HPV diagnosis by molecular methods

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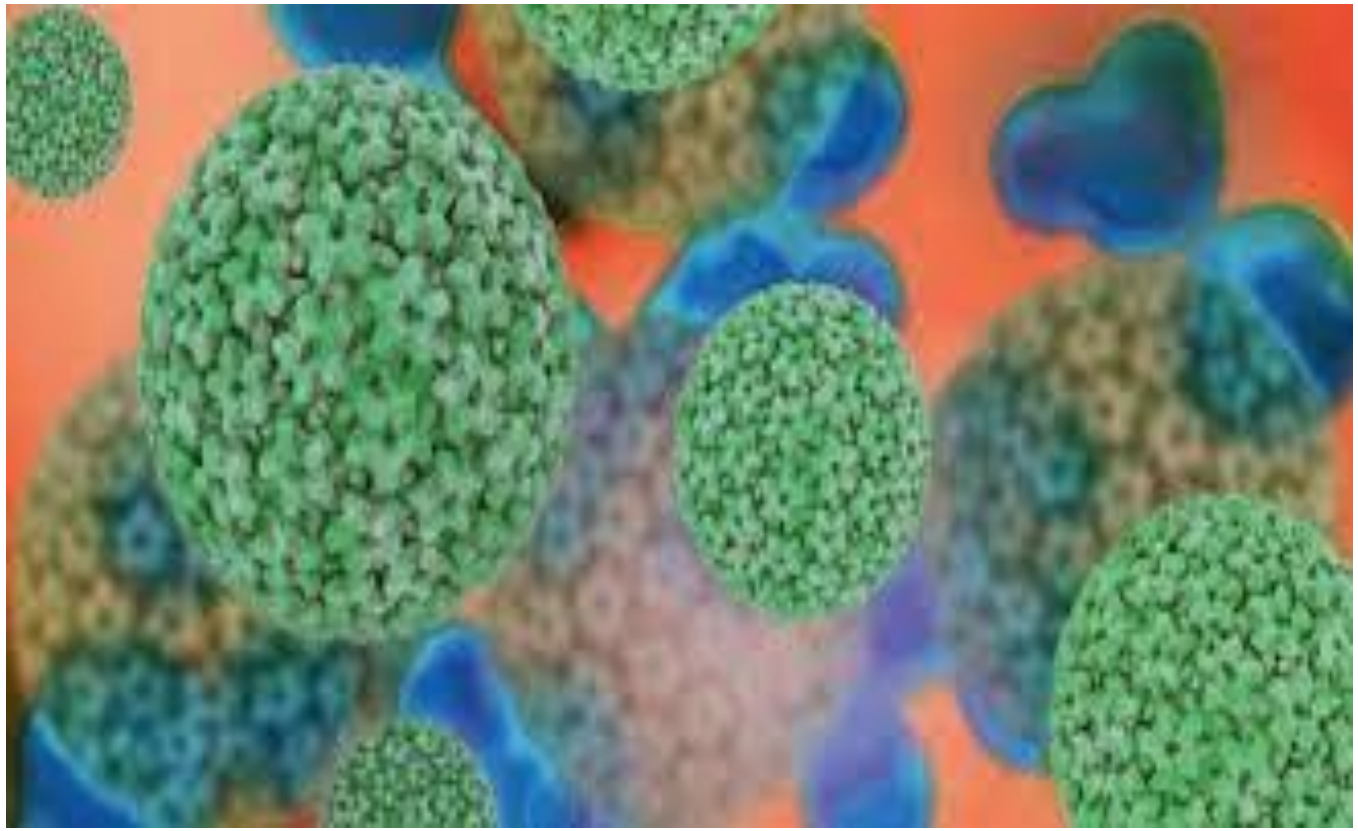
Clinical and anatomical pathologist

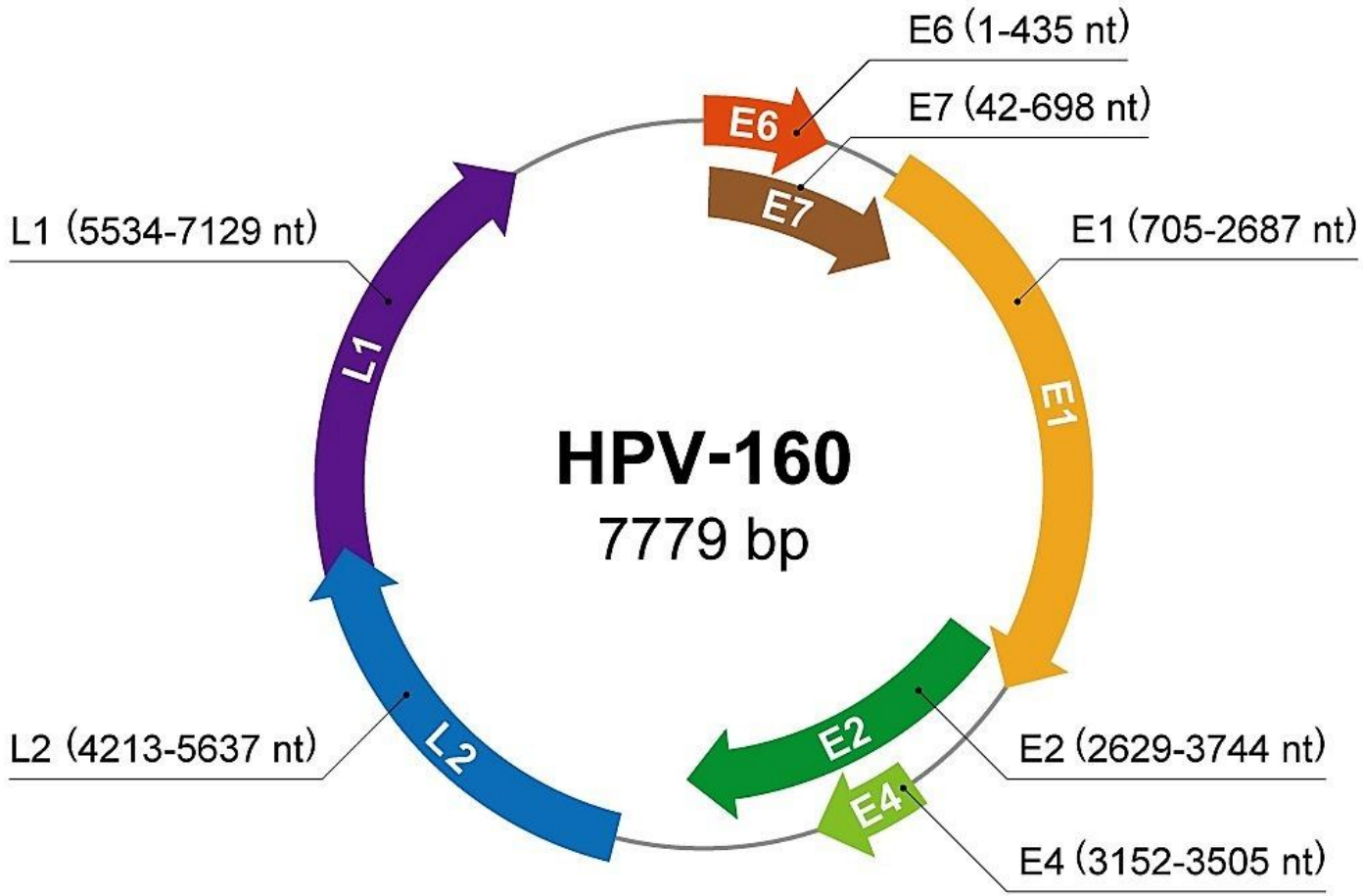
Flow of molecular pathology and cytogenetic

Raz Lab



Biology and natural history of HPV

- The HPV virion has a double-stranded, circular DNA genome of approximately 7900 bp, with eight overlapping open reading frames, comprising early (E), and late (L) genes and an untranslated long control region. The L1 and L2 genes encode the major and minor capsid proteins.
- The capsid contains 72 pentamers of L1, and approximately 12 molecules of L2.
- The early genes regulate viral replication and some have transformation potential
- Transcripts encoding the early proteins are detected in basal and suprabasal epithelial cells in the early portion of the viral replication cycle, and encode proteins that interact with the host cell machinery to allow viral replication and transcription to occur


E6 (1-435 nt)
E7 (42-698 nt)
E1 (705-2687 nt)
L1 (5534-7129 nt)
HPV-160
7779 bp
E6
E7
E1
L1
L2
E2
E4
L2 (4213-5637 nt)
E2 (2629-3744 nt)
E4 (3152-3505 nt)

HPV types and variant

- Individual HPVs are referred to as “types”, distinguished based on their genomic sequence, and numbered in order of discovery. Viruses with sequence divergence of less than 10% from a recognized type are “subtypes” (2%–10% divergence) or “variants” (<2%) of that type.

- At present, 118 HPV genotypes have been classified according to their biological niche, oncogenic potential and phylogenetic position
- Both mucosal (anogenital and oral) and cutaneous (skin) HPV types are distinguished and high-risk and low-risk (LR) genotypes are defined, depending on their association with cervical carcinoma or associated precursor lesions.
- HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82 are considered oncogenic or high-risk types, with types 26, 53, and 66 being probably oncogenic

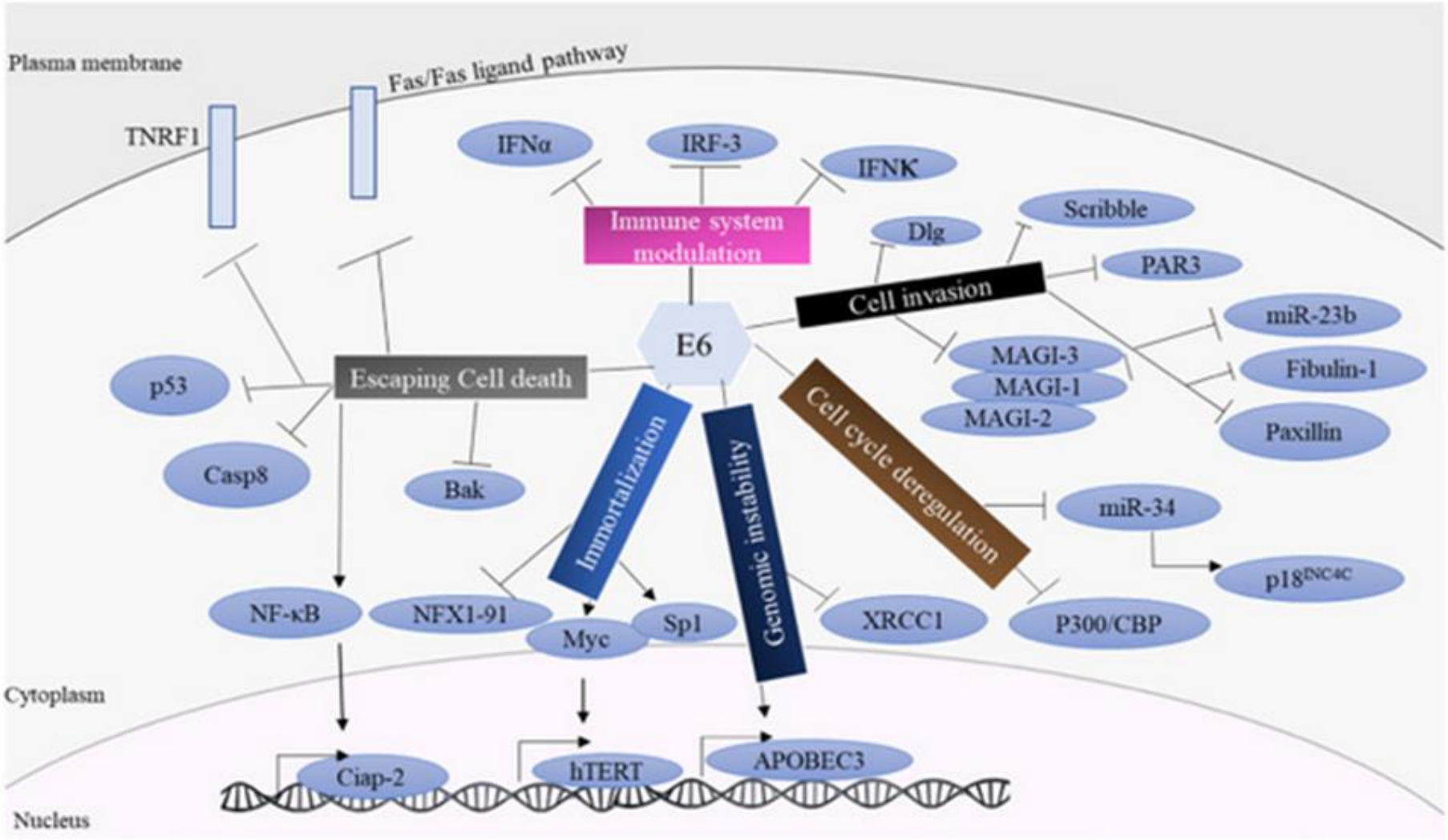
HPV-induced carcinogenesis

The integration of viral DNA into the host's genome represents a dead-end for the viral replication but is essential to drive the process of HPV-induced carcinogenesis.

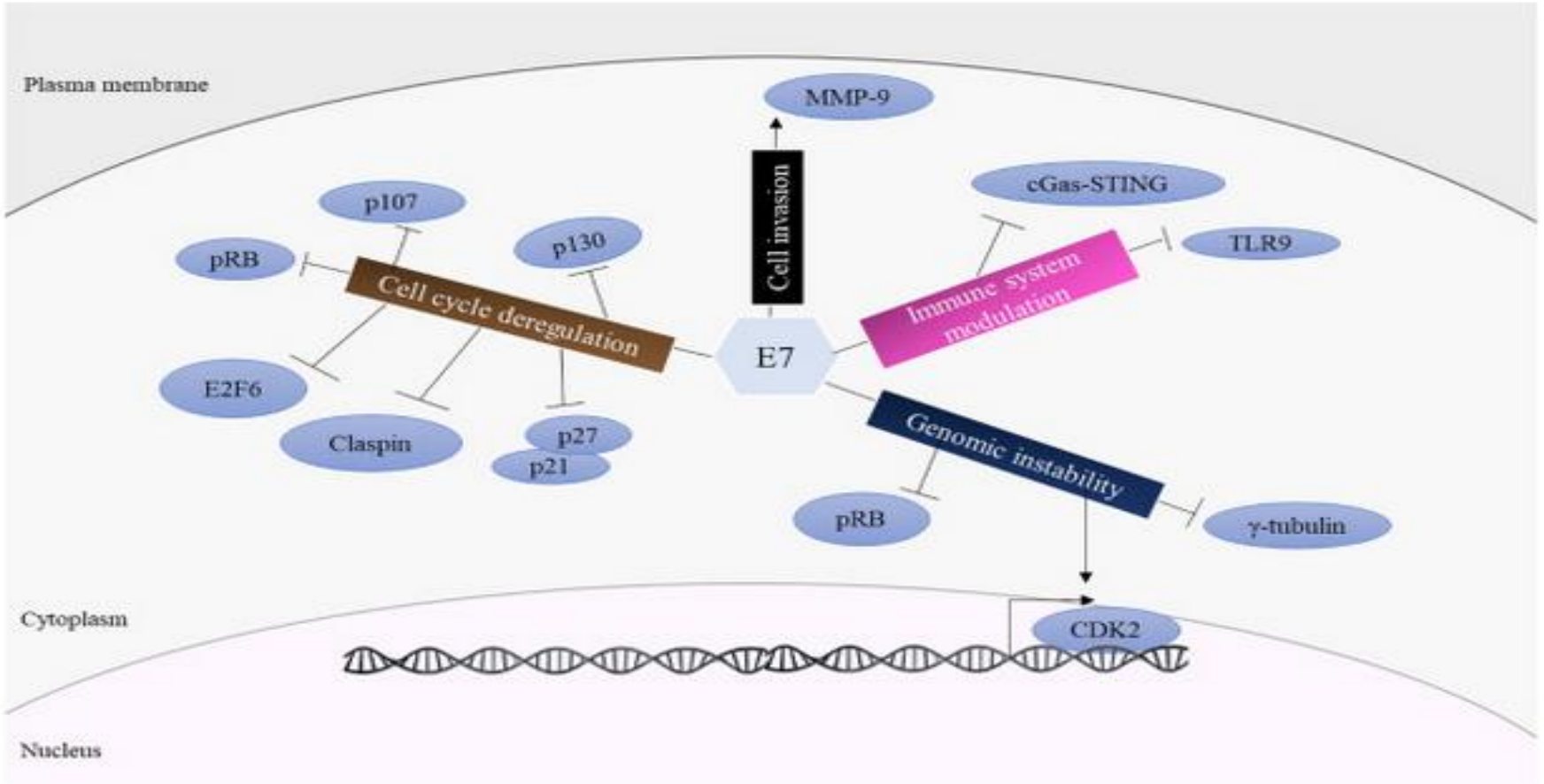
This integration may be triggered by the genomic instability caused by the viral oncoproteins E6 and E7, which increase the occurrence of double strand break in both the host's DNA and the viral genome.

Even though HPV integration can happen across all the human genome, it is more common in fragile chromosomal regions such as *3q28*, *4q13.3*, *8q24.21*, *13q22.1* and *17q21* or near clusters of microRNAs.

However, HPV integration is not the only factor that has been identified as a crucial step in the HPV carcinogenesis. Mechanisms of HPV hypermethylation were found to block the access to the *E2* promoter region, also leading to an unbalanced expression of the *E6* and *E7* viral oncogenes without *E2* disruption.



Molecular targets of the high-risk E6 oncoprotein. E6 is known to interact with a diverse range of molecules that are involved in several cellular pathways namely in immune system modulation, invasion, cell cycle deregulation, genomic instability, cell immortalization and cell death, potentiating cancer development.



Molecular targets of the high-risk E7 oncoprotein. E7 is known to interact with a diversity of molecules that are involved in several cellular pathways and therefore influence the development of cancer, such cell cycle deregulation, cell invasion, immune system modulation and genomic instability.

Inflammation promotion

Both the E6 and the E7 oncoproteins can induce tumour-associated inflammation by up-regulating the expression of the pro-inflammatory cytokines Interleukin-6 (IL-6) and IL-18.

The consequent inflammatory process leads to the up-regulation of pro-angiogenic factors, metalloproteinases and chemokines with pro-tumoral functions that support tumour progression.

The burden of the disease caused by HPV

- HPV has been associated with more than 99% of cervical cancers
- While the type distribution in cervical cancer does vary somewhat worldwide, HPV16 and HPV18 are the most prevalent types, being found in more than 70% of samples from cervical cancer around the world
- The causal relationship between HPV and cervical carcinoma has provided the incentive for the development of vaccines that prevent infection with HPV.

Prevalence of HPV in Iran

Out of 5176 cases from 7 laboratories, 2727 (53%) were positive for HPV

HPV positive patients were younger than negative individuals. Positive rate was higher among age categories 20–40.
Genotyping was performed for 2525 cases. Out of 1219 (48%) patients who contained single genotypes, 566 (22%) and 653 (26%) harboured HR and LR genotypes, respectively.

No substantial associations were found between different age categories and HR/LR and multiple genotypes distribution

Prevalence of human papilloma virus (HPV) genotypes between outpatients males and females referred to seven laboratories in Tehran, Iran

Iman Rezaee Azhar, Mahmood Yaghoobi, Mir Majid Mossalaeie,

Prevalence of HPV in Iran

Totally, the most common low risk HPV genotypes detected in the studied male and female subjects were HPV-6 (77.7% and 43.3%) and HPV-11 (13.7% and 11.4%), and more frequent high risk HPV genotypes were HPV-16 (5.5% and 16.6%) and HPV-52 (3.2% and 9.6%), respectively.

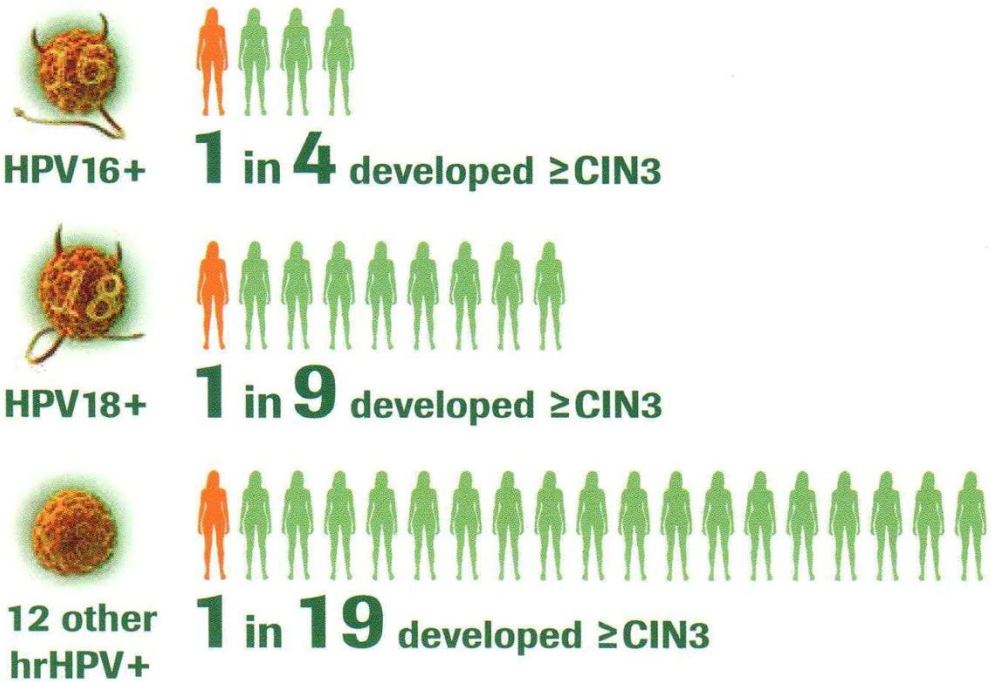
High burden of the HPV infection was observed at ranges of 30 and 44 years (51.8%) with a peak at ranges between 30 and 32 years.

The Prevalence of HPV Genotypes in Iranian Population: An Update

Mina Mobini Kesheh ,Hossein Keyvani

Not all HPV positive women have the same risk

Risk of developing ≥CIN3 within 3 years[4]



HPV16 and HPV18 genotyping allows clinicians to stratify patients into risk groups for appropriate management.[4]

Diagnosis of HPV infections

- Diagnosis of HPV infections HPV cannot be grown in conventional cell cultures, and serological assays have only limited accuracy
- As infection with HPV is followed by a humoral immune response against the major capsid protein with antibodies remaining detectable for many years, serology is not suitable for distinguishing present and past infections.
- Consequently, accurate diagnosis of HPV infection relies on the detection of viral nucleic acid by molecular methods.

- Molecular diagnosis of HPV infection is important for virus screening, and is mainly based on methods such as:
- hybrid capture
- in situ hybridization
- PCR
- These techniques have vary widely in terms of sensitivity and specificity, and **PCR is the most used** today in various areas of molecular diagnostics due to its great ability to detect small fragments of DNA

- Molecular techniques can be broadly divided into those technologies that

1-are not amplified, such as nucleic acid probe tests,

2-and those that utilize amplification, such as polymerase chain reaction (PCR).

Amplification techniques can be further divided into three separate categories:

(1) target amplification, in which the assay amplifies the target nucleic acids (for example, PCR);

(2) signal amplification, in which the signal generated from each probe is increased by a compound-probe or branched-probe technology; and

(3) (3) probe amplification, in which the probe molecule itself is amplified (for example, ligase chain reaction).

- To date, target and signal amplification techniques, in addition to non-amplified techniques, have been applied to the detection of HPV.
- Signal-amplified techniques for detecting HPV include hybrid capture and branched DNA approaches. The most widely used technique is the hybrid capture technology as described below

Hybrid Capture Technology.

Hybrid capture technology (HC), detects nucleic acid targets directly, using signal amplification to provide sensitivity comparable to target amplification methods.

The first-generation Hybrid Capture Tube (HCT) test and the more recent Hybrid Capture II (HCII) assay .

Both assays detect “high-risk” HPV types.

In March 1999, the US FDA approved Digene’s second-generation HPV detection kit (HC II).

The level of detection of the second-generation HC II is rated at 5,000 viral copies per sample, or one picogram of HPV DNA per sample (in contrast to HCT, which detects 10 picograms).

What is PCR

PCR is an exponentially progressing synthesis of the defined target DNA sequences *in vitro*.

It was invented in 1983 by Dr. Kary Mullis, for which he received the Nobel Prize in Chemistry in 1993.

What is PCR
"Reaction" Components

1) Target DNA - contains the sequence to be amplified.

2) Pair of Primers - oligonucleotides that define the sequence to be amplified.

3) dNTPs - deoxynucleotidetriphosphates: DNA building blocks.

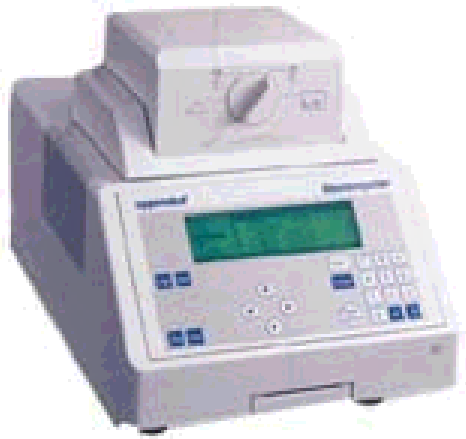
4) Thermostable DNA Polymerase - enzyme that catalyzes the reaction

5) Mg^{++} ions - cofactor of the enzyme

6) Buffer solution – maintains pH and ionic strength of the reaction solution suitable for the activity of the enzyme

The Reaction

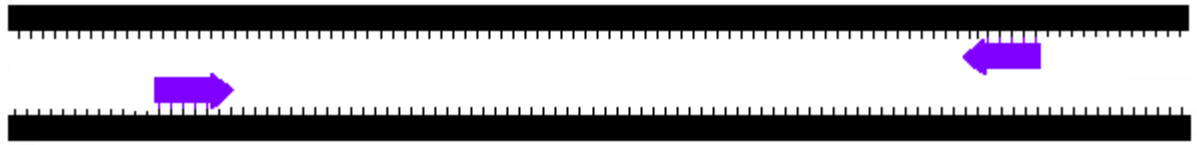
PCR tube



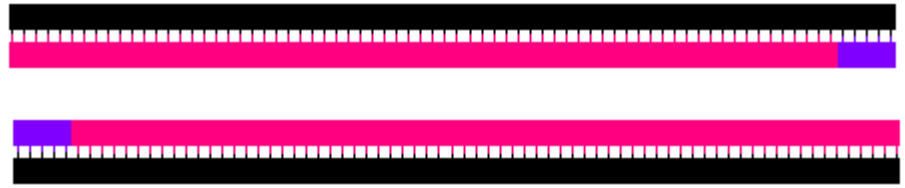
THERMOCYCLER

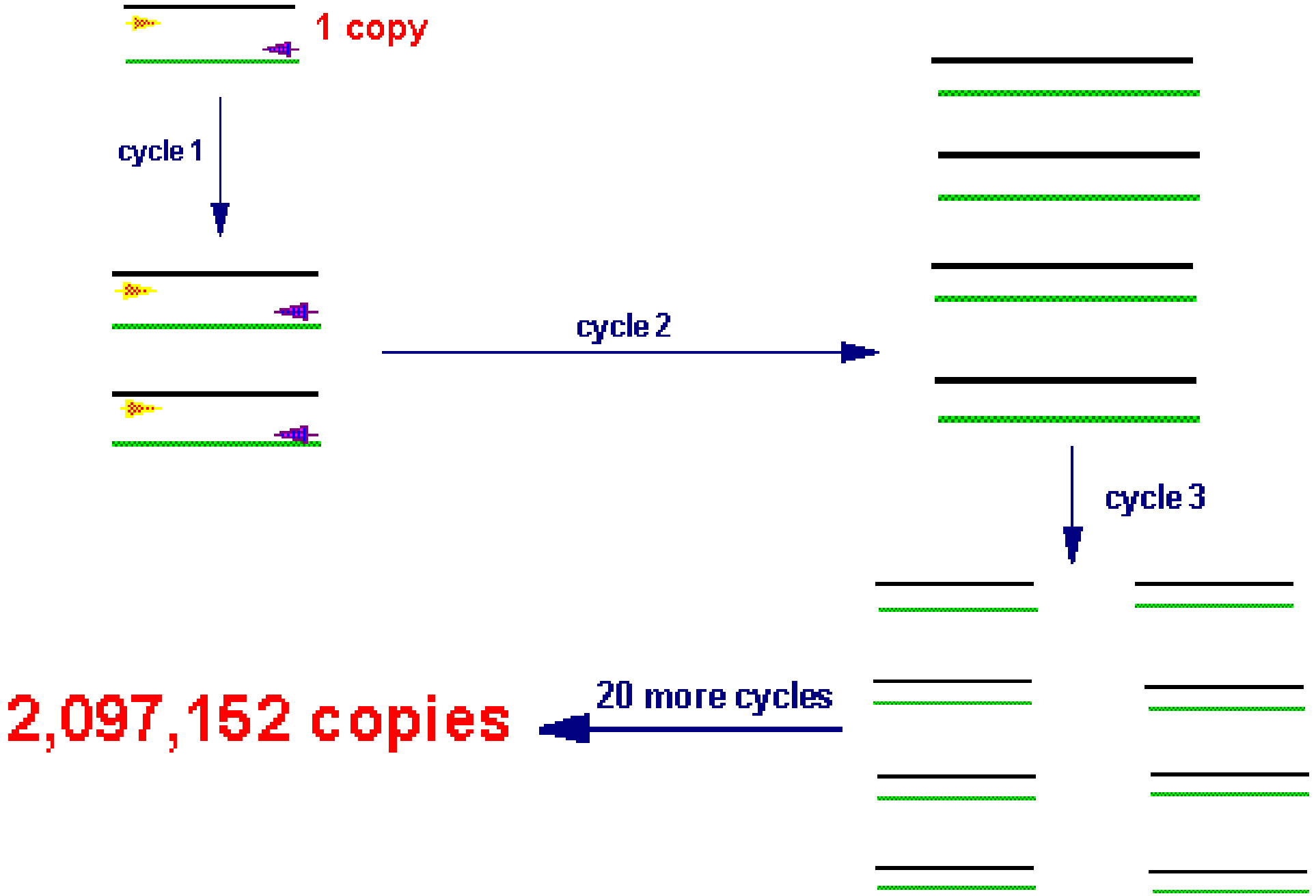
Denature (heat to 95°C)

Lower temperature to 56°C
Anneal with primers



Increase temperature to 72°C
DNA polymerase + dNTPs



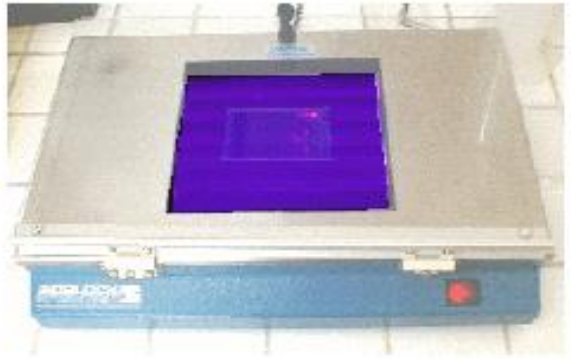

1 copy
cycle 1
cycle 2
cycle 3
20 more cycles
2,097,152 copies

PROCEDURE …..

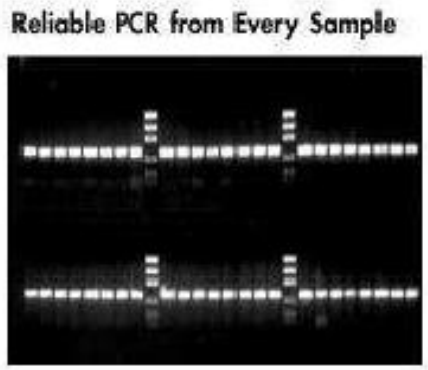
PCR

Agarose gel electrophoresis

3-4 hours



UV visualisation



The final product

Applications of PCR

- **Genotyping**
- **Mutagenesis**
- **Mutation detection**
- **Sequencing**
- **Cancer research**
- **DNA fingerprinting**
- **Drug discovery**
- **Genetic matching**
- **Genetic engineering**
- **Pre-natal diagnosis**
- ***Detection of pathogens***
- ***Classification of organisms***

Molecular Identification:

Detection Of Pathogens

A sample that may contain pathogenic DNA.

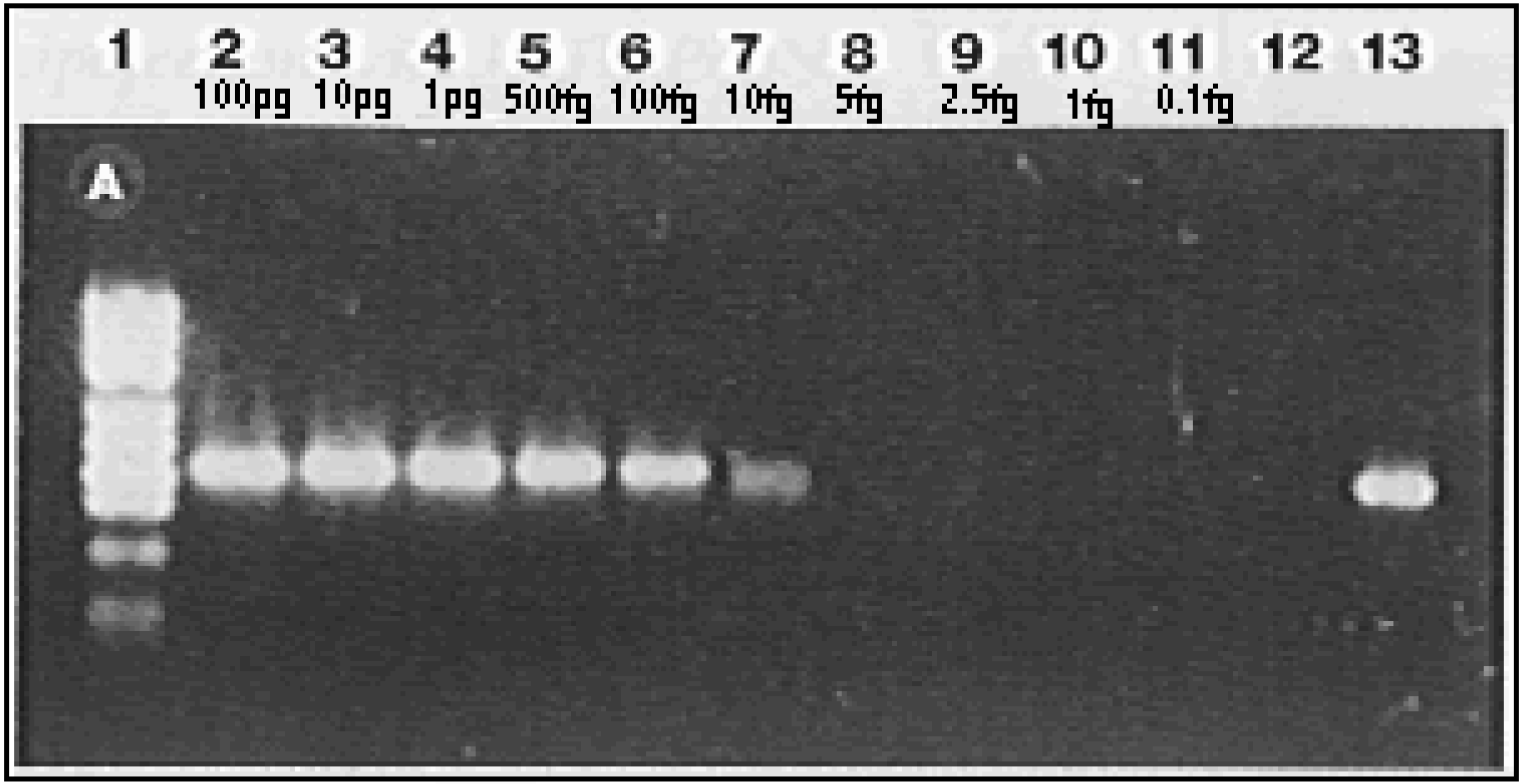
PCR

YES PCR → YES Pathogen

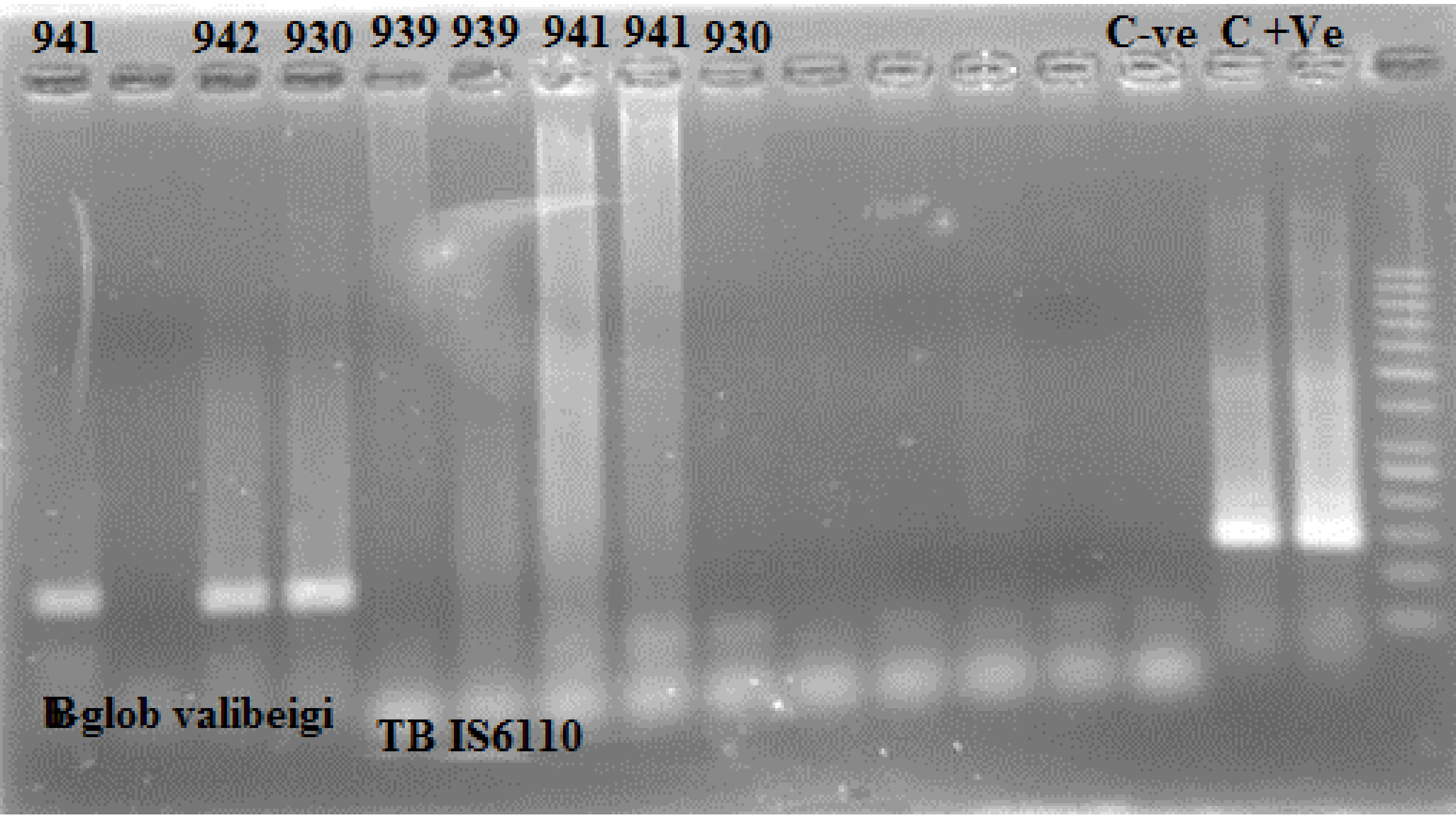
NO PCR → NO Pathogen

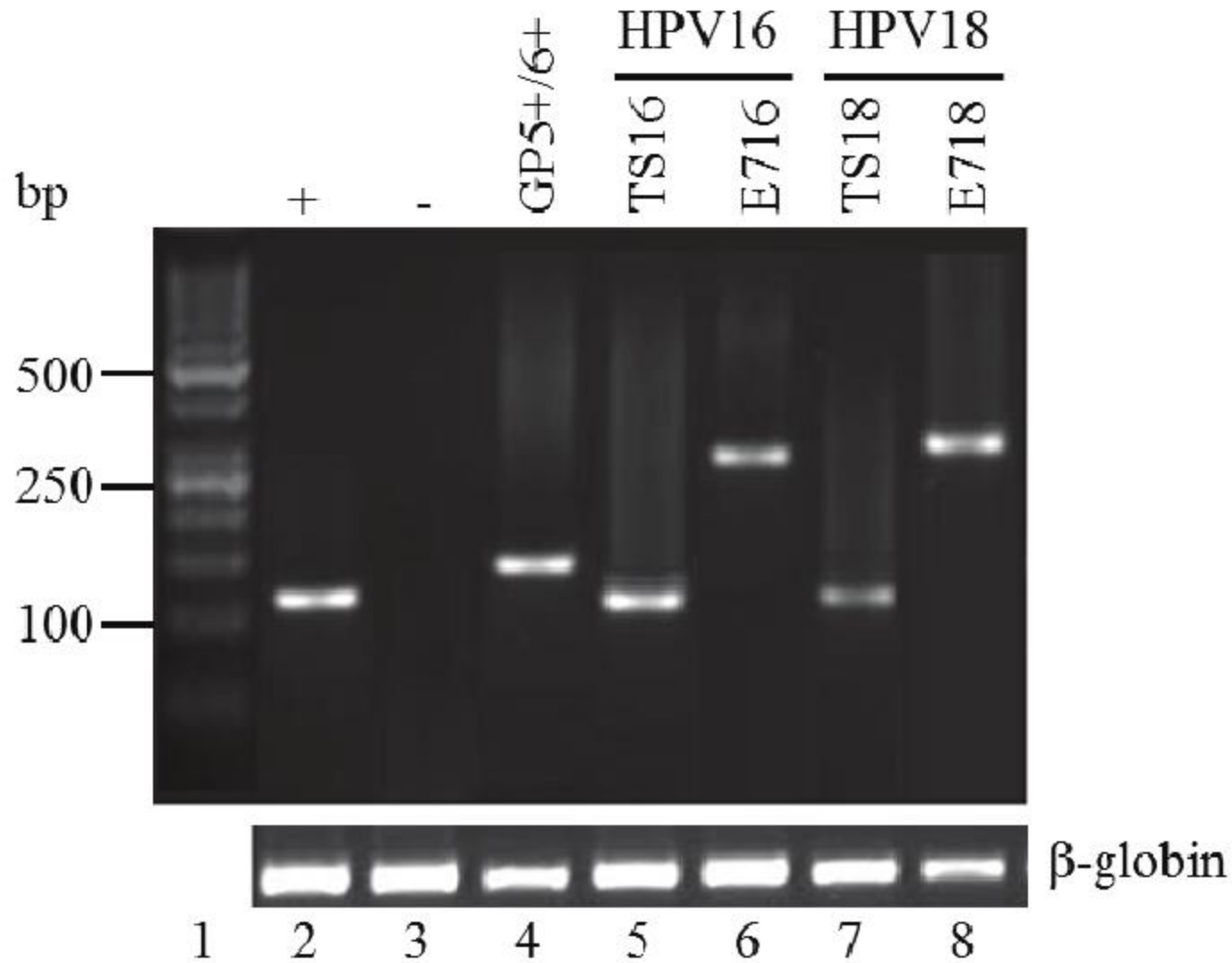
Molecular Identification:

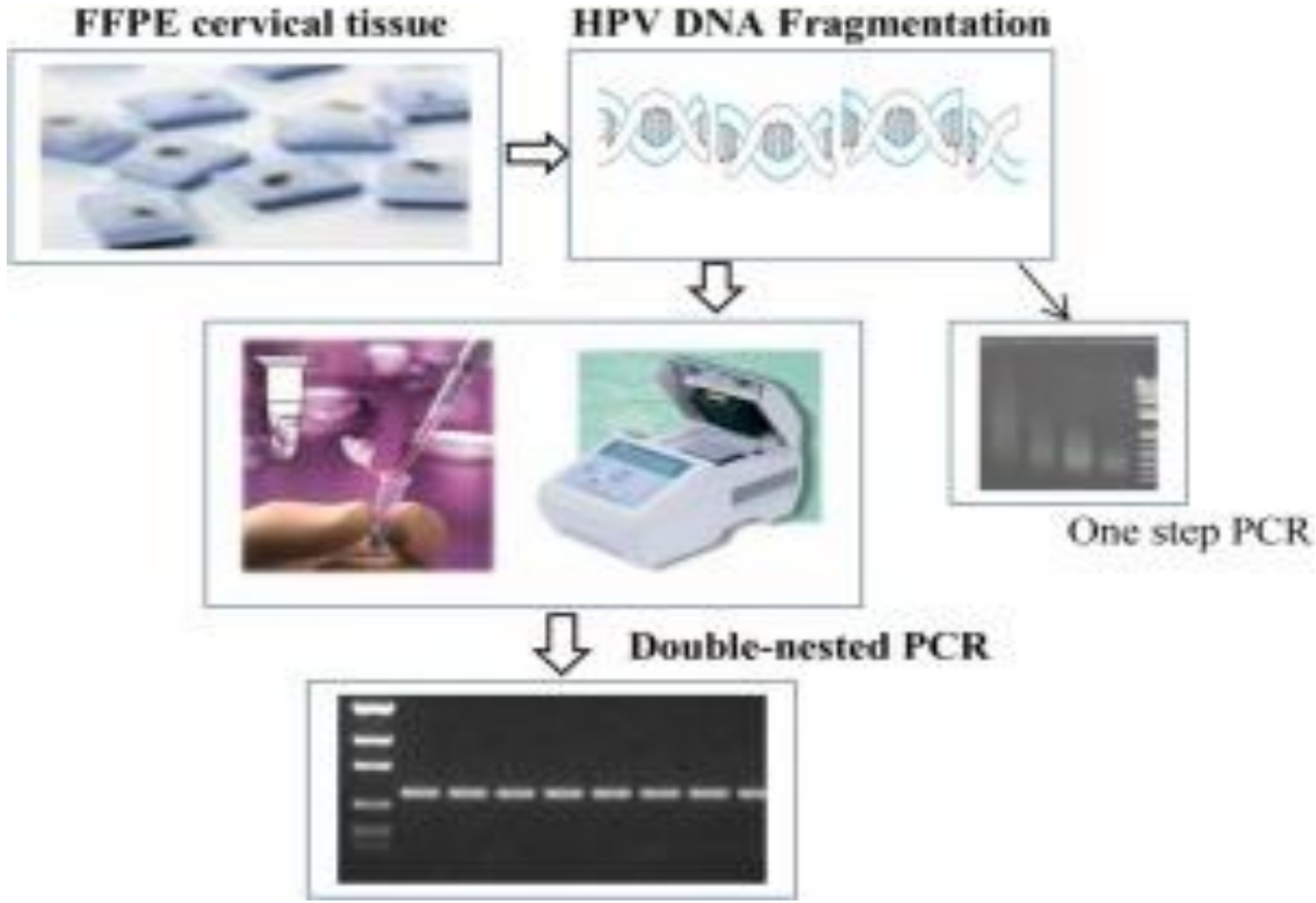
Detection Of Pathogens



Sensitivity of detection of PCR-amplified *M. tuberculosis* DNA. (Kaul *et al*.1994)


941
942
930
939
939
941
941
930
C-ve
C +Ve
B-glob valibeigi
TB IS6110


bp
+
-
GP5+/6+
HPV16
HPV18
TS16
E716
TS18
E718
500
250
100
β-globin
1
2
3
4
5
6
7
8


FFPE cervical tissue
HPV DNA Fragmentation
One step PCR
Double-nested PCR

Hybridization analysis of PCR products

A common way to investigate the sequence of PCR products is hybridization with one or more oligonucleotide probes.

Type-specific PCR products can be confirmed with corresponding type-specific probes.

The original method is Southern blotting, where a PCR product is electrophoresed prior to transfer to a membrane that is subsequently hybridized to a labeled probe

However, Southern blotting is labor-intensive and not suitable for routine application. Therefore, alternative hybridization formats have been developed

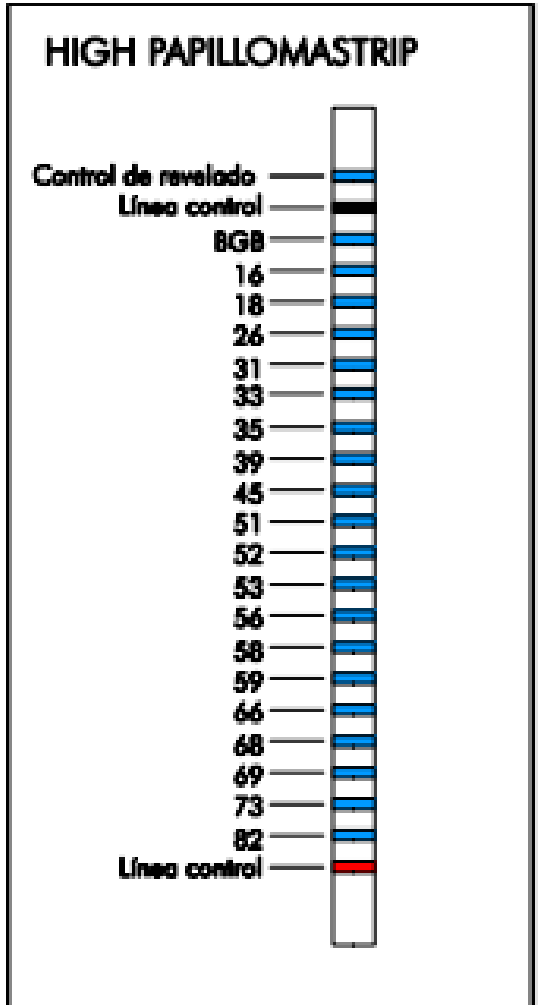
New molecular commercial tests

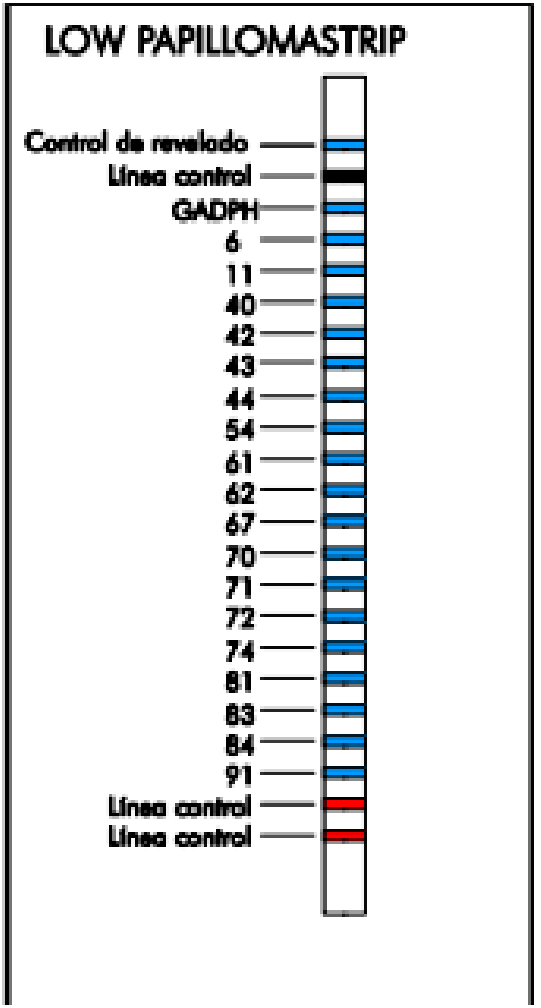
- in three steps:
- a) DNA extraction
- b) amplification by PCR
- c) hybridization/developing

HIGH+LOW PAPILLOMASTRIP Test for the detection of 37 anogenital HPVs

PapillomaStrip test is a test based on the reverse blot technique that allows qualitative detection in DNA samples from cervico-uterine smears or biopsies, of 37 human Papillomavirus subtypes:

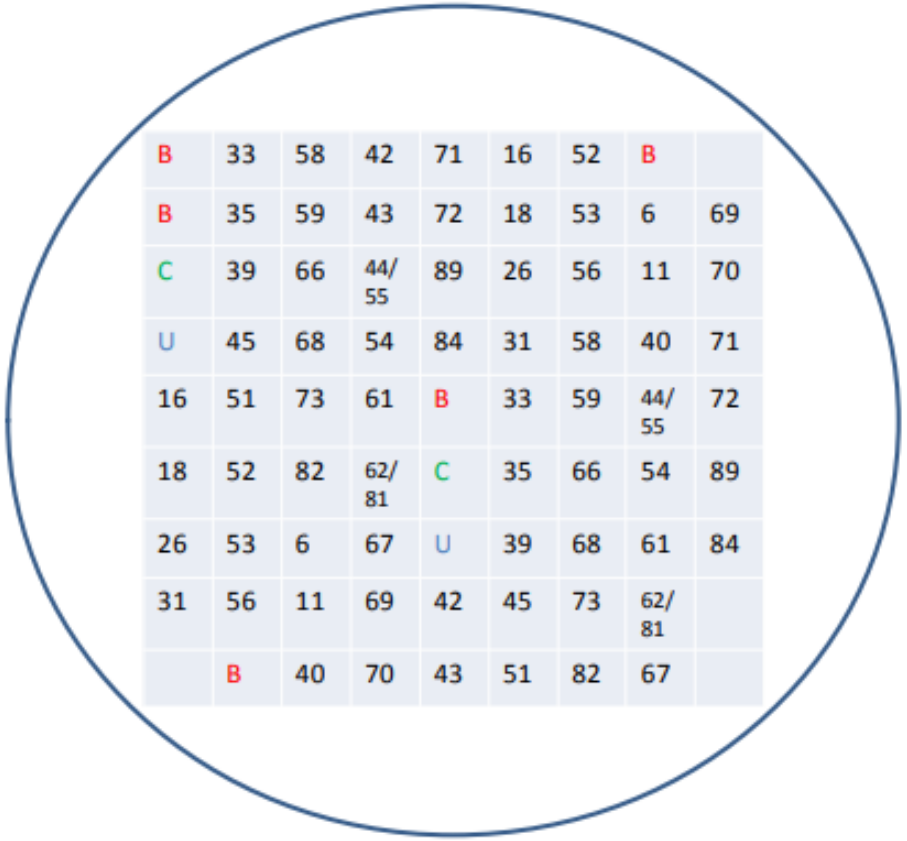
- Medium-high risk: 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73 and 82 (MM4 e IS39).
- Low risk: 6, 11, 40, 42, 43, 44, 54, 61, 62, 67, 70, 71, 72, 74, 81, 83, 84 y 91.





HPV Direct Flow CHIP

Screening and genotyping of human papillomavirus based on PCR amplification and reverse dot blot hybridization



B	33	58	42	71	16	52	B	
B	35	59	43	72	18	53	6	69
C	39	66	44/55	89	26	56	11	70
U	45	68	54	84	31	58	40	71
16	51	73	61	B	33	59	44/55	72
18	52	82	62/81	C	35	66	54	89
26	53	6	67	U	39	68	61	84
31	56	11	69	42	45	73	62/81	
	B	40	70	43	51	82	67	

Current HPV tests

1. **High risk HPV DNA tests without genotyping**
2. **High risk HPV DNA tests with limited/partial genotyping (genotypes 16 and 18)**
3. **HPV DNA tests with full genotyping (Both low risk and high risk genotypes)**
4. **High risk HPV mRNA tests**

*** In situ hybridization-based HPV tests**

FDA-approved hrHPV assays

- At present, there are 5 FDA-approved hrHPV assays that are commercially available, but only one of these assays is now FDA-approved specifically for primary screening.

- Clinicians should not use an FDA-approved test without a specific primary hrHPV screening indication.

FDA-approved hrHPV assays

- **Hybrid Capture® 2 (HC2) HPV DNA test** (Qiagen, USA)
- **Cervista® HPV HR Test** (Hologic, USA)
- **Cervista® HPV 16/18 Test** (Hologic, USA)
- **cobas® 4800 HPV test** (Roche Diagnostics, Switzerland)
- **APTIMA ® HPV Assay** (Hologic, USA)

FDA-approved hrHPV assays

- **FDA** Approves Roche's HPV Test for **First-Line Primary Screening** for Cervical Cancer
- Expanded indication makes cobas HPV Test the only test approved in U.S. that can be used instead of Pap in first-line primary screening in women 25 and older
- The approval follows the March 12 (2014) unanimous recommendation from the Microbiology Devices Panel of the FDA's Medical Devices Advisory Committee, making the cobas HPV Test **the first** and **only** HPV test in the United States approved for **first-line primary screening.**

FDA-approved hrHPV assays

- The Cobas HPV test uses:
  1. amplification of target DNA by PCR

     and subsequently

  2. nucleic acid hybridization for the detection of 14 high-risk HPV types in a single analysis.

The test specifically identifies HPV types 16 and 18 while concurrently detecting the 12 remaining high-risk types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) at clinically relevant infection levels (Cobas HPV test, draft package insert, 2011).