohar-Betancourt A, Dueñas-González A, Contreras-Paredes A, Pérez-Cardenas E, Herrera-Goepfert R, Lizano-Soberón M. Differential splicing of E6 within human papillomavirus type 18 variants and functional consequences. J Gen Virol 86 (2005) : 2459–2468.

[169] López-Saavedra A, González-Maya L, Ponce-de-León S, García-Carrancá A, Mohar A, Lizano M. Functional implication of sequence variation in the long control region and E2 gene among human papillomavirus type 18 variants. Arch Virol 154 (2009) : 747-754.

[170] Arias-Pulido H, Peyton CL, Torrez-Martínez N, Anderson DN, Wheeler CM. Human papillomavirus types 18 variants lineages in United States populations characterized by sequence analysis of LCR-E6, E2 and L1 regions. Virology 338 (2005) : 22-34.

[171] Lizano M, De la Cruz-Hernandez E, Carrillo-Garcia A, Garcia-Carranca A, Ponce-de-Leon-Rosales S, Duenas-Gonzalez A, Hernandez-Hernadez DM, Mohar A. Distribution of HPV16 and 18 intratipic variants in normal cytology, Intraepithelial lesions, and cervical cancer in a Mexican population. Gynecol Oncol 102 (2006) : 230–235.

[172] Michels KB, zur Hausen H. HPV vaccine for all. The Lancet 374 (2009) : 268-270.

[173] Alphs HH, Gambhira R, Karanam B, Roberts JN, Jagu S, Schiller JT, Zeng W, Jackson DC, Roden RBS. Protection against heterologous human papillomavirus challenge by a synthetic lipopeptide vaccine containing a broadly cross-neutralizing epitope of L2. PNAS 105 (2008) : 5850-5855.

[174] Burk RD, Terai M, Gravitt PE, Brinton LA, Kurman RJ, Barnes WA, Greenberg MD, Hadjimichael OC, Fu L, McGowan L, Mortel R, Schwartz PE, Hildesheim A. Distribution of human papillomavirus types 16 and 18 variants in squamous cell carcinomas and adenocarcinomas of the cervix. Cancer Res 63 (2003) : 7215–7220.

[175] Koss LG, The Papanicolaou test for cervical cancer detection. JAMA 261 (1989) : 737-743.

[176] Bekker RI, Massuger LF, Bulten J, Melchers WJ. Epidemiological and clinical aspects of human papillomavirus detection in the prevention of cervical cancer. Rev Med Virol 14 (2004) : 95-105.

[177] Stanley MA. Prognostic factors and new therapeutic approaches to cervical cancer. Virus Res 89 (2002) : 241–248.

[178] Baldwin P, Laskey R, Coleman N. Translational approaches to improving cervical screening. Nat Rev Cancer 3 (2003) : 217–226.

[179] Michalas SP. The Pap test: George N. Papanicolaou (1883 – 1962) A screening test for the prevention of cancer of uterine cervix. Eur J Obstet Gynecol Reprod Biol 90 (2000) : 135–138.

[180] Wright TC. Cervical cancer screening in the 21st century: is it time to retire the PAP smear? Clin Obst Gynecol 50 (2007) : 313–323.

[181] Lie AK, Risberg B, Borge B,Sandstad B, Delabie J, Rimala R, Onsrud M, Thoresen S.DNA-versus RNA-based methods for human papillomavirus detection in cervical neoplasia. Gynecol Oncol 97 (2005) : 908– 915.

[182] Arbyn M, Herbert A, Schenck P, Nieminen P, Jordan J, Mcgoogan E, Patnick J, Bergeron C,

Baldauf JJ, Klinkhamer P, Bulten J, Martin-Hirsch P. European guidelines for quality assurance in cervical cancer screening: recommendations for collecting samples for conventional and liquid-based cytology. Cytopathology 18 (2007) : 133–139.

[183] Franco EL, Ferenczy A. Cervical cancer screening following the implementation of prophylactic human papillomavirus vaccination. Future Oncol 3 (2007) : 319–327.

[184] Bae JH, Lee SJ, Kim CJ, Hur SY, Park YG, Lee WC, Kim YT, Ng TL, Bock HL, Park JS. Human Papillomavirus (HPV) type distribution in Korean women: a Meta-Analysis. J Microlbiol Biotechnol 18 (2008) : 788-794.

[185] Bulk S, Berkhof J, Bulkmans NW, Zielinski GD, Rozendaal L, van Kemenade FJ, Snijders PJ, Meijer CJ. Preferential risk of HPV16 for squamous cell carcinoma and of HPV18 for adenocarcinoma of the cervix compared to women with normal cytology in the Nertherlands. Br J Cancer 94 (2006) : 171-175.

[186] Smith JS, Lindsay L, Hoots B, Keys J, Franceschi S, Winer R, Clifford GM. Human papillomavirus type distribution in invasive cervical cancer and high-grade cervical lesions: A meta-analysis update. Int J Cancer 121 (2007) : 621-632.

[187] Miura S, Matsumoto K, Oki A, Satoh T, Tsunoda H, Yasugi T, Taketani Y, Yoshikawa H. Do we need a different strategy for HPV screening and vaccination in East Asia? Int J Cancer 119 (2006) : 2713-2715.

[188] Lai CH, Chao A, Chang CJ, Chao FY, Huang HJ, Hsueh S, Lin CT, Cheng HH, Huang CC, Yang JE, Wu Ti, Chou HH, Chang TC. Host and viral factors in relation to clearance of human papillomavirus infection: a cohort study in Taiwan. Int J Cancer 123 (2008) : 1685–1692.

[189] Fontaine J, Hankins C, Money D, Rachlis A, Pourreaux K, Ferenczy A; Canadian Women's HIV Study Group, Coutlée. Human papillomavirus type 16 (HPV-16) viral load and persistence of HPV-16 infection in women infected or at risk for HIV. J Clin Virol 43 (2008) : 307–312.

[190] Dalstein V, Riethmuller D, Prétet JL, Le Bail Carval K, Sautière JL, Carbillet JP, Kantelip B, Schaal JP, Mougin C. Persistence and load of high risk HPV are predictors for development of high-grade cervical lesions: a longitudinal French cohort study. Int J Cancer 106 (2003) : 396–403.

[191] van Duin M, Snijders PJ, Schrijnemakers HF, Voorhorst FJ, Rozendaal L, Nobbenhuis MA, van den Brule AJ, Verheijen RH, Helmerhorst TJ, Meijer CJ. Human papillomavirus 16 load in normal and abnormal cervical scrapes: an indicator of CIN II/III and viral clearance. Int J Cancer 98 (2002) : 590–595.

[192] Park JY, Lee KH, Dong SM, Kang S, Park SY, Seo SS. The association of pre-conization

high-risk HPV load and the persistence of HPV infection and persistence/recurrence of cervical intraepithelial neoplasia after conization. Gynecol Oncol 108 (2008) : 549–554.

[193] Song SH, Lee JK, Oh MJ, Hur JY, Na JY, Park YK, Saw HS. Persistent HPV infection after conization in patients with negative margins. Gynecol Oncol 101 (2006) : 418–422.

[194] Molano M, Van den Brule A, Plummer M, Weiderpass E, Posso H, Arslan A, Meijer CJ, Muños N, Franceschi S; HPV Study Group. Determinants of clearance of human papillomavirus infections in Colombian women with normal cytology: a population-based, 5-year follow-up study. Am J Epidemiol 158 (2003) : 486–494.

[195] Kulmala SM, Shabalova IP, Petrovitchev N, Syrjänen KJ, Gyllensten UB, Johansson BC, Syrjänen SM; New Independent States of the former Soviet Union Cohort Study Group. Type-specific persistence of high-risk human papillomavirus infections in the New Independent States of the former Soviet Union Cohort Study. Cancer Epidemiol Biomarkers Prev 16 (2007) : 17–22.

[196] Lillo FB, Lodini S, Ferrari D, Stayton C, Taccagni G, Galli L, Lazzarin A, Uberti-Foppa C. Determination of human papillomavirus (HPV) load and type in high-grade cervical lesions surgically resected from HIV-infected women during follow-up of HPV infection. Clin Infect Dis 40 (2005) : 451-457.

[197] Nindl I, Greinke C, Zahm DM, Stockfleth E, Hoyer H, Schneider A. Human papillomavirus distribution in cervical tissues of different morphology as determined by hybrid capture assay and PCR. Int J Gynecol Pathol 16 (1997) : 197-204.

[198] Swan DC, Tucker RA, Tortolero-Luna G, Mitchell MF, Wideroff L, Unger ER, Nisenbaum RA, Reeves WC, Icenogle JP. Human papillomavirus (HPV) DNA copy number is dependent on grade of cervical disease and HPV type. J Clin Microbiol 37 (1999) : 1030-1034.

[199] Heard I, Tassie JM, Schmitz V, Mandelbrot L, Kazatchkine MD, Orth G. Increased risk of cervical disease among human immunodeficiency virus-infected women with severe immunosuppression and high human papillomavirus load(1). Obstet. Gynecol 96 (2000) : 403-409.

[200] Clavel C, Masure M, Levert M, Putaud I, Mangeonjean C, Lorenzato M, Nazeyrollas P, Gabriel R, Quereux C, Birembaut P. Human papillomavirus detection by the hybrid capture II assay: a reliable test to select women with normal cervical smears at risk for developing cervical lesions. Diagn Mol Pathol 9 (2000) : 145-150.

[201] van Duin M, Snnijders PJ, Schrijnemakers HF, Voorhorst FJ, Rozendaal L, Nobbenhuis MA,

van den Brule AJ, Verheijen RH, Helmerhorst TJ, Meijer CJ. Human papillomavirus 16 load in normal and abnormal cervical scrapes: an indicator of CIN II/III and viral clearance. Int J Cancer 8 (2002) : 590-595.

[202] Crum CP, Beach KJ, Hedley ML, Yuan L, Lee KR, Wright TC, Urbn RG. Dynamics of human papillomavirus infection between biopsy and excision of cervical intraepithelial neoplasia: results from the ZYC101a protocol. J Infect Dis 189 (2004) : 1348-1354.

[203] Xi LF, Hughes JP, Edelstein ZR, Kiviat NB, Koutsky LA, Mao C, Ho Jesse, Schiffman M. Human papillomavirus (HPV) type 16 and type 18 DNA loads at Baseline and persistence of type specific infection during a 2 year follow up. J Infect Dis 11 (2009) : 1789-1797.

APPENDICES

APPENDIX A

Molecular biology techniques

Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a relatively simple technique that amplifies a DNA template to produce specific DNA fragments *in vitro*. PCR is a technique to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. Some traditional methods of cloning a DNA sequence into a vector and replicating it in a living cell often require days or weeks of work, but amplification of DNA sequences by PCR requires only hours. In addition, the PCR process requires very little amount of biological material whereas most biochemical analyses require the input of significant amounts of biological material such as nucleic acid detection with radioisotopes. PCR can achieve more sensitive detection and higher levels of amplification of specific sequences in less time than previously used methods. There is more very advantage of PCR and this technique is very useful, not only in basic research, but also applies in commercial uses, including genetic identity testing, forensics, industrial quality control and *in vitro* diagnostics. Basic PCR has become commonplace in many molecular biology labs where it is used to amplify DNA fragments and detect DNA or RNA sequences within a cell or environment. However, PCR has evolved far beyond simple amplification and detection, and many extensions of the original PCR method have been described.

- **Conventional PCR**

The PCR process was originally developed to amplify short segments of a longer DNA molecule. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction. A typical amplification reaction includes the target biological material (DNA or RNA), a thermostable DNA polymerase, two oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), reaction buffer and magnesium. DNA polymerase is an important component to select and repeat amplification. Once

assembled, the reaction is placed in a thermal cycler, an instrument that subjects the reaction to a series of different temperatures for varying amounts of time. This series of temperature and time adjustments is referred to as one cycle of amplification. Each PCR cycle theoretically doubles the amount of targeted sequence (amplicon) in the reaction. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified.

Each cycle of PCR includes steps for template denaturation, primer annealing and primer extension (Figure 25). There are 3 general steps to process that are repeated for a number of cycles to exponentially increase the number of copies of specific target region. Step1 is to first unzip the double strand DNA, also called denaturation. The initial step denatures the target DNA by heating it to 94°C or higher for 15 seconds to 2 minutes. In the denaturation process, the two intertwined strands of DNA separate from one another, producing the necessary single-stranded DNA template for replication by the thermostable DNA polymerase. Step 2 isolates the target region of the double strand DNA by landing 2 primers, which exactly match two 20-30 unique base pair regions, that bookend the target region. This is called annealing. The temperature is reduced to approximately 40–60°C. At this temperature, the oligonucleotide primers can form stable associations (anneal) with the denatured target DNA and serve as primers for the DNA polymerase. This step lasts approximately 15–60 seconds. Finally, step 3 involves heating the mix to 72 °C at which point a special polymerase builds the DNA strand starting at the primers and continuing in the 5 prime direction. This is called extension. The synthesis of new DNA begins as the reaction temperature is raised to the optimum for the DNA polymerase. For most thermostable DNA polymerases, this temperature is in the range of 68–74°C. The extension step lasts approximately 1–2 minutes. The next cycle begins with a return to 94°C for denaturation.

These three steps are repeated 25-40 times to produce millions of exact copies of the target region of DNA. Because during the second cycle of this process, extension can occur on both the origin copy f genomic DNA and the newest pieces subsequent extensions are quickly limited precisely to the target region. Each step of the cycle should be optimized for each template and primer pair combination. If the temperature during the annealing and extension steps are similar, these two steps can be combined

into a single step in which both primer annealing and extension take place. After 20–40 cycles, the amplified product may then be analyzed for size, quantity, sequence, etc., or used in further experimental procedures.

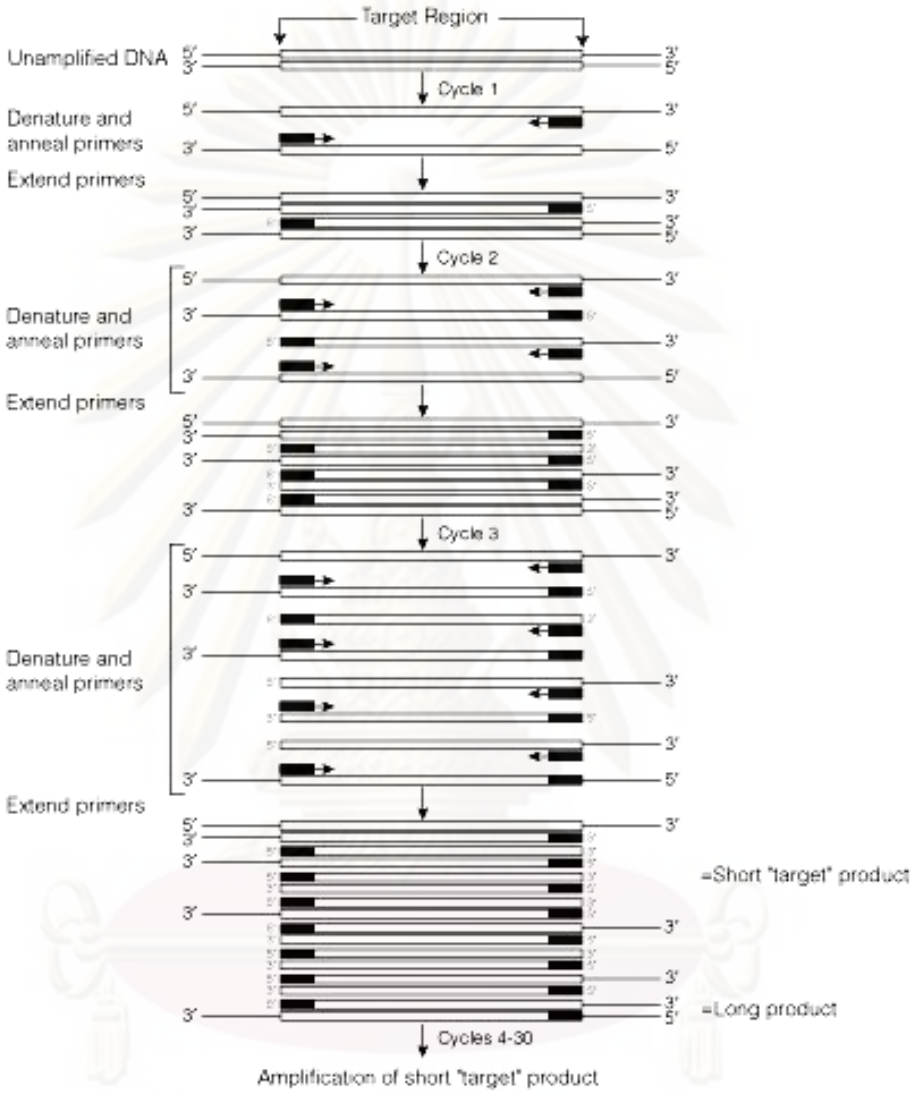


Figure 25 Schematic diagram of the PCR process (http://www.promega.com/paguide/chap1.htm)

Degenerate primer

A group of degenerate oligonucleotides contain related sequence with differences at specific locations. Theses are actually mixtures of similar primer, but not identical primers. Degenerate primers are useful for pulling out one part of gene sequence when you only know the gene sequence in related organisms. These primers

are helpful for organisms that have a high variation nucleotide sequence because the degenerate primers have a widely range amplification. Furthermore, the other use for degenerate primers design is based on protein sequence. As several codon can code for one amino acid. One amino acid can reverse translate this sequence to determine all of the possible nucleotide sequences that could encode that amino acid sequence. A set of degenerate oligonucleotides would be produced matching those DNA sequence.

Real-Time PCR and conventional PCR

Real-time PCR allow for the detection of PCR amplification during early phases of the reaction. Measuring the kinetics of the reaction in the early phases of CR provides a distinct advantage over conventional PCR detection. Conventional PCR methods use agarose gels for detection of PCR amplification at the final phase or end-point of the PCR reaction.

Real-time PCR

Real-time PCR or quantitative real-time PCR or kinetic PCR is a molecular biology technique. The use of fluorescently labeled oligonucleotide probes or primers or DNA binding fluorescent dyes, such as SYBR Green, to detect and quantitate a PCR product allows quantitative PCR to be performed in real time. The key important of this procedure is real-time PCR probe that labeled at the 5'-end with fluorescence donor and a few based downstream or on the 3'-end with a quencher. During PCR the labeled oligonucleotide hybridizes to the target sequence and the 5'-dye is removed by 5' to 3'-exonuclease activity of Taq. The fluorescence of the donor is no longer quenched and can be measures. The more PCR products present the stronger the fluorescent intensity of the reaction. Measuring the increase in signal intensity during the exponential phase of the PCR reaction allows the researcher to determine the amount of genetic material present at the beginning of the reaction. DNA-binding dyes are easy to use but do not differentiate between specific and nonspecific PCR products. Fluorescently labeled nucleic acid probes have the advantage that they react with only specific PCR products.

Using any of the developed chemistries, the increase in fluorescence emission during the PCR reaction can be detected in real-time. The computer software constructs

amplification plots using the fluorescence signal that are collected during each cycle of the PCR amplification (Figure 26).

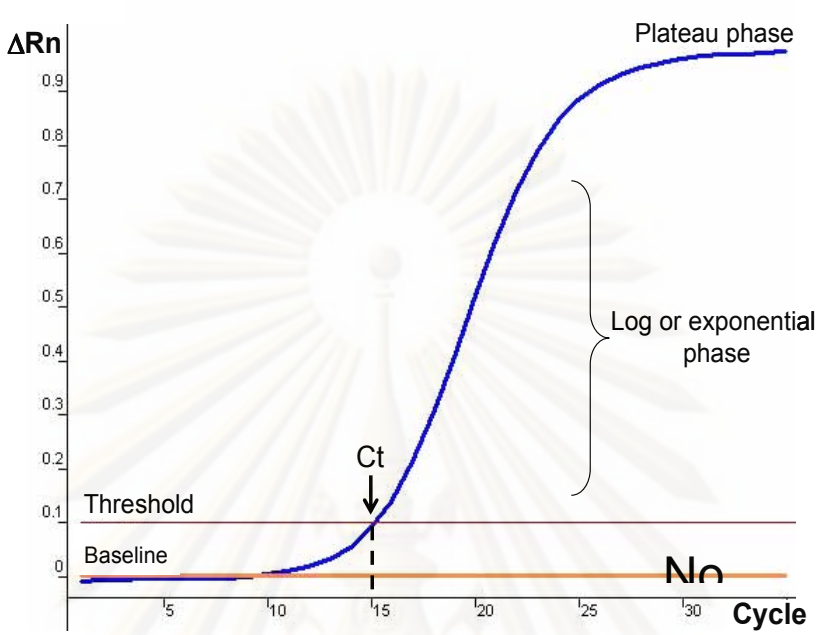


Figure 26 Schematic representative of amplification plot and the important terminology associated with real-time PCR analysis [154].

According to the amplification plot (Figure 26), there are several important terms associated with the data analysis of real-time PCR assay as the following.

- Baseline: the baseline is defined as the PCR cycles in which a reporter fluorescent signal is accumulating but is beneath the limits of detection of the instrument. By default, the software sets the baseline from cycles 3 to 15.
- ΔRn: the software calculates a ΔRn by using ΔRn=Rnf – Rnb, Rnf is the fluorescence emission of the product at each time point and Rnb is the fluorescence emission of the baseline. The ΔRn values are plotted against the cycle number.
- Threshold: an arbitary threshold is chosen by the computers, based on the variability of the baseline. Conventionally, it is calculated as ten-times the standard deviation of the average signal of the baseline fluorescent signal between cycles 3 to 15. A fluorescent signal that is detected above the threshold

level is considered as a real signal which can be used to define the threshold cycle (Ct).

- Threshold cycle (Ct): this is defined as the fractional PCR cycle number at which the reporter fluorescence is greater than the minimal detection (threshold) level. The Ct is the basic principle of real-time PCR and is an essential component in producing accurate and reproducible data. The presence of more template at the beginning of reaction leads to a fewer number of cycles reaching the threshold level.

As reaction components become limiting, the rate of the target amplification decreases until the PCR reaction is no longer generating template at an exponential rate (plateau phase) and there is little or no increase in PCR product. This is the main reason why real-time PCR is a more reliable measurement of starting copy number than an endpoint measurement of the accumulated PCR product.

Commonly used detection chemistries in real-time PCR

As the popularity of Real-time PCR technology has grown over the last several years, a number of companies have developed products to ease the process and time requirements for quantitative real-time PCR (qPCR).

TaqMan probe

TaqMan probes are oligonucleotides that consist of two types of fluorophores, which a fluorescent dye on the 5' end (typically) and a quenching dye on the 3' end. While the probe is attached or unattached to the template DNA and before the polymerase acts, the close proximity of the reporter and the quencher prevent the emission of fluorescence via fluorescence resonance energy transfer (FRET), which is inhibition of one dye caused by another without emission of the proton. TaqMan probes anneal to an internal region of a PCR product, thus when the Taq polymerase with exonuclease activity replicates a template on which TaqMan is bound, the probe is cleaved, releasing the reporter dye from the quencher.

During the high temperature denaturation step there is no binding of the probe to the DNA. As the temperature cools during the renaturation (annealing) step, the primers and the TaqMan probe anneal. At this stage, the TaqMan probe has bound to its specific piece of the template DNA and the primers anneal to the DNA too. The fluorescent signal from the reporter is quenched by the close proximity of the quencher. After that, Taq polymerase addes nucleotides and removes the TaqMan probe from the template DNA. This separates the quencher from the reporter, and the synthesis of DNA results in the displacement and hydrolysis of the labeled probe by the polymerase, thus allows the reporter to release the signal because the reporter is separated from the quencher (Figure 27).

Advantages of TaqMan probe:

- Detects only amplification of specific product
- Hybridization and cleavage does not interfere with accumulation of the product
- Can be used in multi-color labeled probes for multiplex detection

Disadvantages of TaqMan probe:

- Requires that specific probes be generated for each template

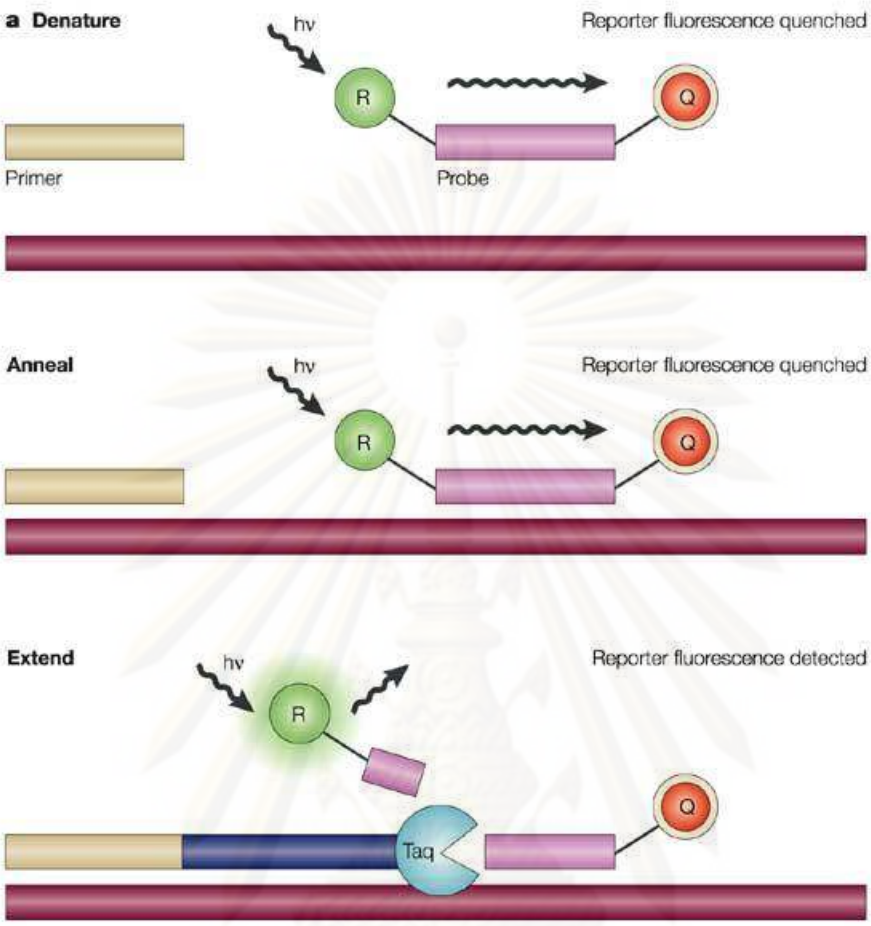


Figure 27 Detection chemistries commonly used in real-time PCR. Detection mechanism of TaqMan probe chemistry (http://www.nature.com/nrd/journal/v3/n9/fig_tab/nrd1496_F1.html)

Multiplex real-time PCR

The term multiplex real-time PCR is used to describe the use of multiple fluorogenic probes for the detection of multiple amplicons in a single tube. Different probes are labeled with dyes that have unique emission spectra. The main advantages of multiplexing over single-target analysis are following these: First, Multiplex real-time PCR reduced reagent cost because target are amplified together instead of separately. Second, this method is reliable results because it has the ability to provide internal controls. Third, this method is conservation of precious samples because more quantification data can be obtained per sample. Finally, multiplex real-time increased

throughput because more target can be analyzed per run on a real-time cycler. The main restrictions of this technique are the limited number of available fluorophores, fluorescence emission from quenching dyes and the common use in real-time instruments of a monochromatic light source. Conventionally, only four-color multiplex reactions are usually possible, of which one color may be used as a passive reference dye control (Table 9).

Table 9 Multiplex fluorescence reporters dyes suitable for each instrument

	Fluorescence dye			Passive
Real-time PCR company	Target 1	Target 2	Target 3	Reference dye
Applied Biosystems (ABI)	FAM	HEX / JOE / VIC	NED	ROX
Corbett Research (Roter Gene)	FAM	HEX / JOE / VIC	Cy5	ROX
Stratagene (Mx)	FAM	HEX / JOE / VIC	Cy5	ROX
BioRad (iCycler iQ)	FAM	HEX / JOE / VIC	Cy5	ROX
Cepheid (Smart Cycler)	FAM	HEX / JOE / VIC	Cy5	ROX
Eppendorf (Mastercycler realplex)	FAM	HEX / JOE / VIC	Cy5	ROX
Roche (Light Cycler)	LC Red 640	LC Red 670	LC Red 705	ROX

Quantitation of nucleic acids by real-time PCR

The quantification strategy is the principal marker in gene quantification. Target nucleic acids can be quantified using either absolute quantitation or relative quantitation. Absolute quantitation determines the absolute amount of a target (expressed as a copy number or concentration), whereas relative quantitation determines the ratio between the amount of a target and the amount of a reference nucleic acid, usually a suitable housekeeping gene. This normalized value can be used to compare, for example, differential gene expression in different samples.

Absolute quantitation

The absolute amount of a target nucleic acid is determined using external standards. Calibration or standard curves are highly reproducible and allow the generation of high specific, sensitive and reproducible data. The sequence of the standards is usually the same as or very similar to the target sequence, but the primer binding sites of the standards must be identical to those in the target sequence. This ensures that both the standards and the target are amplified with equivalent efficiencies, which is essential for absolute quantitation. Standard design, production, determination of the exact standard concentration and stability over long storage time is not straightforward and can be problematic. The concentrations of DNA standard molecule such as DNA plasmid are very stable and generate highly reproducible standard curves even after a long storage time. A standard curve (plot of Ct value against log of initial amount of standard) is a linear line generated by using different dilutions of the standard (Figure 28). The target and each of the standards are amplified in separate tubes. Quantitation of the amount of target in the “unknown” samples of interest is accomplished by measuring Ct and using the standard curve to determine starting amount of the sample.

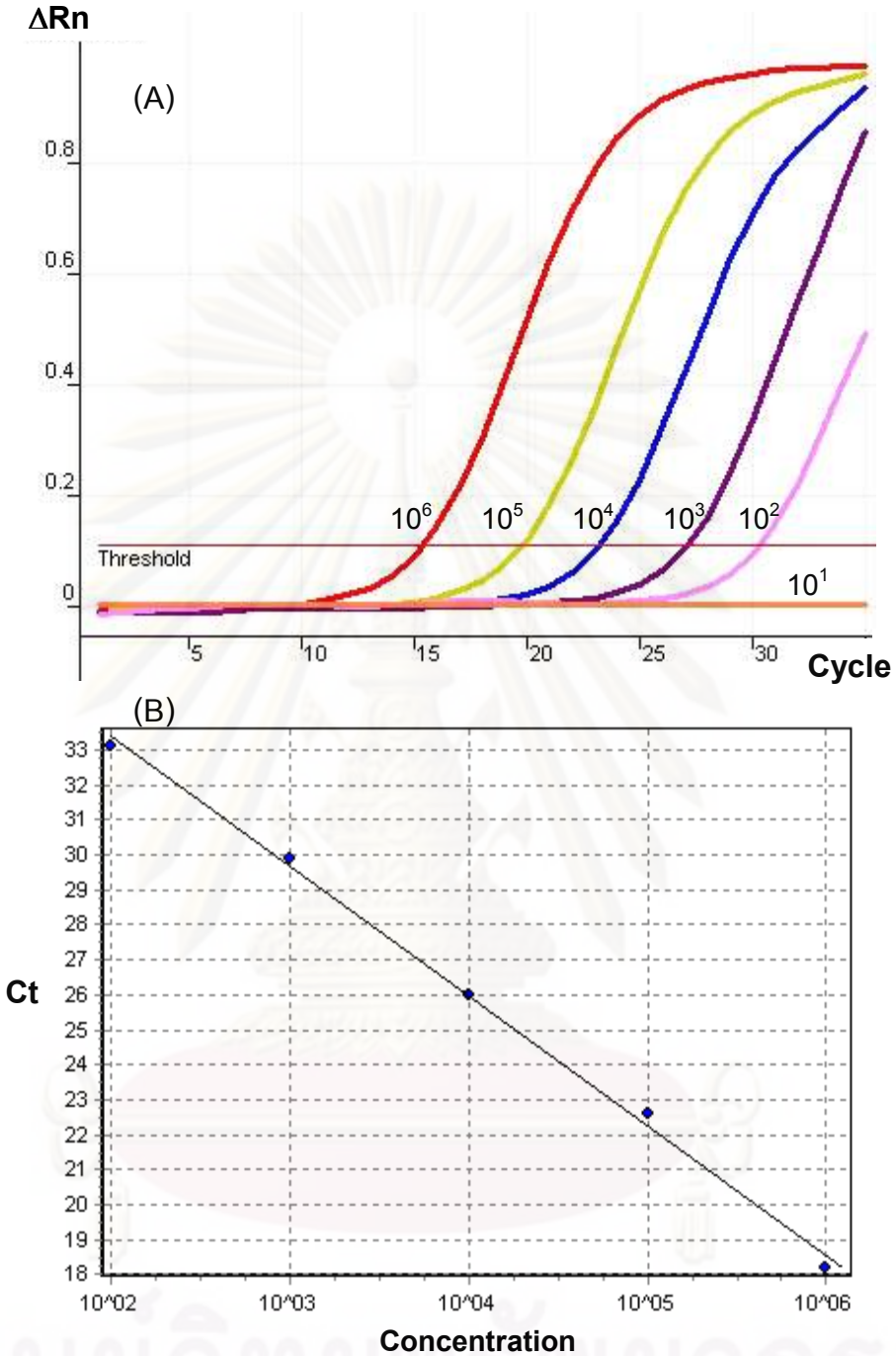


Figure 28 Representative of absolute quantitation by real-time PCR. (A) Amplification plot obtained from 10-fold serial dilution of standard template (10^6-10). (B) Standard curve obtained from plotting Ct values against the concentration of each standard dilution [154].

Generating standard curves

Standard curves can be used in both absolute and relative quantitation. To generate a standard curve, at least 5 different amounts of the standard should be quantified, and the amount of unknown target should fall within the range of the standard curve. Reactions should be carried out in at least triplicate, especially when quantifying standards of low copy number.

For absolute quantitation, the copy number or concentration of the nucleic acids used as standards must be known. In addition, standards should show the following features:

- Primer binding sites identical to the target to be quantified
- Sequence between primer binding sites identical or highly similar to target
- Sequences upstream and downstream from the amplified sequence identical or similar to “natural” target

Plasmid DNA containing the target gene can be used as a standard for generating a standard curve. After determination of plasmid DNA concentration by spectrophotometry (Table 10), the copy number of standard DNA molecules can be calculated using the following formula:

(X g/µl DNA / [plasmid length in nucleotides x 660]) x 6.022 x 10^{23} = Y molecules/µl

Table 10 Spectrophotometric conversions for nucleic acid templates

Types of nucleic acid	Concentration (µg/ml) when A_{260}= 1
Double-stranded DNA (dsDNA)	50
Single-stranded DNA (ssDNA)	33
Single-stranded RNA (ssRNA)	40

In addition, the curve must be linear over the whole concentration range. The linearity is denoted by the R squared (Rsq) value (R^2 or Pearson Correlation Coefficient) and should be very close to 1 (≥ 0.985 is acceptable). Ideally, the efficiency of both the standard curve and sample reactions should be between 90 and 110% (slope between -

3.1 to -3.6) are acceptable. One hundred percent efficiency implies perfect doubling of amplicon each cycle. If the efficiency is significantly less, this implies the reaction is being slowed in some way, either from inhibitors present in the reaction mix or suboptimal primer sets or reaction conditions. Efficiencies significantly above 100% typically indicate experimenter error (e.g. miscalibrated pipettors, PCR inhibitors, probe degradation, formation of nonspecific products, and formation of primer dimers).

Relative quantitation by comparative threshold (delta-delta Ct) method ($2^{-\Delta\Delta_{Ct}}$)

Comparative threshold method is the most commonly used strategy for relative quantitation in real-time PCR. This method eliminates the need for standard curves and mathematical equations are used to calculate the relative expression levels of a target relative to calibrator such as a non treated control or RNA from normal tissue or a sample at time zero in a time-course study. The amount of target gene in the sample, normalized to an endogenous housekeeping gene (reference gene) and relative to the normalized calibrator, is then given by $2^{-\Delta\Delta_{Ct}}$, where

$\Delta\Delta$Ct = ΔCt (sample) - ΔCt (calibrator)

ΔCt (sample) = Ct (target gene of sample) – Ct (reference gene of sample)

ΔCt (calibrator) = Ct (target gene of calibrator) – Ct (reference gene of calibrator)

Ratio (folds of difference) of sample: calibrator = $2^{-\Delta\Delta_{Ct}}$

For this calculation to be valid and in order to obtain reliable results, it is imperative that the amplification efficiencies of the reference and the target gene are approximately equal and at or above 90%. This can be established by calculation of ΔCt (of both and calibrator) varies with template dilution. If the plot of template dilution against ΔCt yield slope < 0.1, it implies that the efficiencies of the target and reference gene are very similar.

Primer design guidelines:-

- Primer length between 18-30 bp.
- Keep GC content in the range of 40-60%.

- The melting temperature (Tm) of primers should be 55-62ºC and within 2ºC of each other. The Tm is calculated by using the formula:
  Tm = 2°C x (number of [A+T]) + 4°C x (number of [G+C])
- G or C at the 3' end of primers will increase priming efficiency.
- Avoid runs of an identical nucleotides (3 or more), especially guanine.
- Avoid secondary structure (hairpin, self-complementary and primer dimers).
- Avoid mismatches between the 3' end of the primer and the target-template.
- The 5 nucleotides at the 3' end should have no more than two G and/or C bases.
- Primers should be searched using BLAST and checked for cross-homology.
- Primers should be specific with the target gene and not anneal with other gene.
- In multiplexing, all primers should be designed using the same settings to ensure that they will work optimally under the same cycling conditions.
- In real-time PCR assay, short amplicon length (70-200) is recommended for high efficiency of amplification.

TaqMan probe design guidelines:-

- Keep G-C content in the 30-80% range.
- The Tm of probe should be 65-72ºC (8-10ºC higher than the Tm of primers.
- Avoid probes with "G" at the 5´ end of the probe. Because "G" adjacent to the reporter dye will quench the reporter fluorescence, even after cleavage.
- Select probes with a Primer Express software–estimated Tm of 65–67 °C.
- Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more should be avoided.
- Design probe as close as possible to the primer without overlapping.

Fluorescence reporters / quenchers selection

- Select the appropriate quencher for each reporter dye (Table 11).
- Select dyes with excitation/emission maxima compatible with the excitation/ detection ranges of the instrument.
- Label least abundant target with the best performing dye (usually FAM)

- For multiplexing, select suitable fluorescence reporters / quenchers for the detector of each real-time PCR instrument in order to avoid spectral overlap between reporter dyes (Table 11).

Table 11 Commonly used fluorescence reporters & quenchers in real-time PCR

Reporters	Excitation (nm)	Emission (nm)	Compatible Quenchers
FAM	494	518	
TET	521	538	TAMRA
JOE	520	548	BHQ1
VIC	538	552	MGB
HEX	535	553	
NED	546	575	
Cy3	552	570	BHQ2
TAMRA	560	582	MGB
ROX	587	607	
Texas Red	596	615	
Cy5	643	667	BHQ3

Cloning of PCR products with pGEM-T Easy vector

The pGEM-T Easy Vector Systems (Promega) is a convenient system for the cloning of PCR products. The vector contains a single 3' terminal thymidine (T) at both ends. These single 3´-T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases including *Taq, Tfl* and *Tth*. Table 12 and Figure 29 show the description of pGEM-T Easy Vector.

Table 12 pGEM-T Easy Vector sequence reference points

Reference points	Positions
T7 RNA polymerase transcription initiation site	1
Multiple cloning region	10-128
SP6 RNA polymerase promoter (–17 to +3)	139-158
SP6 RNA polymerase transcription initiation site	141
M13 Reverse Sequencing Primer binding site	176-197
M13 Forward Sequencing Primer binding site	2949-2972
T7 RNA polymerase promoter (–17 to +3)	2999-3

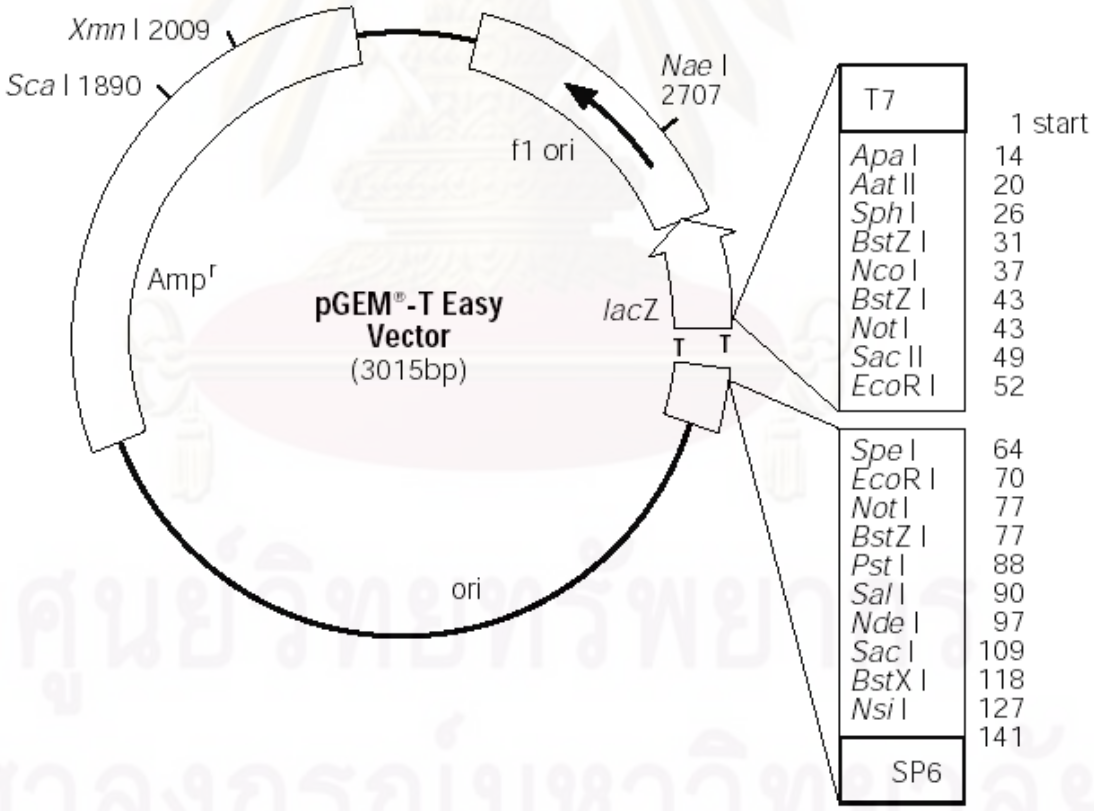


Figure 29 Circle map of pGEM-T Easy vector (Promega) (Technical manual Promega®)

APPENDIX B

Nucleotide sequencing result

All of HPV specimens can be efficiently detected by the PCR were subjected to nucleotide sequencing and BLAST search for confirmation of the HPV genotypes detection. Some sequences obtained in this study have been submitted to GenBank database and assigned accession numbers (table 13).

Table 13 Conclusion of HPV complete genome and partial E1 gene of this study that submitted in GenBank database

Name	HPV genotype	genome	Product size (Base pairs)	Accession number
CU1	HPV16	Complete	7,906	FJ610146
CU2	HPV16	Complete	7,906	FJ610147
CU3	HPV16	Complete	7,906	FJ610148
CU4	HPV16	Complete	7,906	FJ610149
CU5	HPV16	Complete	7,905	FJ610150
CU6	HPV16	Complete	7,906	FJ610151
CU7	HPV16	Complete	7,905	FJ610152
CU8	HPV18	Complete	7,857	GQ180784
CU9	HPV18	Complete	7,857	GQ180785
CU10	HPV18	Complete	7,857	GQ180786
CU11	HPV18	Complete	7,844	GQ180787
CU12	HPV18	Complete	7,857	GQ180788
CU13	HPV18	Complete	7,857	GQ180789
CU14	HPV18	Complete	7,857	GQ180790
CU15	HPV18	Complete	7,857	GQ180791
CU16	HPV18	Complete	7,857	GQ180792
CU8	HPV18	Partial E1 gene	733	GQ161244

Name	HPV genotype	genome	Product size (Base pairs)	Accession number
CU9	HPV18	Partial E1 gene	733	GQ161245
CU10	HPV18	Partial E1 gene	733	GQ161246
CU11	HPV18	Partial E1 gene	733	GQ161247
CU12	HPV18	Partial E1 gene	733	GQ161248
CU13	HPV18	Partial E1 gene	733	GQ161249
CU14	HPV18	Partial E1 gene	733	GQ161250
CU15	HPV18	Partial E1 gene	733	GQ161251
CU16	HPV18	Partial E1 gene	733	GQ161252
CU17	HPV16	Partial E1 gene	639	GQ161253
CU18	HPV16	Partial E1 gene	796	GQ161254
CU19	HPV52	Partial E1 gene	669	GQ161255
CU20	HPV31	Partial E1 gene	759	GQ161256
CU21	HPV16	Partial E1 gene	675	GQ161257
CU22	HPV16	Partial E1 gene	592	GQ161258
CU23	HPV51	Partial E1 gene	641	GQ161259
CU24	HPV56	Partial E1 gene	644	GQ161260
CU25	HPV51	Partial E1 gene	645	GQ161261
CU26	HPV33	Partial E1 gene	662	GQ161262
CU27	HPV31	Partial E1 gene	762	GQ161263
CU28	HPV58	Partial E1 gene	427	GQ161264
CU29	HPV66	Partial E1 gene	646	GQ161265
CU30	HPV59	Partial E1 gene	664	GQ161266
CU31	HPV16	Partial E1 gene	390	GQ161267
CU32	HPV71	Partial E1 gene	657	GQ161268
CU33	HPV31	Partial E1 gene	614	GQ161269
CU34	HPV35	Partial E1 gene	778	GQ161270
CU35	HPV66	Partial E1 gene	765	GQ161271

Name	HPV genotype	genome	Product size (Base pairs)	Accession number
CU36	HPV52	Partial E1 gene	127	GQ161272
CU37	HPV52	Partial E1 gene	669	GQ161273
CU38	HPV73	Partial E1 gene	761	GQ161274
CU39	HPV31	Partial E1 gene	614	GQ161275
CU40	HPV31	Partial E1 gene	619	GQ161276
CU41	HPV51	Partial E1 gene	653	GQ161277
CU42	HPV52	Partial E1 gene	675	GQ161278
CU43	HPV31	Partial E1 gene	96	GQ161279
CU44	HPV73	Partial E1 gene	761	GQ161280
CU45	HPV40	Partial E1 gene	649	GQ161281
CU46	HPV52	Partial E1 gene	669	GQ161282
CU47	HPV66	Partial E1 gene	651	GQ161283
CU48	HPV33	Partial E1 gene	662	GQ161284
CU49	HPV33	Partial E1 gene	363	GQ161285
CU50	HPV33	Partial E1 gene	662	GQ161286
CU51	HPV52	Partial E1 gene	673	GQ161287
CU52	HPV74	Partial E1 gene	648	GQ161288
CU53	HPV16	Partial E1 gene	652	GQ161289
CU54	HPV30	Partial E1 gene	659	GQ161290
CU55	HPV56	Partial E1 gene	683	GQ161291
CU56	HPV31	Partial E1 gene	627	GQ161292
CU57	HPV16	Partial E1 gene	662	GQ161293
CU58	HPV16	Partial E1 gene	671	GQ161294
CU59	HPV52	Partial E1 gene	674	GQ161295
CU60	HPV31	Partial E1 gene	626	GQ161296
CU61	HPV16	Partial E1 gene	683	GQ161297
CU62	HPV31	Partial E1 gene	626	GQ161298

Name	HPV genotype	genome	Product size (Base pairs)	Accession number
CU63	HPV52	Partial E1 gene	673	GQ161299
CU64	HPV55	Partial E1 gene	661	GQ161300
CU65	HPV31	Partial E1 gene	626	GQ161301
CU66	HPV42	Partial E1 gene	651	GQ161302
CU67	HPV16	Partial E1 gene	683	GQ161303
CU68	HPV18	Partial E1 gene	531	GQ161304
CU69	HPV66	Partial E1 gene	157	GQ161305
CU70	HPV6	Partial E1 gene	655	GQ161306
CU71	HPV59	Partial E1 gene	668	GQ161307
CU72	HPV59	Partial E1 gene	666	GQ161308
CU73	HPV42	Partial E1 gene	573	GQ161309
CU74	HPV51	Partial E1 gene	645	GQ161310
CU75	HPV31	Partial E1 gene	626	GQ161311
CU76	HPV51	Partial E1 gene	645	GQ161312
CU77	HPV31	Partial E1 gene	626	GQ161313
CU78	HPV16	Partial E1 gene	528	GQ161314
CU79	HPV59	Partial E1 gene	616	GQ161315
CU80	HPV56	Partial E1 gene	671	GQ161316
CU81	HPV68	Partial E1 gene	658	GQ161317
CU82	HPV39	Partial E1 gene	654	GQ161318
CU83	HPV51	Partial E1 gene	653	GQ161319
CU84	HPV56	Partial E1 gene	670	GQ161320
CU85	HPV70	Partial E1 gene	677	GQ161321
CU86	HPV58	Partial E1 gene	659	GQ161322
CU87	HPV42	Partial E1 gene	652	GQ131323
CU88	HPV66	Partial E1 gene	656	GQ161324
CU89	HPV35	Partial E1 gene	636	GQ161325

Name	HPV genotype	genome	Product size (Base pairs)	Accession number
CU90	HPV68	Partial E1 gene	611	GQ161326
CU91	HPV52	Partial E1 gene	570	GQ161327
CU92	HPV16	Partial E1 gene	610	GQ161328
CU93	HPV31	Partial E1 gene	624	GQ161329
CU94	HPV66	Partial E1 gene	567	GQ161330
CU95	HPV31	Partial E1 gene	625	GQ161331
CU96	HPV30	Partial E1 gene	656	GQ161332
CU97	HPV74	Partial E1 gene	432	GQ161333
CU98	HPV39	Partial E1 gene	657	GQ161334
CU99	HPV16	Partial E1 gene	526	GQ161335
CU100	HPV16	Partial E1 gene	684	GQ161336
CU101	HPV42	Partial E1 gene	602	GQ161337
CU102	HPV16	Partial E1 gene	684	GQ161338
CU103	HPV74	Partial E1 gene	531	GQ161339
CU104	HPV34	Partial E1 gene	662	GQ161340
CU105	HPV52	Partial E1 gene	673	GQ161341
CU106	HPV16	Partial E1 gene	524	GQ161342
CU107	HPV34	Partial E1 gene	671	GQ161343
CU108	HPV39	Partial E1 gene	626	GQ161344
CU109	HPV35	Partial E1 gene	635	GQ161345
CU110	HPV39	Partial E1 gene	649	GQ161346
CU111	HPV68	Partial E1 gene	669	GQ161347
CU112	HPV34	Partial E1 gene	573	GQ161348
CU113	HPV52	Partial E1 gene	674	GQ161349
CU114	HPV52	Partial E1 gene	673	GQ161350
CU115	HPV90	Partial E1 gene	638	GQ161351
CU116	HPV90	Partial E1 gene	640	GQ161352

Name	HPV genotype	genome	Product size (Base pairs)	Accession number
CU117	HPV31	Partial E1 gene	626	GQ161353
CU118	HPV31	Partial E1 gene	626	GQ161354
CU119	HPV59	Partial E1 gene	663	GQ161355
CU120	HPV68	Partial E1 gene	496	GQ161356
CU121	HPV52	Partial E1 gene	509	GQ161357
CU122	HPV42	Partial E1 gene	652	GQ161358
CU123	HPV16	Partial E1 gene	669	GQ161359
CU124	HPV68	Partial E1 gene	631	GQ161360
CU125	HPV59	Partial E1 gene	630	GQ161361
CU126	HPV53	Partial E1 gene	672	GQ161362
CU127	HPV35	Partial E1 gene	587	GQ161363
CU128	HPV16	Partial E1 gene	134	GQ161364
CU129	HPV34	Partial E1 gene	679	GQ161365
CU130	HPV39	Partial E1 gene	670	GQ161366
CU131	HPV33	Partial E1 gene	672	GQ161367
CU132	HPV66	Partial E1 gene	653	GQ161368
CU133	HPV90	Partial E1 gene	665	GQ161369
CU134	HPV30	Partial E1 gene	657	GQ161370
CU135	HPV16	Partial E1 gene	681	GQ161371
CU136	HPV16	Partial E1 gene	669	GQ161372
CU137	HPV53	Partial E1 gene	672	GQ161373
CU138	HPV16	Partial E1 gene	681	GQ161374
CU139	HPV90	Partial E1 gene	625	GQ161375
CU140	HPV34	Partial E1 gene	672	GQ161376
CU141	HPV66	Partial E1 gene	659	GQ161377
CU142	HPV16	Partial E1 gene	669	GQ161378
CU143	HPV31	Partial E1 gene	597	GQ161379

Name	HPV genotype	genome	Product size (Base pairs)	Accession number
CU144	HPV56	Partial E1 gene	666	GQ161380
CU145	HPV53	Partial E1 gene	633	GQ161381
CU146	HPV18	Partial E1 gene	540	GQ161382
CU147	HPV39	Partial E1 gene	655	GQ161383
CU148	HPV39	Partial E1 gene	626	GQ161384
CU149	HPV58	Partial E1 gene	104	GQ161385
CU150	HPV70	Partial E1 gene	666	GQ161386
CU151	HPV16	Partial E1 gene	178	GQ161387
CU152	HPV31	Partial E1 gene	590	GQ161388
CU153	HPV59	Partial E1 gene	66	GQ161389
CU154	HPV31	Partial E1 gene	615	GQ161390
CU155	HPV51	Partial E1 gene	630	GQ161391
CU156	HPV66	Partial E1 gene	627	GQ161392
CU157	HPV39	Partial E1 gene	618	GQ161393
CU158	HPV42	Partial E1 gene	652	GQ161394
CU159	HPV18	Partial E1 gene	540	GQ161395
CU160	HPV16	Partial E1 gene	671	GQ161396
CU161	HPV16	Partial E1 gene	671	GQ161397
CU162	HPV16	Partial E1 gene	678	GQ161398
CU163	HPV34	Partial E1 gene	678	GQ161399
CU164	HPV59	Partial E1 gene	657	GQ161400
CU165	HPV68	Partial E1 gene	670	GQ161401
CU166	HPV16	Partial E1 gene	676	GQ161402
CU167	HPV52	Partial E1 gene	657	GQ161403
CU168	HPV90	Partial E1 gene	653	GQ161404
CU169	HPV39	Partial E1 gene	618	GQ161405
CU170	HPV52	Partial E1 gene	589	GQ161406

Name	HPV genotype	genome	Product size (Base pairs)	Accession number
CU171	HPV68	Partial E1 gene	670	GQ161407
CU172	HPV66	Partial E1 gene	627	GQ161408
CU173	HPV66	Partial E1 gene	659	GQ161409
CU174	HPV51	Partial E1 gene	653	GQ161410
CU175	HPV16	Partial E1 gene	669	GQ161411
CU176	HPV32	Partial E1 gene	629	GQ161412
CU177	HPV42	Partial E1 gene	649	GQ161413
CU178	HPV42	Partial E1 gene	649	GQ161414
CU179	HPV16	Partial E1 gene	682	GQ161415
CU180	HPV52	Partial E1 gene	590	GQ161416
CU181	HPV66	Partial E1 gene	659	GQ161417
CU182	HPV16	Partial E1 gene	681	GQ161418
CU183	HPV16	Partial E1 gene	669	GQ161419
CU184	HPV66	Partial E1 gene	659	GQ161420
CU185	HPV39	Partial E1 gene	654	GQ161421
CU186	HPV6	Partial E1 gene	348	GQ161422
CU187	HPV53	Partial E1 gene	420	GQ161423
CU188	HPV16	Partial E1 gene	666	GQ161424
CU189	HPV52	Partial E1 gene	676	GQ161425
CU190	HPV56	Partial E1 gene	619	GQ161426
CU191	HPV68	Partial E1 gene	461	GQ161427
CU192	HPV52	Partial E1 gene	671	GQ161428
CU193	HPV16	Partial E1 gene	682	GQ161429
CU194	HPV52	Partial E1 gene	436	GQ161430
CU195	HPV90	Partial E1 gene	308	GQ161431
CU196	HPV16	Partial E1 gene	624	GQ161432
CU197	HPV16	Partial E1 gene	669	GQ161433

Name	HPV genotype	genome	Product size (Base pairs)	Accession number
CU198	HPV16	Partial E1 gene	511	GQ161434
CU199	HPV66	Partial E1 gene	661	GQ161435
CU200	HPV16	Partial E1 gene	671	GQ161436
CU201	HPV68	Partial E1 gene	269	GQ161437
CU202	HPV35	Partial E1 gene	640	GQ161438
CU203	HPV32	Partial E1 gene	255	GQ161439
CU204	HPV31	Partial E1 gene	294	GQ161440
CU205	HPV32	Partial E1 gene	410	GQ161441
CU206	HPV31	Partial E1 gene	624	GQ161442
CU207	HPV31	Partial E1 gene	376	GQ161443
CU208	HPV16	Partial E1 gene	355	GQ161444
CU209	HPV74	Partial E1 gene	650	GQ161445
CU210	HPV82	Partial E1 gene	396	GQ161446
CU211	HPV31	Partial E1 gene	698	GQ161447
CU212	HPV90	Partial E1 gene	593	GQ161448
CU213	HPV90	Partial E1 gene	644	GQ161449
CU214	HPV16	Partial E1 gene	84	GQ161450
CU215	HPV59	Partial E1 gene	662	GQ161451
CU216	HPV90	Partial E1 gene	665	GQ161452
CU217	HPV16	Partial E1 gene	671	GQ161453
CU218	HPV42	Partial E1 gene	651	GQ161454
CU219	HPV52	Partial E1 gene	661	GQ161455
CU220	HPV68	Partial E1 gene	672	GQ161456
CU221	HPV31	Partial E1 gene	601	GQ161457
CU222	HPV31	Partial E1 gene	667	GQ161458
CU223	HPV31	Partial E1 gene	626	GQ161459
CU224	HPV30	Partial E1 gene	659	GQ161460

Name	HPV genotype	genome	Product size (Base pairs)	Accession number
CU225	HPV68	Partial E1 gene	679	GQ161461
CU226	HPV16	Partial E1 gene	667	GQ161462
CU227	HPV31	Partial E1 gene	625	GQ161463
CU228	HPV31	Partial E1 gene	624	GQ161464
CU229	HPV68	Partial E1 gene	665	GQ161465
CU230	HPV66	Partial E1 gene	660	GQ161466
CU231	HPV34	Partial E1 gene	585	GQ161467
CU232	HPV71	Partial E1 gene	771	GQ161468
CU233	HPV16	Partial E1 gene	689	GQ161469
CU234	HPV90	Partial E1 gene	672	GQ161470
CU235	HPV34	Partial E1 gene	801	GQ161471
CU236	HPV34	Partial E1 gene	646	GQ161472
CU237	HPV90	Partial E1 gene	781	GQ161473
CU238	HPV66	Partial E1 gene	659	GQ161474
CU239	HPV66	Partial E1 gene	653	GQ161475
CU240	HPV51	Partial E1 gene	650	GQ161476
CU241	HPV51	Partial E1 gene	654	GQ161477
CU242	HPV39	Partial E1 gene	668	GQ161478
CU243	HPV68	Partial E1 gene	671	GQ161479
CU244	HPV31	Partial E1 gene	625	GQ161480
CU245	HPV31	Partial E1 gene	624	GQ161481
CU246	HPV39	Partial E1 gene	663	GQ161482
CU247	HPV16	Partial E1 gene	580	GQ161483
CU248	HPV31	Partial E1 gene	625	GQ161484
CU249	HPV68	Partial E1 gene	652	GQ161485
CU250	HPV66	Partial E1 gene	653	GQ161486
CU251	HPV16	Partial E1 gene	679	GQ161487

Name	HPV genotype	genome	Product size (Base pairs)	Accession number
CU252	HPV16	Partial E1 gene	720	GQ161488
CU253	HPV71	Partial E1 gene	658	GQ161489
CU254	HPV16	Partial E1 gene	772	GQ161490
CU255	HPV31	Partial E1 gene	692	GQ161491
CU256	HPV16	Partial E1 gene	677	GQ161492
CU257	HPV74	Partial E1 gene	659	GQ161493
CU258	HPV35	Partial E1 gene	626	GQ161494
CU259	HPV34	Partial E1 gene	657	GQ161495
CU260	HPV39	Partial E1 gene	665	GQ161496
CU261	HPV52	Partial E1 gene	672	GQ161497
CU262	HPV32	Partial E1 gene	649	GQ161498
CU263	HPV52	Partial E1 gene	663	GQ161499
CU264	HPV35	Partial E1 gene	630	GQ161500
CU265	HPV51	Partial E1 gene	642	GQ161501
CU266	HPV68	Partial E1 gene	643	GQ161502
CU267	HPV31	Partial E1 gene	473	GQ161503
CU268	HPV35	Partial E1 gene	587	GQ161504
CU269	HPV56	Partial E1 gene	559	GQ161505
CU270	HPV52	Partial E1 gene	443	GQ161506
CU271	HPV16	Partial E1 gene	261	GQ161507
CU272	HPV31	Partial E1 gene	395	GQ161508
CU273	HPV6	Partial E1 gene	520	GQ161509
CU274	HPV66	Partial E1 gene	656	GQ161510
CU275	HPV31	Partial E1 gene	399	GQ161511
CU276	HPV31	Partial E1 gene	626	GQ161512
CU277	HPV31	Partial E1 gene	627	GQ161513
CU278	HPV39	Partial E1 gene	666	GQ161514

Name	HPV genotype	genome	Product size (Base pairs)	Accession number
CU279	HPV39	Partial E1 gene	620	GQ161515
CU280	HPV66	Partial E1 gene	146	GQ161516
CU281	HPV16	Partial E1 gene	501	GQ161517
CU282	HPV16	Partial E1 gene	628	GQ161518
CU283	HPV16	Partial E1 gene	686	GQ161519
CU284	HPV31	Partial E1 gene	629	GQ161520
CU285	HPV35	Partial E1 gene	587	GQ161521
CU286	HPV55	Partial E1 gene	669	GQ161522
CU287	HPV51	Partial E1 gene	651	GQ161523
CU288	HPV31	Partial E1 gene	383	GQ161524
CU289	HPV31	Partial E1 gene	624	GQ161525
CU290	HPV31	Partial E1 gene	615	GQ161526
CU291	HPV71	Partial E1 gene	390	GQ161527
CU292	HPV71	Partial E1 gene	738	GQ161528
CU293	HPV35	Partial E1 gene	620	GQ161529
CU294	HPV31	Partial E1 gene	572	GQ161530
CU295	HPV66	Partial E1 gene	660	GQ161531
CU296	HPV31	Partial E1 gene	616	GQ161532
CU297	HPV56	Partial E1 gene	679	GQ161533
CU298	HPV16	Partial E1 gene	461	GQ161534
CU299	HPV56	Partial E1 gene	684	GQ161535
CU300	HPV82	Partial E1 gene	540	GQ161536
CU301	HPV31	Partial E1 gene	626	GQ161537
CU302	HPV56	Partial E1 gene	652	GQ161538
CU303	HPV31	Partial E1 gene	733	GQ161539
CU304	HPV66	Partial E1 gene	659	GQ161540
CU305	HPV31	Partial E1 gene	625	GQ161541

Name	HPV genotype	genome	Product size (Base pairs)	Accession number
CU306	HPV51	Partial E1 gene	651	GQ161542
CU307	HPV68	Partial E1 gene	626	GQ161543
CU308	HPV16	Partial E1 gene	680	GQ161544
CU309	HPV39	Partial E1 gene	622	GQ161545
CU310	HPV16	Partial E1 gene	679	GQ161546
CU311	HPV90	Partial E1 gene	643	GQ161547
CU312	HPV34	Partial E1 gene	664	GQ161548
CU313	HPV16	Partial E1 gene	664	GQ161549
CU314	HPV68	Partial E1 gene	670	GQ161550
CU315	HPV51	Partial E1 gene	633	GQ161551
CU316	HPV59	Partial E1 gene	416	GQ161552
CU317	HPV59	Partial E1 gene	320	GQ161553
CU318	HPV16	Partial E1 gene	659	GQ161554
CU319	HPV16	Partial E1 gene	440	GQ161555
CU320	HPV16	Partial E1 gene	563	GQ161556
CU321	HPV52	Partial E1 gene	532	GQ161557
CU322	HPV35	Partial E1 gene	626	GQ161558
CU323	HPV66	Partial E1 gene	185	GQ161559
CU324	HPV6	Partial E1 gene	618	GQ161560
CU325	HPV66	Partial E1 gene	289	GQ161561
CU326	HPV70	Partial E1 gene	122	GQ161562
CU327	HPV68	Partial E1 gene	659	GQ161563
CU328	HPV66	Partial E1 gene	656	GQ161564
CU329	HPV35	Partial E1 gene	623	GQ161565
CU330	HPV6	Partial E1 gene	655	GQ161566
CU331	HPV16	Partial E1 gene	681	GQ161567
CU332	HPV42	Partial E1 gene	642	GQ161568

Name	HPV genotype	genome	Product size (Base pairs)	Accession number
CU333	HPV81	Partial E1 gene	400	GQ161569
CU334	HPV16	Partial E1 gene	681	GQ161570
CU335	HPV31	Partial E1 gene	639	GQ161571
CU336	HPV16	Partial E1 gene	681	GQ161572
CU337	HPV52	Partial E1 gene	663	GQ161573
CU338	HPV52	Partial E1 gene	669	GQ161574
CU339	HPV16	Partial E1 gene	671	GQ161575
CU340	HPV52	Partial E1 gene	663	GQ161576
CU341	HPV16	Partial E1 gene	649	GQ161577
CU342	HPV31	Partial E1 gene	624	GQ161578
CU343	HPV34	Partial E1 gene	669	GQ161579
CU344	HPV52	Partial E1 gene	669	GQ161580
CU345	HPV16	Partial E1 gene	681	GQ161581
CU346	HPV52	Partial E1 gene	670	GQ161582
CU347	HPV34	Partial E1 gene	670	GQ161583
CU348	HPV66	Partial E1 gene	164	GQ161584
CU349	HPV59	Partial E1 gene	664	GQ161585
CU350	HPV90	Partial E1 gene	648	GQ161586
CU351	HPV31	Partial E1 gene	521	GQ161587
CU352	HPV71	Partial E1 gene	653	GQ161588
CU353	HPV16	Partial E1 gene	670	GQ161589
CU354	HPV16	Partial E1 gene	676	GQ161590
CU355	HPV33	Partial E1 gene	655	GQ161591
CU356	HPV31	Partial E1 gene	613	GQ161592
CU357	HPV16	Partial E1 gene	670	GQ161593
CU358	HPV31	Partial E1 gene	758	GQ161594
CU359	HPV31	Partial E1 gene	251	GQ161595

Name	HPV genotype	genome	Product size (Base pairs)	Accession number
CU360	HPV68	Partial E1 gene	638	GQ161966
CU361	HPV42	Partial E1 gene	444	GQ161597
CU362	HPV58	Partial E1 gene	661	GQ161598
CU363	HPV16	Partial E1 gene	820	GQ161599
CU364	HPV31	Partial E1 gene	757	GQ161600
CU365	HPV16	Partial E1 gene	671	GQ161601
CU366	HPV16	Partial E1 gene	874	GQ161602
CU367	HPV52	Partial E1 gene	656	GQ161603
CU368	HPV16	Partial E1 gene	808	GQ161604
CU369	HPV56	Partial E1 gene	664	GQ161605
CU370	HPV71	Partial E1 gene	638	GQ161606
CU371	HPV59	Partial E1 gene	665	GQ161607
CU372	HPV16	Partial E1 gene	672	GQ161608
CU373	HPV73	Partial E1 gene	681	GQ161609
CU374	HPV55	Partial E1 gene	831	GQ161610
CU375	HPV31	Partial E1 gene	599	GQ161611
CU376	HPV70	Partial E1 gene	661	GQ161612
CU377	HPV16	Partial E1 gene	734	GQ161613
CU378	HPV52	Partial E1 gene	643	GQ161614
CU379	HPV16	Partial E1 gene	671	GQ161615
CU380	HPV16	Partial E1 gene	680	GQ161616
CU381	HPV16	Partial E1 gene	746	GQ161617
CU382	HPV16	Partial E1 gene	677	GQ161618
CU383	HPV16	Partial E1 gene	678	GQ161619
CU384	HPV16	Partial E1 gene	682	GQ161620
CU385	HPV74	Partial E1 gene	656	GQ161621
CU386	HPV16	Partial E1 gene	680	GQ161622

Name	HPV genotype	genome	Product size (Base pairs)	Accession number
CU387	HPV16	Partial E1 gene	671	GQ161623
CU388	HPV16	Partial E1 gene	489	GQ161624
CU389	HPV16	Partial E1 gene	674	GQ161625
CU390	HPV16	Partial E1 gene	668	GQ161626
CU391	HPV16	Partial E1 gene	682	GQ161627
CU392	HPV44	Partial E1 gene	658	GQ161628
CU393	HPV56	Partial E1 gene	683	GQ161629
CU394	HPV16	Partial E1 gene	681	GQ161630
CU395	HPV52	Partial E1 gene	284	GQ161631
CU396	HPV31	Partial E1 gene	622	GQ161632
CU397	HPV16	Partial E1 gene	665	GQ161633
CU398	HPV31	Partial E1 gene	752	GQ161634
CU399	HPV52	Partial E1 gene	643	GQ161635
CU400	HPV31	Partial E1 gene	769	GQ161636
CU401	HPV16	Partial E1 gene	668	GQ161637
CU402	HPV16	Partial E1 gene	668	GQ161638
CU403	HPV16	Partial E1 gene	668	GQ161639
CU404	HPV52	Partial E1 gene	670	GQ161640
CU405	HPV56	Partial E1 gene	652	GQ161641
CU406	HPV16	Partial E1 gene	668	GQ161642
CU407	HPV52	Partial E1 gene	670	GQ161643
CU408	HPV52	Partial E1 gene	670	GQ161644
CU409	HPV16	Partial E1 gene	668	GQ161645
CU410	HPV16	Partial E1 gene	681	GQ161646
CU411	HPV16	Partial E1 gene	681	GQ161647
CU412	HPV73	Partial E1 gene	761	GQ161648
CU413	HPV31	Partial E1 gene	769	GQ161649

Name	HPV genotype	genome	Product size (Base pairs)	Accession number
CU414	HPV58	Partial E1 gene	670	GQ161650
CU415	HPV90	Partial E1 gene	653	GQ161651
CU416	HPV31	Partial E1 gene	769	GQ161652
CU417	HPV16	Partial E1 gene	681	GQ161653
CU418	HPV16	Partial E1 gene	681	GQ161654
CU419	HPV39	Partial E1 gene	478	GQ161655
CU420	HPV16	Partial E1 gene	681	GQ161656
CU421	HPV31	Partial E1 gene	681	GQ161657
CU422	HPV42	Partial E1 gene	653	GQ161658
CU423	HPV52	Partial E1 gene	673	GQ161659
CU424	HPV16	Partial E1 gene	350	GQ161660
CU425	HPV16	Partial E1 gene	669	GQ161661
CU426	HPV16	Partial E1 gene	580	GQ161662
CU427	HPV16	Partial E1 gene	669	GQ161663
CU428	HPV31	Partial E1 gene	622	GQ161664
CU429	HPV42	Partial E1 gene	653	GQ161665
CU430	HPV35	Partial E1 gene	636	GQ161666
CU431	HPV16	Partial E1 gene	669	GQ161667
CU432	HPV66	Partial E1 gene	656	GQ161668
CU433	HPV90	Partial E1 gene	665	GQ161669
CU434	HPV71	Partial E1 gene	647	GQ161670
CU435	HPV66	Partial E1 gene	656	GQ161671
CU436	HPV35	Partial E1 gene	626	GQ161672
CU437	HPV53	Partial E1 gene	475	GQ161673
CU438	HPV90	Partial E1 gene	658	GQ161674
CU439	HPV16	Partial E1 gene	260	GQ161675
CU440	HPV16	Partial E1 gene	618	GQ161676

Name	HPV genotype	genome	Product size (Base pairs)	Accession number
CU441	HPV71	Partial E1 gene	645	GQ161677
CU442	HPV74	Partial E1 gene	647	GQ161678
CU443	HPV52	Partial E1 gene	666	GQ161679
CU444	HPV90	Partial E1 gene	653	GQ161680
CU445	HPV16	Partial E1 gene	671	GQ161681
CU446	HPV39	Partial E1 gene	618	GQ161682
CU447	HPV39	Partial E1 gene	657	GQ161683
CU448	HPV42	Partial E1 gene	641	GQ161684
CU449	HPV16	Partial E1 gene	672	GQ161685
CU450	HPV31	Partial E1 gene	554	GQ161686
CU451	HPV52	Partial E1 gene	640	GQ161687
CU452	HPV90	Partial E1 gene	658	GQ161688
CU453	HPV16	Partial E1 gene	674	GQ161689
CU454	HPV34	Partial E1 gene	676	GQ161690
CU455	HPV16	Partial E1 gene	683	GQ161691
CU456	HPV11	Partial E1 gene	674	GQ161692
CU457	HPV71	Partial E1 gene	646	GQ161693
CU458	HPV34	Partial E1 gene	666	GQ161694
CU459	HPV59	Partial E1 gene	657	GQ161695
CU460	HPV66	Partial E1 gene	657	GQ161696
CU461	HPV55	Partial E1 gene	662	GQ161697
CU462	HPV71	Partial E1 gene	617	GQ161698
CU463	HPV42	Partial E1 gene	603	GQ161699
CU464	HPV16	Partial E1 gene	630	GQ161700
CU465	HPV71	Partial E1 gene	646	GQ161701
CU466	HPV59	Partial E1 gene	630	GQ161702
CU467	HPV66	Partial E1 gene	661	GQ161703

Name	HPV genotype	genome	Product size (Base pairs)	Accession number
CU468	HPV66	Partial E1 gene	530	GQ161704
CU469	HPV91	Partial E1 gene	214	GQ161705
CU470	HPV71	Partial E1 gene	652	GQ161706
CU471	HPV53	Partial E1 gene	674	GQ161707
CU472	HPV16	Partial E1 gene	626	GQ161708
CU473	HPV71	Partial E1 gene	627	GQ161709
CU474	HPV39	Partial E1 gene	662	GQ161710
CU475	HPV16	Partial E1 gene	670	GQ161711
CU476	HPV31	Partial E1 gene	383	GQ161712
CU477	HPV71	Partial E1 gene	658	GQ161713
CU478	HPV52	Partial E1 gene	652	GQ161714
CU479	HPV56	Partial E1 gene	669	GQ161715
CU480	HPV39	Partial E1 gene	695	GQ161716
CU481	HPV32	Partial E1 gene	639	GQ161717
CU482	HPV52	Partial E1 gene	530	GQ161718
CU483	HPV30	Partial E1 gene	275	GQ161719
CU484	HPV53	Partial E1 gene	257	GQ161720
CU485	HPV68	Partial E1 gene	657	GQ161721
CU486	HPV16	Partial E1 gene	484	GQ161751
CU487	HPV51	Partial E1 gene	644	GQ161722
CU488	HPV85	Partial E1 gene	658	GQ161723
CU489	HPV90	Partial E1 gene	658	GQ161724
CU490	HPV16	Partial E1 gene	681	GQ161725
CU491	HPV90	Partial E1 gene	655	GQ161726
CU492	HPV66	Partial E1 gene	671	GQ161727
CU493	HPV52	Partial E1 gene	661	GQ161728
CU494	HPV68	Partial E1 gene	670	GQ161729

Name	HPV genotype	genome	Product size (Base pairs)	Accession number
CU495	HPV71	Partial E1 gene	634	GQ161730
CU496	HPV90	Partial E1 gene	655	GQ161731
CU497	HPV90	Partial E1 gene	660	GQ161732
CU498	HPV16	Partial E1 gene	668	GQ161733
CU499	HPV16	Partial E1 gene	678	GQ161734
CU500	HPV16	Partial E1 gene	672	GQ161735
CU501	HPV90	Partial E1 gene	671	GQ161736
CU502	HPV90	Partial E1 gene	653	GQ161737
CU503	HPV90	Partial E1 gene	594	GQ161738
CU504	HPV90	Partial E1 gene	647	GQ161739
CU505	HPV90	Partial E1 gene	579	GQ161740
CU506	HPV90	Partial E1 gene	646	GQ161741
CU507	HPV90	Partial E1 gene	655	GQ161742
CU508	HPV90	Partial E1 gene	655	GQ161743
CU509	HPV90	Partial E1 gene	613	GQ161744
CU510	HPV16	Partial E1 gene	675	GQ161745
CU511	HPV34	Partial E1 gene	677	GQ161746
CU512	HPV31	Partial E1 gene	642	GQ161747
CU513	HPV52	Partial E1 gene	663	GQ161748
CU514	HPV35	Partial E1 gene	637	GQ161749
CU515	HPV32	Partial E1 gene	642	GQ161750

BIOGRAPHY

NAME: Woradee Lurchachaiwong

SEX: Female

DATE OF BIRTH: April 20th, 1983

PLACE OF BIRTH: Bangkok, Thailand

NATIONALITY: Thai

E-MAIL ADDRESS: peach_angle@hotmail.com

EDUCATION:

2001-2005: Bachelor of Science (1st Class Honors) in Biochemistry, Chulalongkorn University, Thailand

2005-Present: Ph.D. student, Department of Biomedical Science, Faculty of Graduate School, Chulalongkorn University, Thailand

RESEARCH GRANT: Royal Golden Jubilee Ph.D. program, Thailand Research Fund

PROFESSIONAL EXPERIENCE: A guest researcher in Division of Virology, Department of Pathology, University of Cambridge, Cambridge, (2009-2010)

INTERNATIOANAL PRESENTATION:

1. **Poster Presentation:** "Correlation between cervical cytology and high-risk human papillomavirus genotypes among Thai women by analysis of E1 genes" Eurogin 2008 Joining Forces for Cancer Prevention. November 12-15, 2008. France.
2. **Poster Presentation:** "Rapid detection and strain identification of porcine reproductive and respiratory syndrome virus (PRRSV) by real-time RT-PCR" RGJ-Ph.D. Congress VIII. April 20-22, 2007. Thailand,
3. **Poster Presentation:** "Rapid detection and strain identification of porcine reproductive and respiratory syndrome virus (PRRSV) by real-time RT-PCR" 32th Congress on Science and Technology of Thailand 2006, October 10-12, 2006. Thailand.

PUBLICATIONS (2005-2010): 5 international publications and 1 revising publication