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In the name of Allah, the Most Beneficent the Most Merciful

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HPV TESTING TECHNOLOGIES & METHOD VALIDATION

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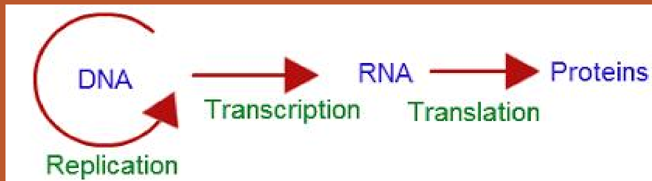
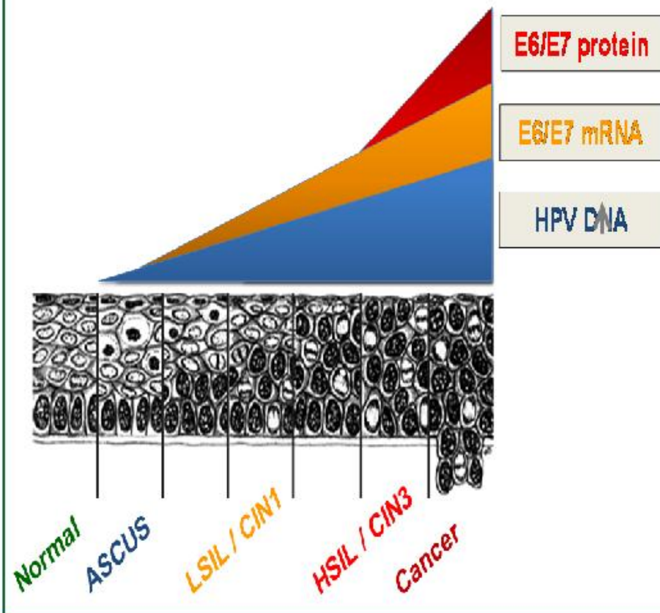
April 2018





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HPV Testing

- HPV cannot be grown in culture
- detection of the virus relies on a variety of techniques used in,

✓ immunology,

✓ serology

✓ molecular biology.

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Diagnosis of human papillomavirus (HPV) infection immunohistochemistry(IHC)

- Antibodies against E6 or E7
- ✓ Direct visualization In cells or tissues

Limitations:

- ✓ Restricted to productive infection
- ✓ Time consuming
- ✓ Error in HPV typing

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Diagnosis of human papillomavirus (HPV) infection
Immunoassay(WB, IP)

- Viral proteins

Immunological detection of HPV in human cells or tissues has been hindered by five main factors:

(a) the late, capsid proteins are only expressed in productive infections

(b) the early proteins are often expressed in low amounts in infected tissues

(c) the lack of high-quality, sensitive, and specific antibodies against the viral proteins

(d) Low sensitivity

(e) not always with consistent results

(f) fail to discriminate between HPV types

Diagnosis of human papillomavirus (HPV) infection
enzyme-linked immunosorbent assay (ELISA)

- type-specific HPV VLP ELISA
- Luminex beads

- Limitation :

Diagnosis of human papillomavirus (HPV) infection
Serology

- About half of the individuals exposed to HPV never develop measurable titers of antibodies
- At present there is no agreed standard methodology for serological assays: they must be standard and consistent

Diagnosis of human papillomavirus (HPV) infection
Hybridization assay

- hybridization procedures that include Southern and Northern blots, dot blots, in situ hybridization (ISH), Hybrid Capture (Digene), is an excellent procedure that can generate information with quality.

Limitations :

- Time- consuming
- Requires large amounts of highly purification nucleic acid
- Requires well preserved, ideally full-size molecules, and therefore cannot be done with any biological specimen, particularly those derived from fixed tissues in which DNA degradation is often observed
- Error in HPV typing because of probe cross-hybridization

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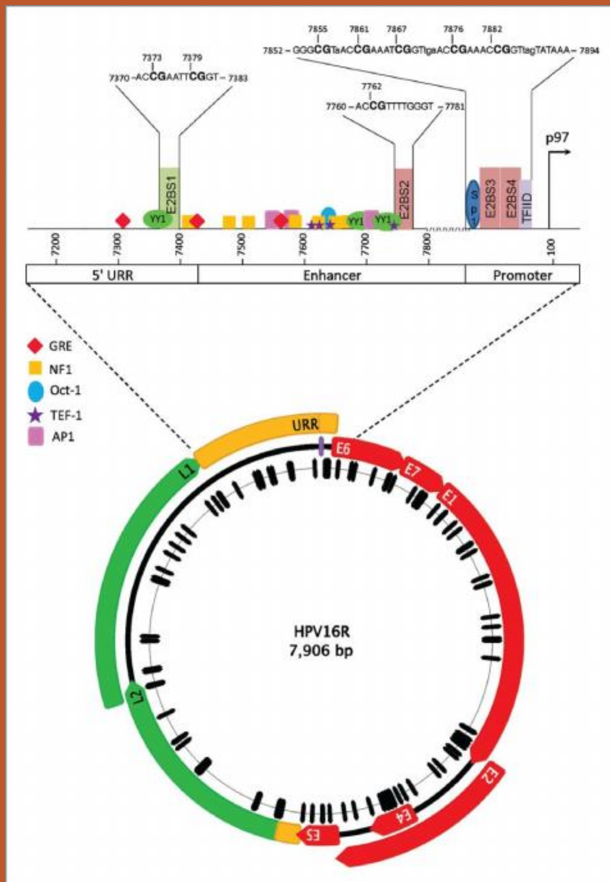
Diagnosis of human papillomavirus (HPV) infection

Molecular assay



- A well-known physical structure and gene organization, tests of choice for detecting HPV from clinical specimens are based on nucleic acid probe and primer technology

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Diagnosis of human papillomavirus (HPV) infection RNA Methods

- Testing for viral RNA aims to evaluate the HPV genome expression
- Design for HPV oncogenes E6/E7 transcripts
- Highly specific
- RNA is a much more labile molecule than DNA, and therefore less available in most biological specimens depending on the time and type of storage conditions
- Need well developed Liquid specimen preservative .

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Diagnosis of human papillomavirus (HPV) infection
DNA Methods

HPV Detection performed by molecular assays

- Signal Amplification (HC2 ; Cervista)

- Target Amplification (PCR)

Both assays are suitable for high-throughput testing, with automated execution and reading, which is a necessary step to be considered for use in large epidemiological studies and in clinical settings.

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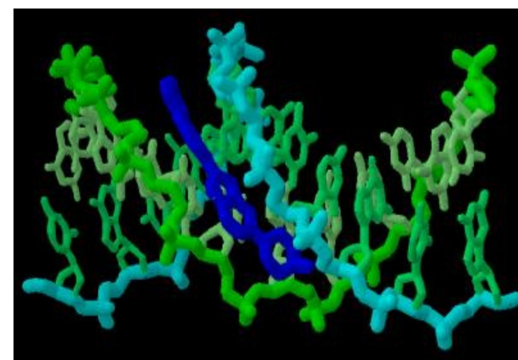
Limitations of Signal amplification

- False-positives due to cross reactivity with low-risk HPV DNA
- HPV DNA not amplified
  - Negative predicted value may be compromised in cases in which HPV DNA copy number is low
- No internal control used for sample sufficiency
  - Not possible to determine if results are due to insufficient DNA or true negative

Target Amplification (PCR)

PCR generic / consensus

- **GP 5 and 6**
- **PGYM**
- **MY09/11**
- **SPF_{10}**
- **GP5 and 6 + SYBR green**



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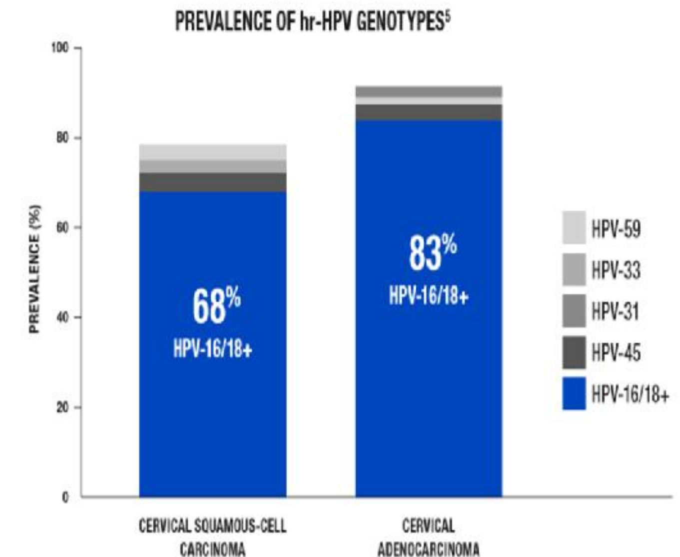
Target Amplification (PCR)

Screening assays
- Designed to detect the group of High and Low Risk HPV Genotypes

❖Consensus assays ,
- **Generally target L1 region**

Genotyping assays
- Some assays also have limited genotyping capacity for types 16 and 18
- Designed to Genotype the majority of the HPV types infecting the Genital Tract-particularly the High Risk Genotypes
- Usually based on either a line blot assay format or micro-array system

❖Type specific assays
- **Generally target E6/E7 region**
- Sequencing



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Detecting HPV Infection in Men

- **PCR has emerged as the most sensitive method to define HPV infection in men.**

Specimen Collection Site: Single and multiple anatomical sites of the male genitalia are often sampled. They include the penile shaft, coronal sulcus/glans (including the prepuce in uncircumcised men), scrotum, urethra, as well as urine and semen (Aguilar et al., 2 006; Benevolo et al., 2 008; Fife et al., 2 003; Giuliano et al., 2008a ; Nielson et al., 2007) . It has been shown that specimens from the urethra and semen contribute little toward the analysis of HPV DNA prevalence and that specimens should be collected from the shaft, glans, and scrotum in a combined sample (Giuliano et al., 2 007).

Caution for Collection Method: Another most important aspect when analyzing HPV infection in men is the variability attributable to the collection method

>220 commercially available HPV test systems

Testsystem	Firma	Bemerkung	FDA	Hochrisiko- (fett)/ mögliche Hochrisiko-HPV-Typen[§]:	Zahl HR-Typen
		Signal Amplification		16 18 26 30 31 33 34 35 39 45 51 52 53 56 58 59 66 67 68 69 70 73 82 85	
Cervista HPV 16/18	Hologic	Invader® Chem.	2009		2
Cervista HPV HR	Hologic	® Chem.	2009		(14)
hc2 HPV DNA Test	QIAGEN	apture 2			(13)
hc2 High-Risk HPV-DNA Test	QIAGEN	apture 2	2003		(13)
digene HPV Genotyping PS Test[1]	QIAGEN	Hybrid Capture 2			3
		PCR		16 18 26 30 31 33 34 35 39 45 51 52 53 56 58 59 66 67 68 69 70 73 82 85	
HPV Easy-Typing Kit	AID Autoimmun Dia.	PCR / Line Blot			3 (10)
f-HPV typing	Nimagen (Genomed)	multiplex PCR			13
AMPLICOR HPV Test	Roche	PCR / 96er Platte			13
cobas® HPV Test	Roche	rt-PCR	2011		2 (12)
RealTime HR HPV	Abbott	rt-PCR			2 (12)
Xpert HPV	Cepheid	PCR / Kartusche			3 (11)
BD Onclarity HPV-Test	Becton Dickinson	rt-PCR			6 (8)
ProDect Chip HPV typing	bcs Biot				14
HPV Genotypes 14 Real-TM Quant	Sacace				14
HPV	DDL Dia				17
PapilloCheck	greiner				18
Anyplex™ II HPV28 Detection	Seegene	rt-PCR / cyclic CMTA			20
HPV Direct Flow Chip	medac	PCR / Chip			20
INNO-LiPA HPV Genotyp. Extra	Fujirebio	PCR / Line Blot			20
CLART HPV2	Genomica	PCR / Microarray			20
Linear Array HPV Genotyp.	Roche	PCR / Line Blot			21
Infiniti HPV Genotyping Assay	AutoGenomics	Microarray			24
		NASBA / TMA		16 18 26 30 31 33 34 35 39 45 51 52 53 56 58 59 66 67 68 69 70 73 82 85	
NucliSens EasyQ HPV	BioMérie	E7 mRNA			5
APTIMA HPV-Test	Hologic	E7 mRNA	2011		(14)
APTIMA HPV 16 18/45 genotype[2]	Hologic	E7 mRNA	2012		1 (2)
OncoE6TM Cervical Test	Arbor Vi	eral flow			2

probes

DNA, PCR

RNA

Protein

[1] zur Genotypisierung HC2 hr HPV DNA-positiver Proben
[2] zur Genotypisierung APTIMA HPV-positiver Proben

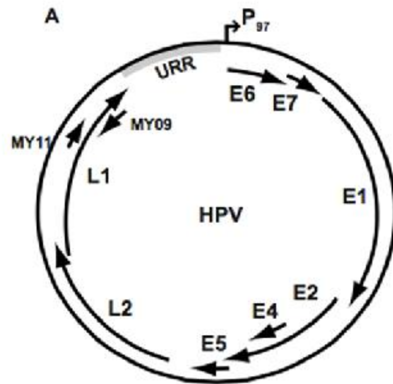
= DNA-Typisierung
= RNA-Typisierung
= Proteinnachweis
= Sonde vorhanden, aber keine individuelle Typisierung
= FDA (Food and Drug Administration, USA) zugelassen (Jahr)

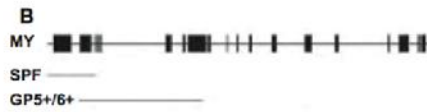
§ Guidelines recommend only HR-HPV testing as LR-HPV testing has no clinical use in CxCa screening or triage of positive cytology.

Human Papillomavirus Laboratory Manual

Immunization, Vaccines and Biologicals

First edition, 2009





A. Genetic organization of HPV

HPV16 is illustrated, but all HPV genomes have similar organization. The L1 region, upstream regulatory region (URR) and the E6/E7 regions are seldom lost in malignancies, so these regions are most often targeted in assays. The L1 region is most conserved among all HPV types.

B. L1 region targeted by most frequently-used consensus PCR assays

The region of L1 delimited by the MY09 and MY11 primers is illustrated. The vertical bars define 10 base sliding domains sharing 80% homology among the 32 most prevalent types of mucosal HPVs. Targets of the SPF and GP5+/6+ primer sets are illustrated below allowing a comparison of the relative size of the fragments.

PGMY Primer PCR

High risk (oncogenic types)

16,18,31,33,35,39,45,51,56,58,59,68,69,82

Low risk (non-oncogenic types)

6,11,40,42,43,44,54,61,72,8

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Quality Control for HPV Testing

1. **Lab Infrastructure (contamination risk, PCR problem)**
2. **Validation Test**
3. **Controls: Internal control for cellular DNA and positive controls**

1. **Monitoring of processes**
2. **Intra/inter Lab reproducibility**
3. **Proficiency Testing of reference laboratory** WHO HPV LabNet proficiency studies

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Quality Control 1. Lab Infrastructure

Individual Compartments should be considered

1. Sample Processing

2. Pre-PCR

3. Post-PCR

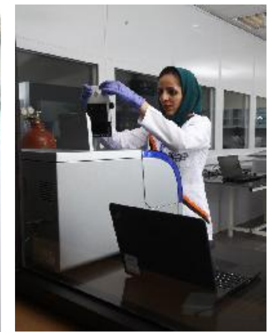
Sample Preparation

Registration

Automated PCR master mix preparation

Automated DNA Extraction

PCR



Automated Capillary Electrophoresis

Quality Control 3. Method Validation

- **Analytical Sensitivity**
- **Analytical Specificity**
- **Matrix Effects**
- **Clinical Sensitivity**

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Method Validation Material

1. Plasmid controls.

- high-risk (HR) -16 , -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66
- low-risk HPV-6, -11,

2. Cell lines controls.

- Hela: 40 Copies of HPV-18
- CaSki: 600 Copies of HPV-16
- SiHa: 3 Copies of HPV-16

3. Microorganism that commonly detected in genital (From FDA List)

Organisms	
Bacteria:	**Human Papillomaviruses:**
Lactobacillus acidophilus	alpha-HPV genotypes.
Staphylococcus epidermidis	
Staphylococcus aureus	
Streptococcus faecalis	**Viruses:**
Streptococcus pyogenes	Adenovirus
Streptococcus agalactiae	Cytomegalovirus
Corynebacterium spp.	Epstein Barr virus
Chlamydia trachomatis	Herpes simplex virus 1
Neisseria gonorrhoeae	Herpes simplex virus 2
Escherichia coli	
Enterococcus spp.	**Other:**
Clostridium spp.	Candida albicans
Peptostreptococcus spp.	Trichomonas vaginalis
Klebsiella spp.	
Enterobacter spp.	
Proteus spp.	
Pseudomonas spp	
Bacteroides spp.	
Bifidobacterium spp.	
Fusobacterium spp.	

Quality Control 3. Method Validation

Analytical Sensitivity:

- The assay's ability to detect very low concentrations of a given substance in a biological specimen.
- Analytical sensitivity is often referred to as the limit of detection (LoD).
- LoD is the actual concentration of an analyte in a specimen that can be consistently detected ≥ 95% of the time

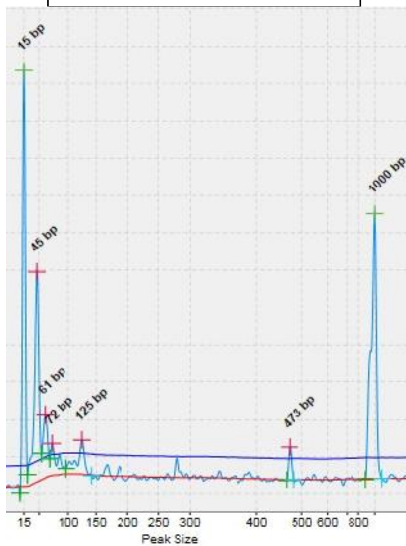
Analytical Sensitivity: Cervical Cancer Cell Line

2. Cell lines controls.

- Hela: 40 Copies of HPV-18
- CaSki: 600 Copies of HPV-16
- SiHa: 3 Copies of HPV-16
  - **LOD: 5000 copies/ ml of liquid**

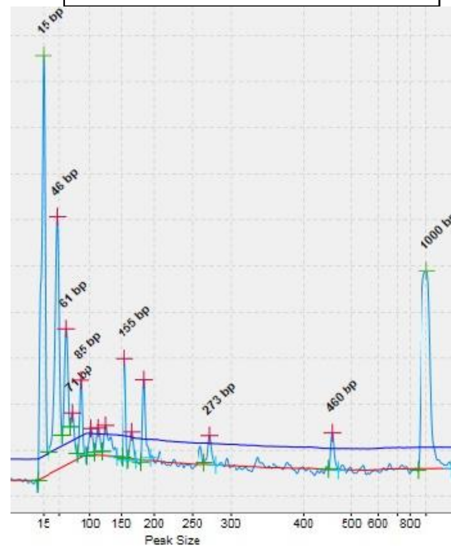
Analytical Sensitivity using Control Plasmids: Screening

50 Copies/µl of PCR reaction



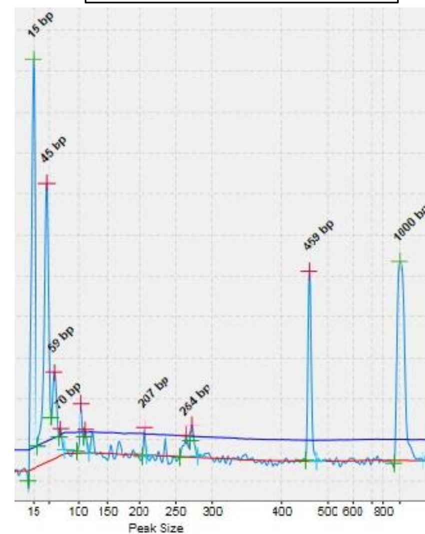
67% Positive

100 Copies/µl of PCR reaction



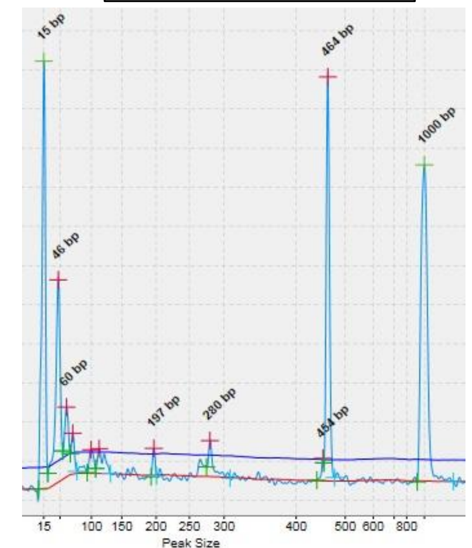
95% Positive

500 Copies/µl of PCR reaction



100% Positive

5000 Copies/µl of PCR reaction

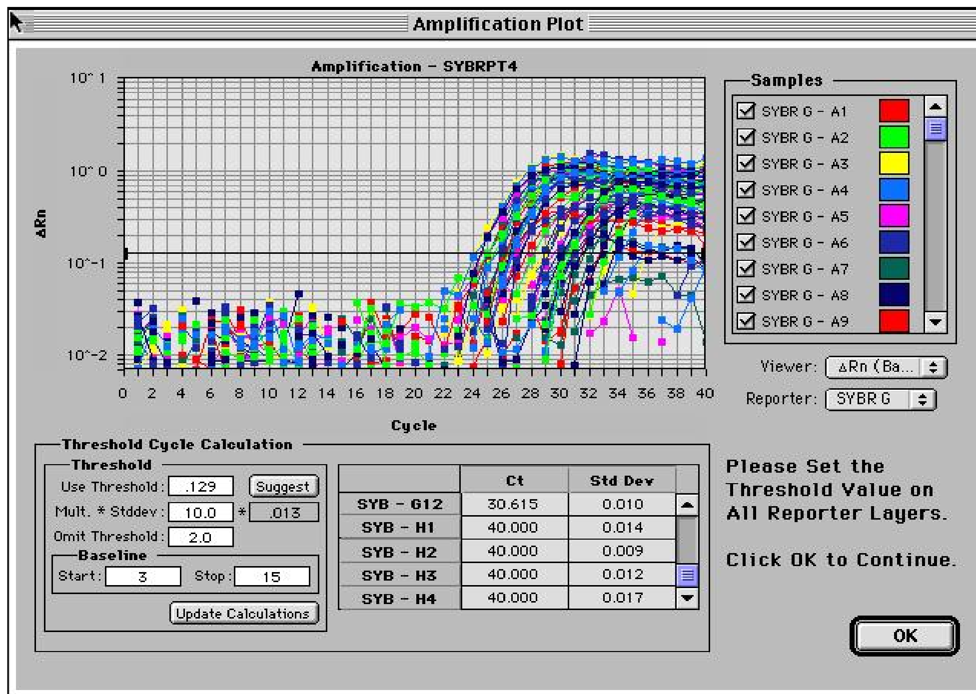


100% Positive

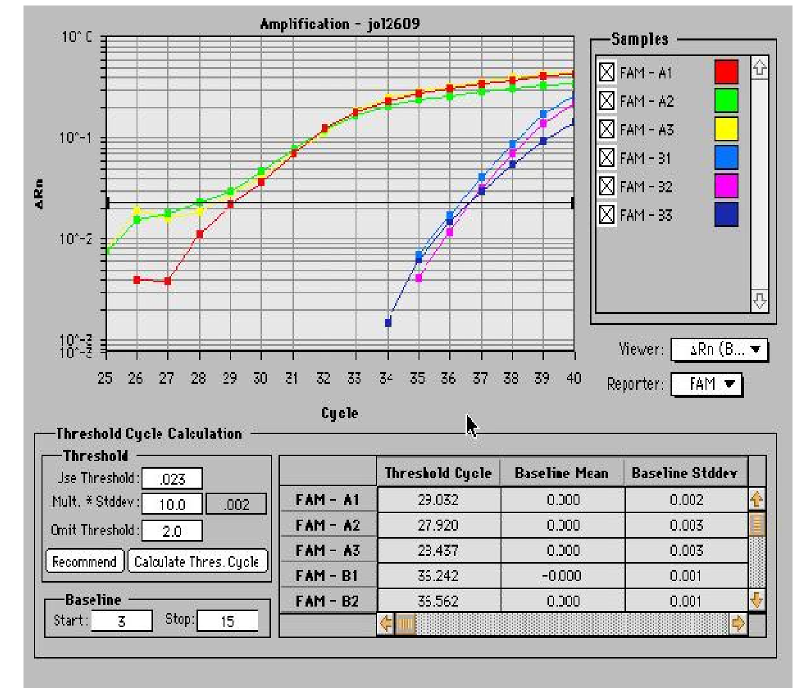
Analytical Sensitivity using Control Plasmids: Screening

SYBR-green

Taq Man PCR



Detection sensitivity 5-10 copies/reaction



Detection sensitivity = 1-2 copies per reaction

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Method validation: Analytical Specificity

Analytical specificity" refers to the ability of an assay to measure on particular organism or substance, rather than others,

Microorganism that commonly detected in genital (From FDA List)

Organisms	
Bacteria:	**Human Papillomaviruses:**
Lactobacillus acidophilus	alpha-HPV genotypes.
Staphylococcus epidermidis	
Staphylococcus aureus	
Streptococcus faecalis	**Viruses:**
Streptococcus pyogenes	Adenovirus
Streptococcus agalactiae	Cytomegalovirus
Corynebacterium spp.	Epstein Barr virus
Chlamydia trachomatis	Herpes simplex virus 1
Neisseria gonorrhoeae	Herpes simplex virus 2
Escherichia coli	
Enterococcus spp.	**Other:**
Clostridium spp.	Candida albicans
Peptostreptococcus spp.	Trichomonas vaginalis
Klebsiella spp.	
Enterobacter spp.	
Proteus spp.	
Pseudomonas spp	
Bacteroides spp.	
Bifidobacterium spp.	
Fusobacterium spp.	

Method Validation: Matrix effect

1. Cervical Cancer cell lines

2. Clinical samples

Procedure: Spike in Collection Media as well as PBS

Check for Sensitivity and specificity

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Method Validation: Clinical Sensitivity

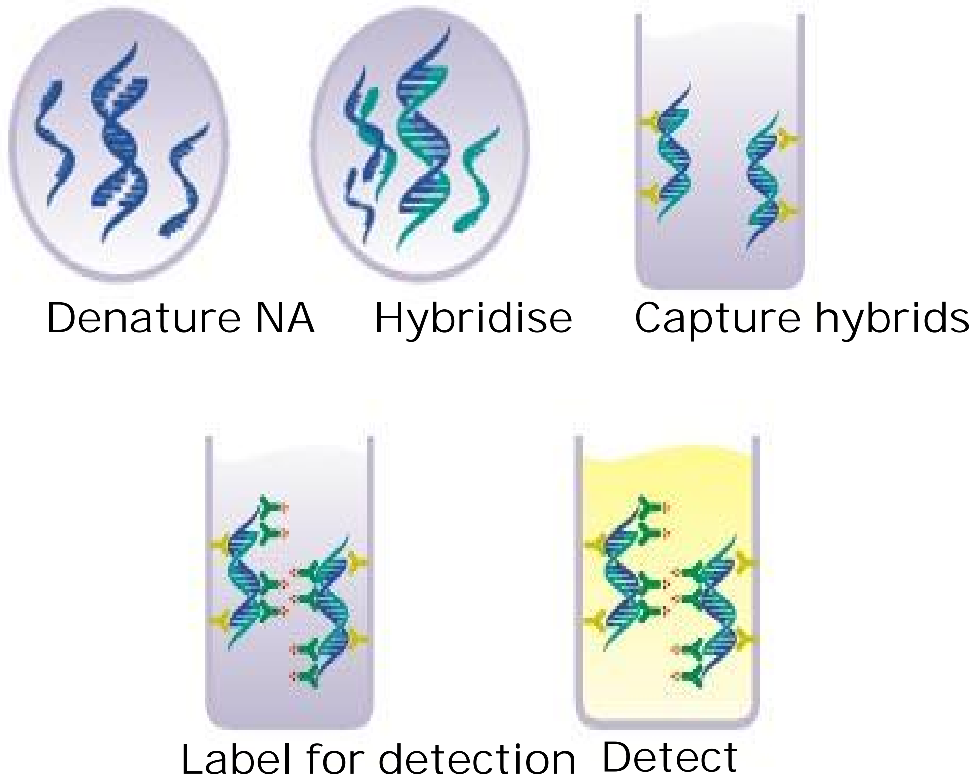
- In the case of HPV infections there is a big difference between analytical sensitivity and clinical sensitivity /specificity
- Several HPV infections do not persist and therefore do not lead to clinically relevant disease (Snijders et al., 2 003)

FDA: Clinically validated versus Hybrid Capture 2 (HC2) and/or GP5+/GP6+ PCR-(EIA) (Diassay)

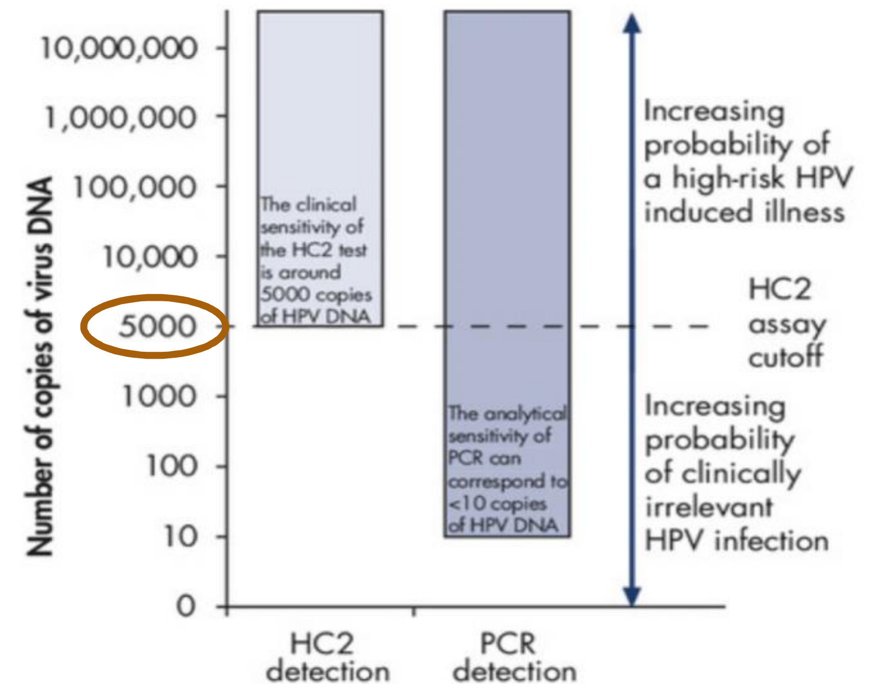
HC2 und Diassay validated in large prospective clinical studies (women >30 years)

FDA comments: Valid your HPV test

Principle of Hybrid Capture II



The U.S. Food and Drug Administration recommended cutoff value for test-positive results **is 1.0 relative light unit(RLU) (equivalent to 1 pg HPV DNA per 1 ml of sampling buffer).**



FDA comments: Valid your HPV test

Shows clin. **Sensitivity** for CIN2+ of 95%
Shows clin. **Specificity** for CIN2+ of 90.7 to 94.1%

New HPV test formats should have relative:
90% of HC2 sensitivity
98% of HC2 specificity

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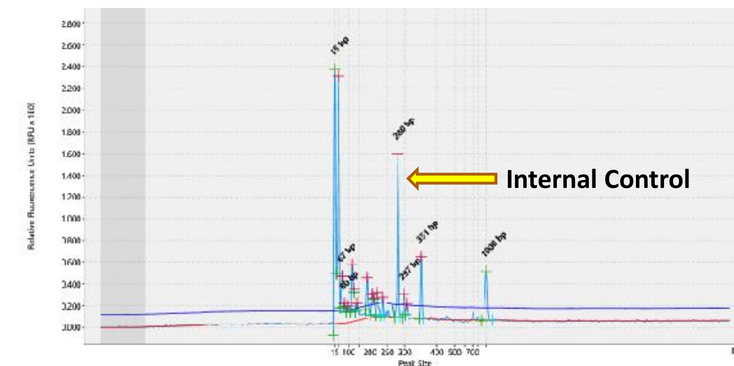
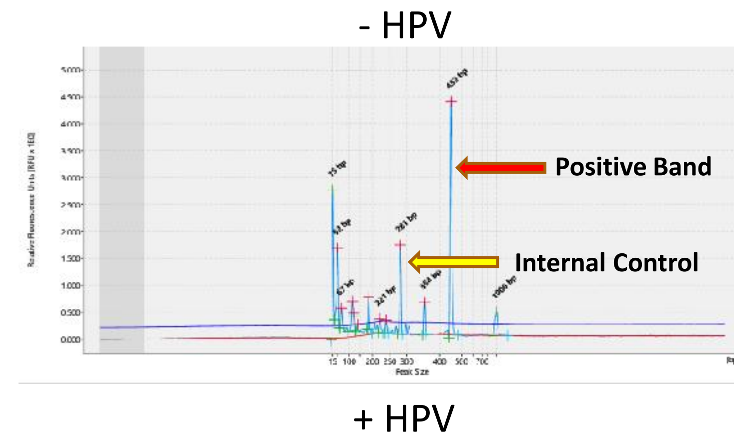
Qulity Control 2. Risk of False Negative

- Small portion of genome targeted
  - Allows testing of samples with poor quality DNA
  - Small changes in virus (variants or integration) may give false negative results
- Amount of DNA assayed varies (limits number of cells sampled)

✓ **Internal control for cellular DNA like beta globin**



- HPV



+ HPV

Qulity Control: How to prepare positive controls

2. Cell lines controls.

1 pg CaSki DNA ≈ 100 HPV16 GE

to prepare the 1000 HPV16 GE positive control, dilute CaSki cell DNA to 2 pg per µL in DNA carrier
(10 ng 293 cell DNA per µL). This stock
≈200 HPV16 GE per µL in background of≈
1670 copies of cellular DNA;

Page 43 WHO Manual

- Positive control for sensitivity assessment: DNA from tissue-culture cell lines is used as control. Propagation of these lines should follow standard tissue-culture methods using media and conditions recommended by ATCC (9). CaSki cells are HPV16 positive and 293 cells are HPV negative human cells. Purify DNA (should not contain RNA) from harvested tissue cultures following the protocol that your laboratory uses for sample extraction. Determine DNA concentration by optical density measurement in a spectrophotometer (for instance the Nanodrop® system) using 50 µg/mL as 1 OD_{260}. The 260/280 ratio should be in the range of 1.7–1.9 and should not exceed 2 as this indicates contamination of the preparation by RNA or low molecular weight nucleic acids that leads to overestimation of DNA concentration.

> **Note:** For copy number evaluation it is assumed that one diploid cell contains 6 pg DNA and that one CaSki cell contains in the order of 600 HPV16 GE; therefore 1 pg CaSki DNA ≈ 100 HPV16 GE. The positive control for sensitivity is not a secondary standard. It serves as a guide for day-to-day assay performance.

- To prepare the 1000 HPV16 GE positive control, dilute CaSki cell DNA to 2 pg per µL in DNA carrier (10 ng 293 cell DNA per µL). This stock ≈ 200 HPV16 GE per µL in background of ≈ 1670 copies of cellular DNA; giving input of ≈ 1000 HPV16 GE and 8400 cell equivalents per 5 µL. The 1000 HPV16 GE control should be stored in 100 µL aliquots at -20°C. **Freezing and thawing cycles must be minimized to avoid degradation of this control.** The 100 and 10 HPV16 GE controls are freshly prepared for each set of PCR reactions by making two consecutive tenfold dilutions of this stock in molecular biology-grade water. **The diluted DNA is lost/degraded even upon short-term storage.**

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Method validation: Proficiency test

WHO HPV *LabNet* proficiency studies

Method Validation: References

1. WHO : Human papillomavirus laboratory manual, Edition 2009.

2. Meijer CJ *et al* have recently developed guidelines for high-risk HPV test requirements for primary cervical screening and validation guidelines for candidate HPV assays

   Int J Cancer 2009 Feb 1 124 (3) 516-20

New Test Systems and Molecular Triage

- Reflex cytology? (co-testing!)
- All colposcopy?
- additional biomarker?

$\Rightarrow$ Molecular Triage?

- Biomarker
- Methylation marker
- HPV oncoprotein expression strength

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Oral presentation. FC 21-10

Cervical Cancer Screening in Iran: Developing a New Method

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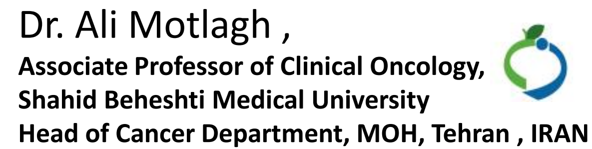

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Thanks

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