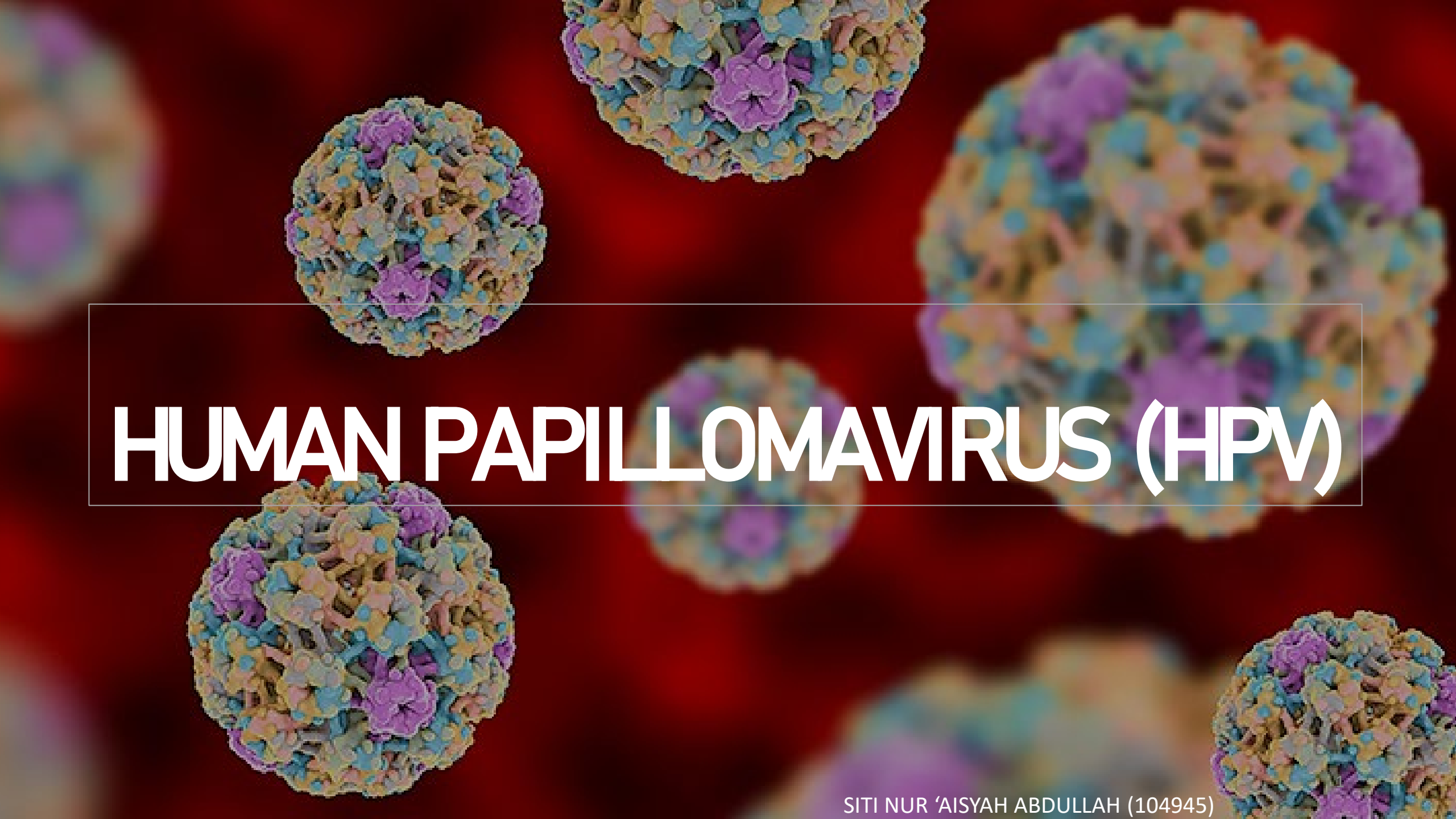
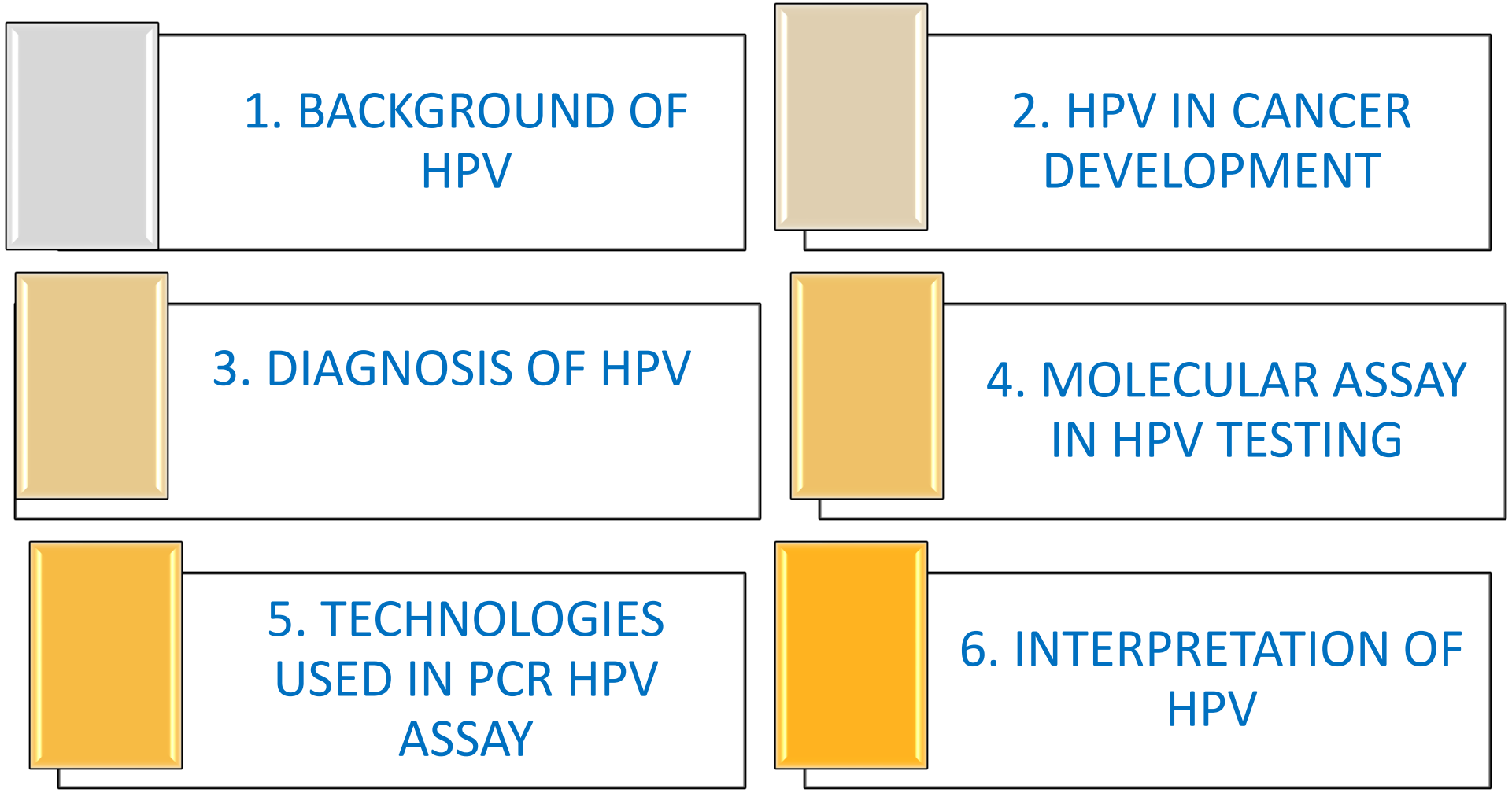


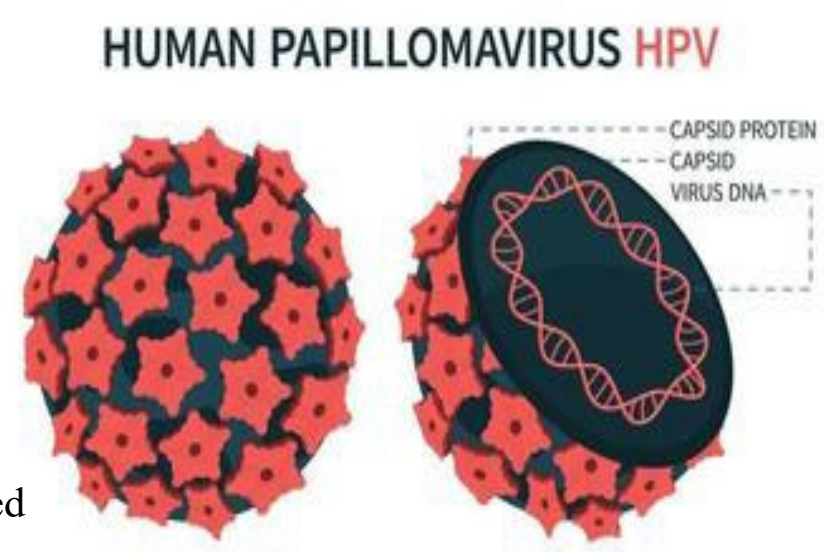
HUMAN PAPILLOMAVIRUS (HPV)

SITI NUR 'AISYAH ABDULLAH (104945)

OVERVIEW


1. BACKGROUND OF HPV
2. HPV IN CANCER DEVELOPMENT
3. DIAGNOSIS OF HPV
4. MOLECULAR ASSAY IN HPV TESTING
5. TECHNOLOGIES USED IN PCR HPV ASSAY
6. INTERPRETATION OF HPV

1.1 PAPILLOMAVIRUSES



- Papillomaviruses are small double-stranded DNA viruses.
- Family: *Papillomaviridae* [1]
- Non-enveloped, epitheliotropic as they initiate productive infections only within stratified epithelia of the skin, examples; the anogenital tract and the oral cavity.
- Contains of DNA molecule of about 8000 base-pairs (bp) that is bound to cellular histones and contained in a protein capsid composed of 72 pentameric capsomers. [2]
- Composed of three genome segments; early (E1-E2, E4-E8), late (L1 & L2) and genomic regions. [2]

1. Zheng, Z. M., & Baker, C. C. (2006). Papillomavirus genome structure, expression, and post-transcriptional regulation. *Frontiers in bioscience : a journal and virtual library*, *11*, 2286–2302.

2. Kirnbauer *et al.* (1992); Hagensee *et al.*, (1993), IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Human Papillomaviruses. Lyon (FR): International Agency for Research on Cancer; (2007), Human Papillomavirus (HPV) Infection

Table 1. Function of HPV Gene Products

Protein	Function [Reference]
E1	DNA helicase activity, DNA-dependent ATP-binding, ATPase activity, Role in replication and replication repressor [5,9]
E2	Regulator of viral transcription and replication, control of early region viral gene expression, necessary for efficient viral DNA replication together with E1 [8,9,10]
E5	Transforming activity , Presumably stimulates benign cell proliferation *in vivo* but might have a role in the innitiator of carcinogenesis. Downregulation MHC class I expression [14,15,16]
E6	Role in transformation process together with E7, Transcriptional activation properties E6 of high-risk HPVs inactivates p53 by including its degradation, Together with E7 provides a cellular enviroment for viral DNA replication [17,18,19,20]
E7	Transactivating properties similar to the adenovirus E2 promoter, induced DNA synthesis in quiescent cells,, role in rodent cell transformation in co-operation with an activated ras oncogene. E7 binds to the hypophosphorylated from of retinoblastoma protein (pRB) resulting in its functional inactivation permitting cell progression to S phase of cell cycle. E7 proteins from the low-risk HPV type 6 and 11 bind less efficiently than the E7 protein from high-risk HPVs (type 16 and 18) [17,18,19]
E4	Expressed as a late gene primarily in differentiating epithelium, role in productive infection associated with the keratin cytoskeleton of cultured epithelial cells, role in viral egress, G2 cell cycle arrest [12,13]
L1	Major capsid protein
L2	Major capsid protein

[3]. Kim, Sang-Woo & Yang, Joo-Sung. (2006). Human Papillomavirus Type 16 E5 Protein as a Therapeutic Target. Yonsei medical journal, page 3.

1.2 INTRODUCTION TO HPV

- Human Papillomavirus (HPV) is one of the most common sexually transmitted diseases in worldwide. [4]

- The route of transmission is from person to person by close intimate contact during vaginal, penile, anal, or oral sex. Both males and females can be infected. [4]

- HPV is usually asymptomatic and resolve spontaneously. But there is a risk that HPV infection may lead to chronic and pre-cancerous lesions which progress to invasive cervical cancer.

- The oncogenicity of HPV, immune system, coinfection of other sexually transmitted infections (STI) , or parity or young age first-born are some of the risk factors for cervical cancer development.

4. Burd E. M. (2003). Human papillomavirus and cervical cancer. *Clinical microbiology reviews*

1.3 TYPES OF HPV

- The HPV genotype can be divided into **High-Risk(HR)** and **Low-Risk(LR).**

High-Risk(HR) 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, 82

- HPV16 and HPV18 are responsible for most HPV-related cancers. [5]
- Types 26, 53, and 66 are likely oncogenic [6]

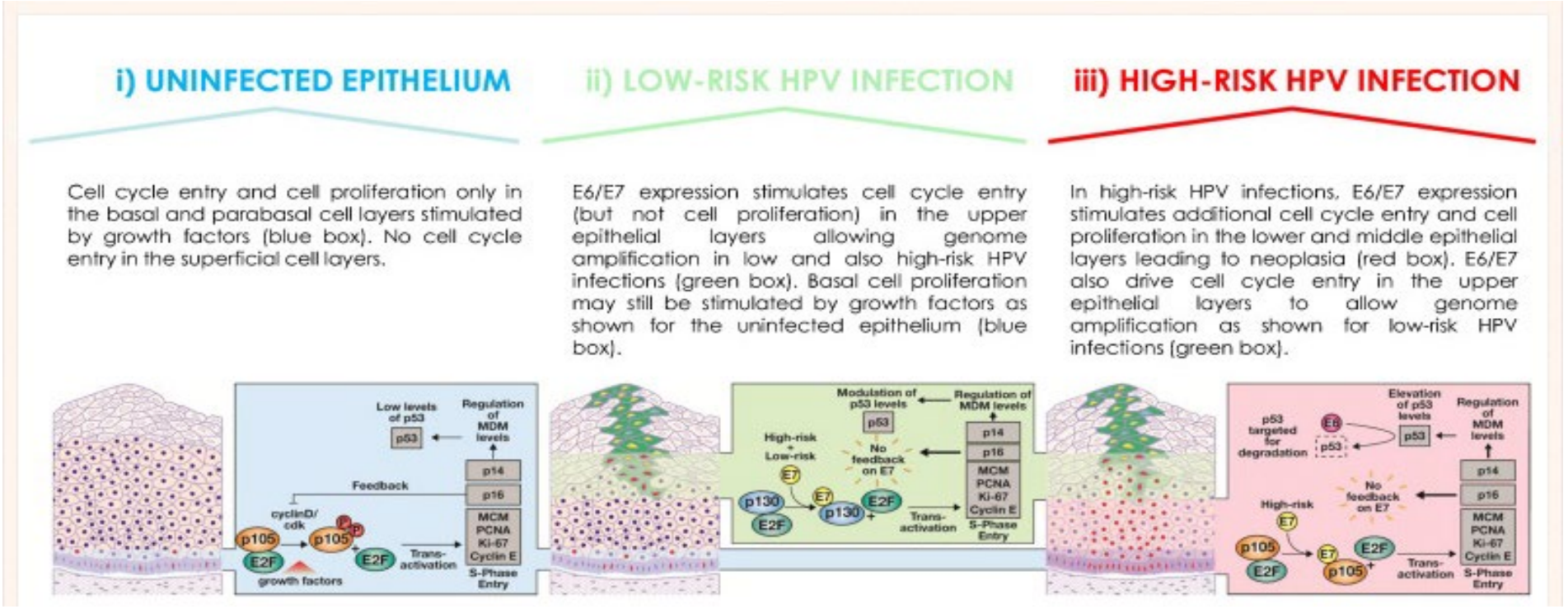
Low-Risk(LR) 6, 11, 40, 42, 43, 44, 54, 61, 70

- Type 6 & 11 are commonly found cause warts on or around the genitals, anus, mouth, or throat. [7]

5. Hildesheim A, Schiffman M, Bromley C, Wacholder S, Herrero R, Rodriguez A, Bratti MC, Sherman ME, Scarpidis U, Lin QQ, Terai M, Bromley RL, Buetow K, Apple RJ, Burk RD, (2001) Human papillomavirus type 16 variants and risk of cervical cancer.
6. Munoz et al., (2003), Journal of Clinical Virology 32S (2005) S44)
7. Adrian Mindel, Dominic Dwyer, Belinda Hering and Anthony L. Cunningham, (2013), Sexual Transmitted Disease

2.1 HPV IN CANCER DEVELOPMENT

- Generally HPV infection causes cell destruction as well as cell transformation and tumor development.
- HPV interfere with cell cycle regulation and prevent apoptosis in cells with unscheduled DNA replication.
- The development of cervical cancer is considered to be a multistep process, where HPV is necessary but in itself an insufficient cause the disease can only develop if there is persistent HPV infection of the cervical epithelium. [8]



8. Walboomers et al., 1999; Steenbergen et al., A. Molijn et al., (2005) , Journal of Clinical Virology 32S S43–S51.

2.2 PATHOGENESIS

- The infection of HPV occurs specifically at the basal cells of stratified epithelium as the virus cannot bind to live tissue.
- It infects the epithelial tissues through micro-abrasions or other epithelial trauma that exposes segments of basement membrane.
- In benign HPV lesions, viral DNA is located episomal in the nucleus.
- Malignant lesions which are high-grade intraepithelial neoplasia and cancer, the DNA is integrated into cell chromosomes.
- When HPV DNA is not integrated into cell genome, E2 becomes active and suppresses the function of E6 and E7.
- Meanwhile, when HPV DNA is integrated , E6 and E7 are active and suppress the activity of E2.

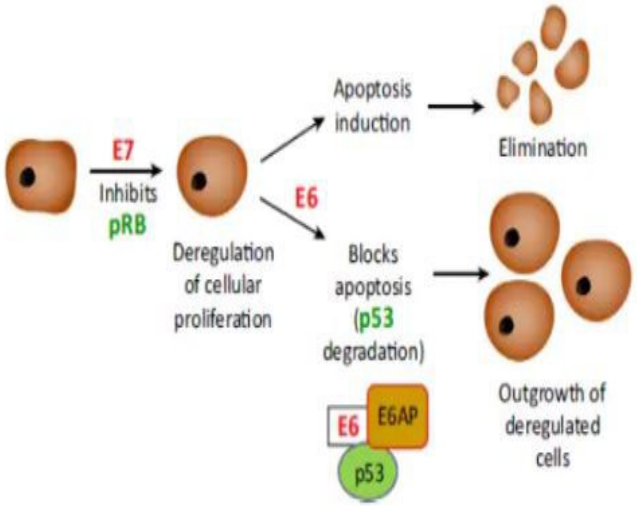
[9]. Stanley M.,(2010),Pathology and epidemiology of HPV infection in females. Gynecol Oncol.

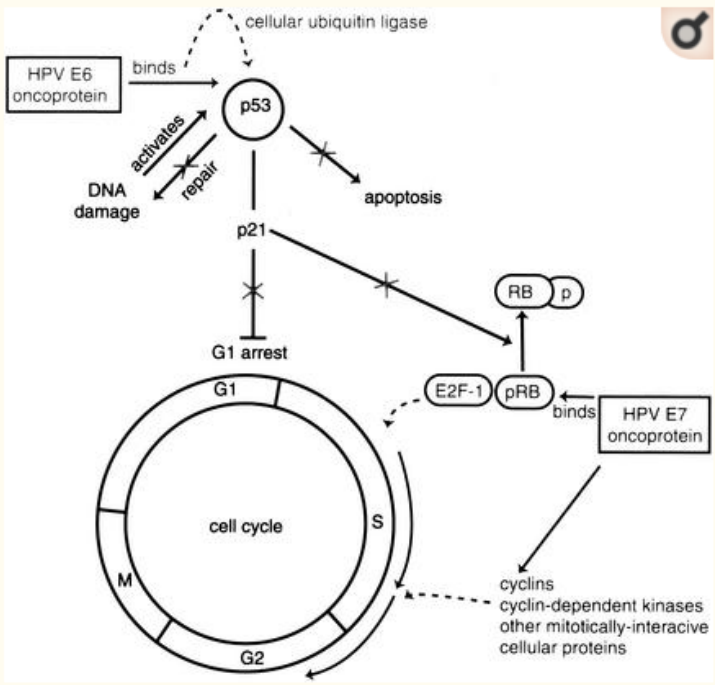
- HPV integrates into host DNA and caused over expression of viral protein E6 and E7. Both early proteins have high affinity for host cells.

- E7 functions as primary transforming protein, competes with retinoblastoma protein (pRb) binding, freeing the transcription factor E2F to activate its target, thus pushing the cell cycle forward. [4]

- Tumor suppressor protein p53 is inactivated by early protein E6 and pRb is inactivated by E7. [4]

- E6 attaches with host E6-associated protein which has ubiquitin ligase activity performed to ubiquitinate p53 and leads to degradation of p53. [4]

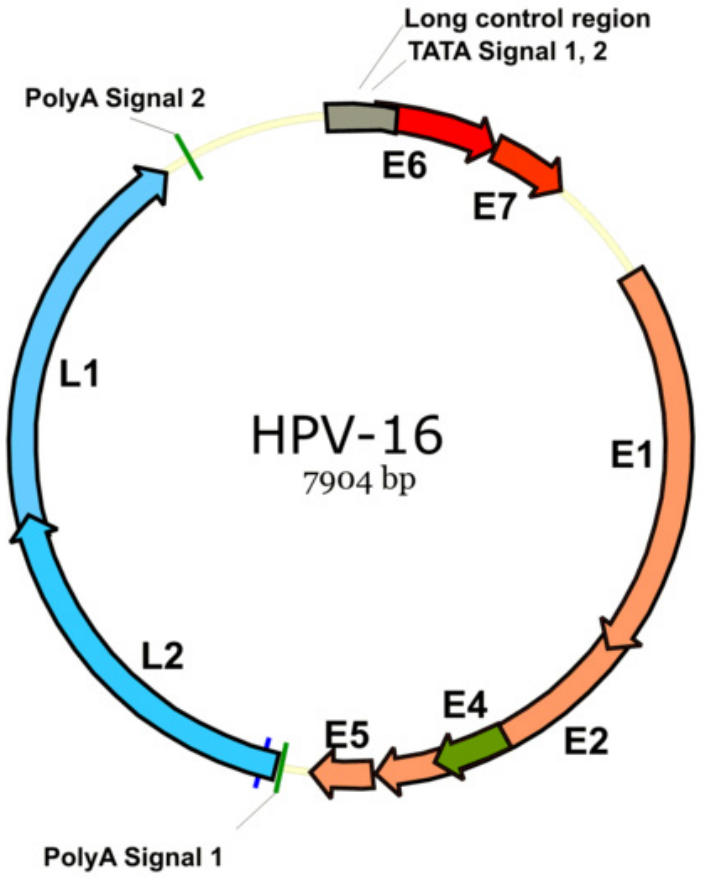




4. Burd E. M. (2003). Human papillomavirus and cervical cancer. *Clinical microbiology reviews*

Genomic structure of HPV Type 16

The binding affinity of E6 and E7 proteins of HPV genotype 16 and 18 to these tumor suppressor proteins (p53 & pRb) are very high which explains why these two genotype are highly oncogenic.



2.3 HUMAN PAPILLOMAVIRUS VACCINES

- Cervical cancer is the 4th most recurrent cancer in women with 7.5% of cancer deaths. [11]
- The global burden of cervical cancer has increased awareness on HPV vaccination programs in many countries.
- The vaccines functioning to produce antibodies which encounters with HPV, bind to the virus and prevent it from infecting cells.
- The vaccines are formed by HPV surface components with lack of virus's DNA. They closely resemble the natural virus, known as virus-like particles (VLP). [12]
- Vaccines are highly effective by the factor of VLP characteristic which is immunogenic – induction of high levels of antibody produced by the body. [12]

11. National Cancer Institute.
12. Renjie Wang, Wei Pan, Lei Jin, Weiming Huang, Yuehan Li, Di Wu, Chun Gao, Ding Ma, Shujie Liao, (2020), Human papillomavirus vaccine against cervical cancer: Opportunity and challenge, Cancer Letters

3. DIAGNOSIS OF HPV

	TEST	TECHNIQUES
CELL MORPHOLOGY	• **Pap smear** • **Colposcopy** • Visual inspection	
DETECTION OF HPV PROTEINS	• **Immunocyto/histochemistry** • Electron microscopy • Western blots	
DETECTION OF HPV GENOMES	• Direct methods	1. Southern blot 2. In situ hybridization 3. Dot blot
	• Signal amplification	1. Hybrid Capture
	• Target amplification	**1. Real-Time PCR**
DETECTION OF ANTI-HPV ANTIBODIES	• ELISA peptides • VLP • Fused E6/E7	

13. Published by Woodhead Publishing Limited, (2012)

Pap smear

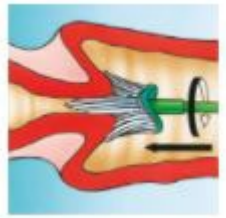
- A screening test first describe by Papanicolaou and Traut.
- To identify cellular changes in the cervix, which could be caused by HPV. [4]
- Apart from premalignant and malignant changes, viral infections like HPV infection and Herpes can also be detected.
- Abnormal microscopic examination requires further confirmatory tests like coloscopy, cervical biopsy, and PCR.
- Normal Pap-smear shows no abnormal cells changes.
- Abnormal pap-smear indicates several levels of abnormalities; atypia,mild,moderate,severe dysplasia,carcinoma in situ.

4. Burd E. M. (2003). Human papillomavirus and cervical cancer. *Clinical microbiology reviews*

	THINPREP	SUREPATH
Collecting Device	Brush is washed in the fixative and discarded	Bristle is detached into the fixative
Name of Fixative	PreserveCyt fluid	CytoRich fluid
Fixative Component	Methanol	Ethanol
Vortex	No vortex	Vortex mixed
Gradient Centrifuge	No gradient centrifugation	gradient centrifugation
Sedimentation	No sedimentation	Sedimentation
Filter	Filter used	No Filter used
Staining	Standard automated staining	Integral part of procedure
Smear Area	20mm	13mm

1. In the thinprep pap test, cell samples from the vagina, cervix and cervical canal are collected using a broom-like device. [14]

2. The central bristles of the brush will be insert into the endocervical canal deep enough to allow the shorter bristles to fully contact the ectocervix. The brush is rotated in a clockwise direction five times for a sufficient cells obtain. [14]



3. The brush is immediately put into the preservecyt fluid vial by pushing it into the bottom of the vial 10 times, forcing the bristles apart. As a final step, swirl the brush vigorously to further release material before discarding the brush. [14]

14. Dr.Premalatha.A. (2015), Diagnostic efficacy of thin prep preparation(liquid based cytology) in Comparison to conventional pap smears as a primary screeningtool for cervical lesions

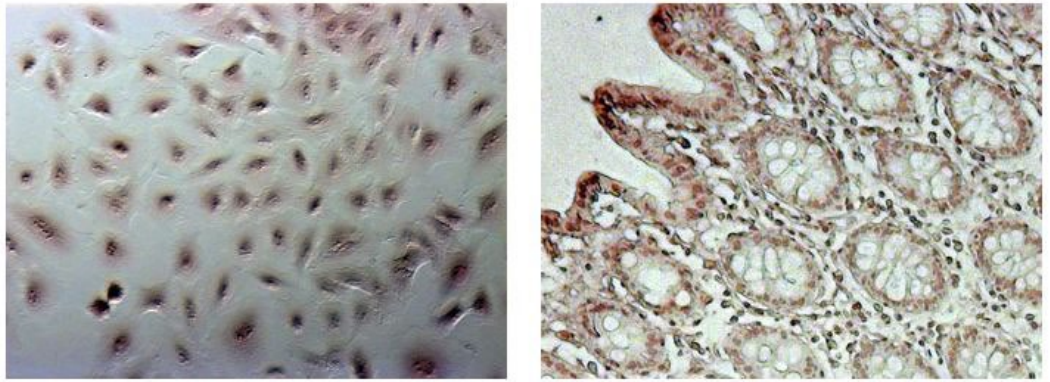
Colposcopy & acetic acid test

- Colposcopy is a procedure performed using low-powered microscope which is colposcope.
- Provides a magnified and illuminated view of vulva, vaginal walls and uterine cervix.
- To examine the cervix with abnormal pap smear result or during the pap smear collection.
- Colposcopy includes: acetic acid wash, color filters and biopsy.[4]
- By apply acid solution such as vinegar or of acetic acid towards the suspected lesions, a white stain will form called “acetowhite lesions”
- Color filters help to examine tiny blood vessels in squamocolumnar junction area. Blue or green filtered light shows abnormal blood vessels, usually more obvious in the acetowhite area.
- Biopsy is a tissue sampling at the targeted abnormal areas.

4. Burd E. M. (2003). Human papillomavirus and cervical cancer. *Clinical microbiology reviews*

Immunocyto/histochemistry

- To identify proteins and other macromolecules in tissues and cells.
- Immunocytochemistry (ICC) or Immunohistochemistry (IHC) are protein marker detection methods used histologically on tissue or cell samples.
- Based on a study shows p16/Ki-67 and p16/MCM2 staining are promising to triage High-Risk HPV-positive women with high sensitivity and low risk for negative women. $P16^{INK4a}$ is a cyclin-dependent kinase inhibitor that has been proven to be significantly overexpressed in transforming infections with High-Risk HPV. As a negative regulator of cell proliferation, p16 protein can downregulate the activity of CDK4 and CDK6 once the retinoblastoma protein has been inactivated. It has been recognized to be a surrogate marker for cervical precancerous lesions. Cross-sectional studies have reported the accuracy of p16/Ki-67 and p16/MCM2 dual staining as a primary or triaging test for detecting high-grade cervical lesions [15].



Comparison of immunocytochemistry (ICC) and immunohistochemistry (IHC) staining. A549 human adenocarcinoma cells (left panel; ICC) and a human colon carcinoma tissue section (right panel; IHC) were fixed and stained for protein phosphatase 2 (PP2A) using an unconjugated mouse anti-human PP2A primary antibody and an HRP-conjugated goat anti-mouse secondary antibody. Enzyme-mediated staining was completed using our Metal Enhanced DAB Substrate Kit (34065), resulting in brown deposits at sites where PP2A was localized.

15. Yu-Cong Li, Yu-Qian Zhao, Ting-Yuan Li, Wen Chen, Guang-Dong Liao, Hai-Rui Wang, Hai-Ke Lei, Yue Guo, Qi Zhou, (2020),"The Performance of Immunocytochemistry Staining as Triaging Tests for High-Risk HPV-Positive Women: A 24-Month Prospective Study", *Journal of Oncology*

Real Time-PCR Detection of HPV DNA

TEST	PRINCIPLES	
Real-time PCR	PCR assay is a technique of amplifying the target DNA by using specific and or general primers. It involves four repeated cycles of denaturation of DNA template, the annealing of target-specific primers to target DNA, the extension of complementary DNA sequences and the aim of to generate amplification of target HPV DNA.	Advantages Capable of recognizing all HPV types and variants present in a biologic specimen is DNA sequencing of the viral genome Accurate method of estimating viral load Disadvantages Expensive equipment Labor intensive

The main method used for HPV DNA detection in clinical laboratories is Real-Time PCR [17].

17. Yoshida T, Sano T, Kanuma T, Owada N, Sakurai S, Fukuda T, Nakajima T Int J Gynecol Cancer, (2008), Quantitative real-time polymerase chain reaction analysis of the type distribution, viral load, and physical status of human papillomavirus in liquid-based cytology samples from cervical lesions.

18. *Jin Y, Li JP, He D, Tang LY, Zee CS, Guo SZ, Zhou J, Chen JN, Shao CK Asian Pac J Cancer Prev.,(2011),* Clinical significance of human telomerase RNA gene (hTERC) amplification in cervical squamous cell lesions detected by fluorescence in situ hybridization. – Can be removed

4. MOLECULAR ASSAY IN HPV TESTING

- In SJMC Molecular Diagnostic Laboratory, the diagnosis of PCR HPV is using a semi-quantitative real-time PCR test kit (Anyplex II HPV28 Detection Kit, Ver. 1.05, CE-IVD) with BioRad CFX96.

- In conjunction with gynaecological cytology testing, a better diagnosis can be obtained.
- There are two package of testing; (i).HPV DNA PCR
  (ii). Thin Prep & HPV DNA PCR

- **Sample type:**

Cervical Scrape and Endocervical Brush for both Thin Prep + HPV DNA PCR, Liquid-Based Cytology for HPV DNA PCR

- **Method use:**

DNA extraction using automated Seegene-NIMBUS instrument and PCR kit using Seegene Anyplex II HPV28

- **Limitation & sensitivity:**

The assay detects 28 HPV types at a limit of detection of 50 copies per reaction

Extraction of Nucleic Acid

- The aim of extraction process is to aim high quality of genomic DNA for HPV testing. Thus, there are a few method that can be use for extraction of nucleic acid process.

FFPE tissue:

- Formalin-fixed, paraffin-embedded tissue is one of the specimen types used for PCR HPV DNA test.
- This method used formaldehyde for the tissue sample preservation and fixation in order to preserve the tissue architecture and cells component shape . The image below shows extraction steps of DNA from FFPE sample

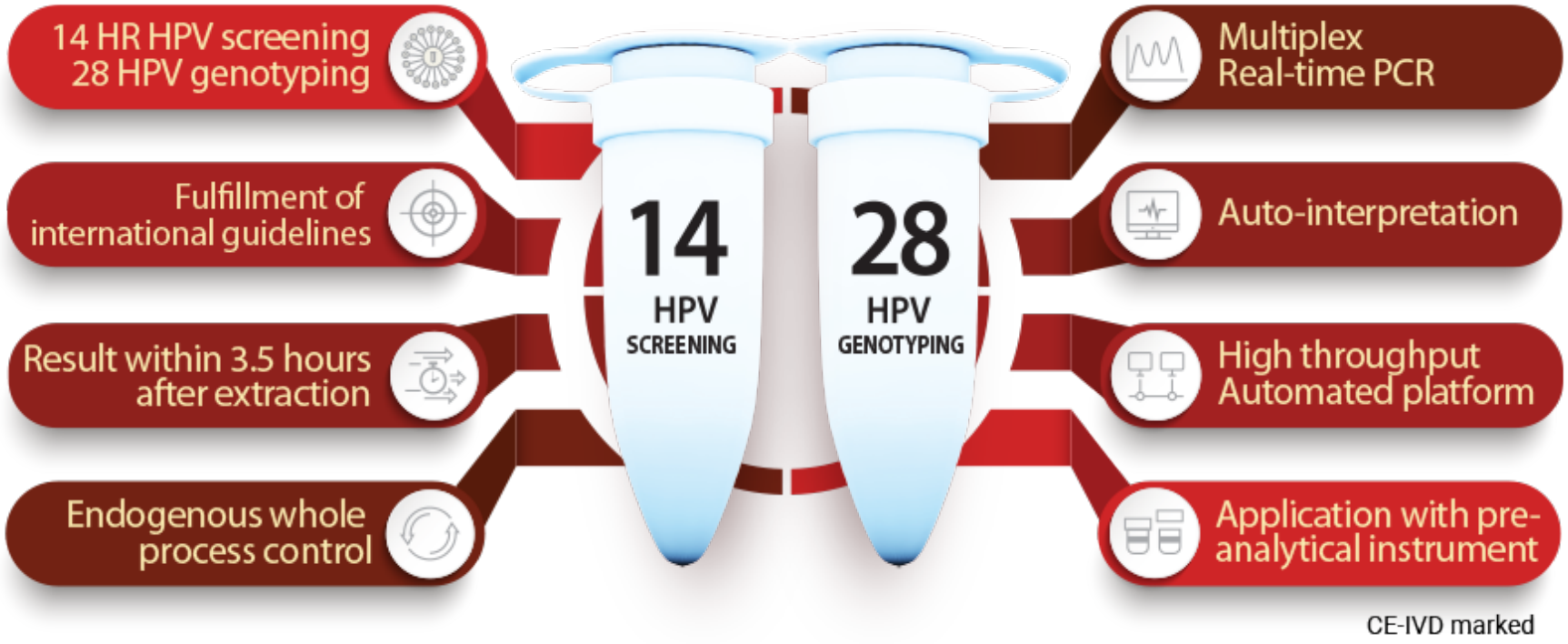
Extraction of Nucleic acid using the MICROLAB Nimbus IVD system (Hamilton):

- The automated MICROLAB Nimbus system is intended to be used for universal isolation of nucleic acid from various type of lab specimens including specimen for HPV testing – liquid based cytology (LBC).
- The procedure involves STARMAG 96 x 4 Universal Cartridge Kit that use magnetic beads principle for a convenient nucleic acid purification. It encompass of four steps which are sample lysis, nucleic acid bind to magnetic beads, wash debris and purified nucleic acid elution.
- The method begin with sample lysis using Proteinase K solution that contains Lysis Buffer in order to break the cell membranes and obtain an efficient amount of cells.
- Then the Binding Buffer will be mix first with magnetic beads before added to the lysate. The magnetic beads separation will be perform by using Wash Buffers (there are three Wash Buffer- WB1, WB2, WB3) in order to remove the contaminants during the process. Lastly, Elution Buffer will be added as it helps in obtaining a highly purified nucleic acid.[19]

19. (STARMag 96x4 Universal Catridge Kit Product Insert, 01/2020, V1.08, Page 3-5)

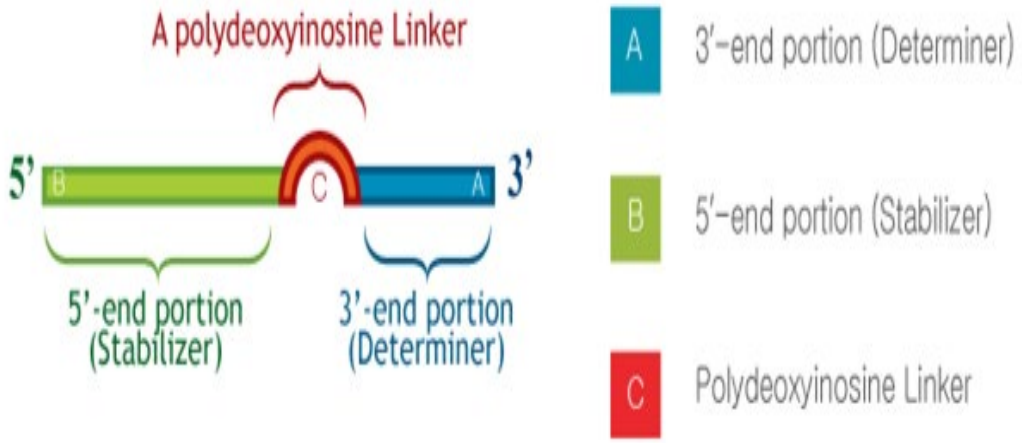
5. TECHNOLOGIES USED IN PCR HPV ASSAY

Seegene Anyplex II HPV28 can detect all 28 HPV genotype at once using advanced technologies such TOCE and DPO.



DPO (Dual Priming Oligonucleotide)

- A tool for blocking extension of non-specifically primed templates
- A new and novel primer concept that fundamentally blocks the non-specific priming and consequently delivers a reliable Multiplex PCR
- Has structure that contains a Polydeoxyinosine Linker (inserted into the primer sequence)
- This separates a single primer into two functional regions that have characteristic annealing temperature
- Free from primer competition , primer dimerization and the formation of complex secondary structure (aerosol can be avoid that can make contamination)
- High specificity , multiplex ability and minimal optimization

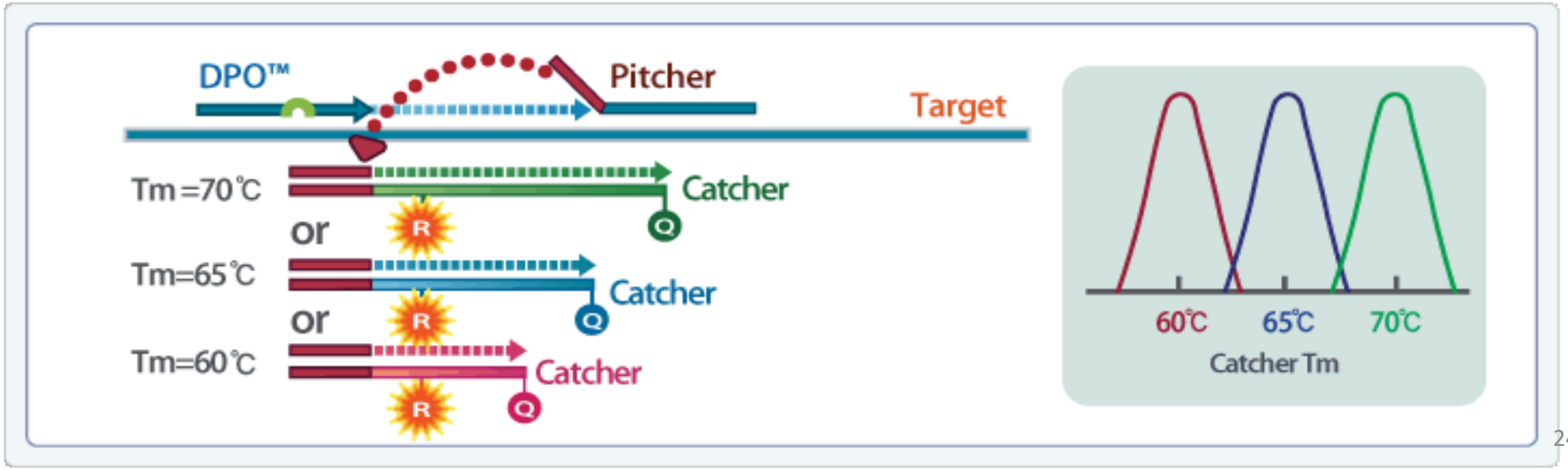


TOCE (Tagging Oligonucleotide Cleavage & Extension)

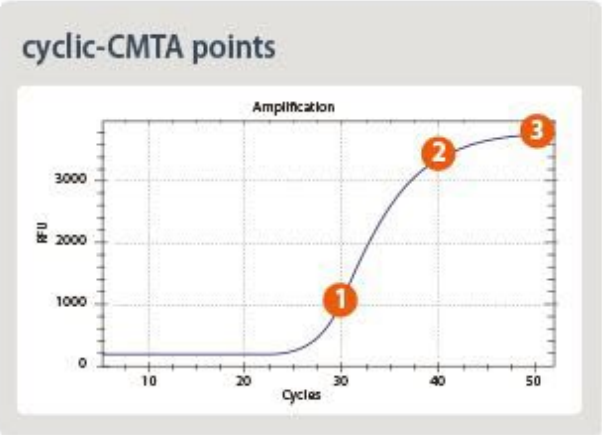
- High multiplex target detection (allows to detect 5 targets per channel)
- High multiplex quantitation
- High multiplex single base discrimination

Keypoints :

- DPO (Seegene proprietary specific primers that provides highly specific amplication of the target region.)
- Catcher (fluorescence labelled artificial template)
- Pitcher (tagging oligonucleotide that hybridize specifically to the target region)



- The DPO primer and the pitcher hybridize specifically to the target sequence. The tagging portion of the pitcher is designed not to hybridize to the target sequence. During the DPO primer extension, the pitcher is cleaved by the exonuclease activity of DNA polymerase and then the tagging portion is released.
- The released tagging portion hybridizes to the catcher which has a complementary sequence of the tagging portion. Extension the tagging portion separates the fluorescent reporter (R) from the quencher (Q) generates signal.



Titer	cyclic-CMTA points			Interpretation
	1st (30 cycle)	2nd (40 cycle)	3rd (50 cycle)	
High	65℃	65℃	65℃	+++
Intermediate	65℃	65℃	65℃	++
Low	65℃	65℃	65℃	+
Not detected	65℃	65℃	65℃	Not detected

- The signal can be analyzed in manners of real-time or melting curve analysis. In the left panel, Catcher Melting Temperature Analysis (CMTA) points, preselected cycles during the amplification process where melting temperature analysis is performed, are indicated (①,②,③). The right panel shows the appearance of the melting peak for three different titers of the target. The melting peak appears at the first CMTA point for the high titer, at the second CMTA point for the intermediate titer, and at the third CMTA point for the low titer. [19]

SEEGENE ANYPLEX II HPV28 DETECTION

ANALYTES DETECTED	19 High-risk HPV genotypes (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, 82) 9 Low-risk HPV genotypes (6, 11, 40, 42, 43, 44, 54, 61, 70) 5 controls (Positive Controls – PC1, PC2,PC3) ,Negative Controls and Internal Controls.
VALIDATED SPECIMENS	Liquid based cytology specimen (ThinPrep® and SurePath™)
PRINCIPLE	The Anyplex II HPV28 test involved multiplex semi-quantitative real-time PCR assay by either End point-CMTA or cyclic-CMTA method that permits simultaneous amplification, detection and differentiation of 19 high risk HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, 82) and 9 low risk HPV types (6, 11, 40, 42, 43, 44, 54, 61, 70) including Internal Control (IC) in assessed nucleic acid isolation, PCR inhibition and also verification of PCR reaction.

SEEGENE ANYPLEX II HPV28 DETECTION

FEATURES	❖ Accurate genotyping of 28 HPV types in a single reaction ❖ Multiplex real-time PCR with high sensitivity and specificity by utilization of DPO™ and TOCE™ technologies ❖ Amenable to automated sample handling and assay systems ❖ Utilization of the UDG system to prevent carry-over contamination ❖ Endogenous whole process control for assay validity ❖ Convenient data interpretation by Seegene Viewer
ADVANTAGES	❖ Quick and easy data analysis & interpretation ❖ Interface specialized for multiplex testing ❖ Interlocked with LIS ❖ Patient information input via barcode scanning system or LIS system ❖ Printable in various formats ❖ Downloadable results in a CSV file ❖ Convenient read out for quantitative analysis result

6. INTERPRETATION OF HPV

HPV DNA PCR	CYTOLOGY
Detected ❑ High-risk ❑ Low-risk	Negative for Intraepithelial Lesion and Malignancy (NILM)
Not Detected	High-grade Squamous Intraepithelial Lesion (HSIL)
	Low-grade Squamous Intraepithelial Lesion (LSIL)
	Atypical Squamous Cells of Undetermined Significance (ASCUS)
	Vaginal intraepithelial neoplasia (VAIN)
	Atypical glandular cells (AGC)

Standard reporting, as defined by The Bethesda System (TBS)