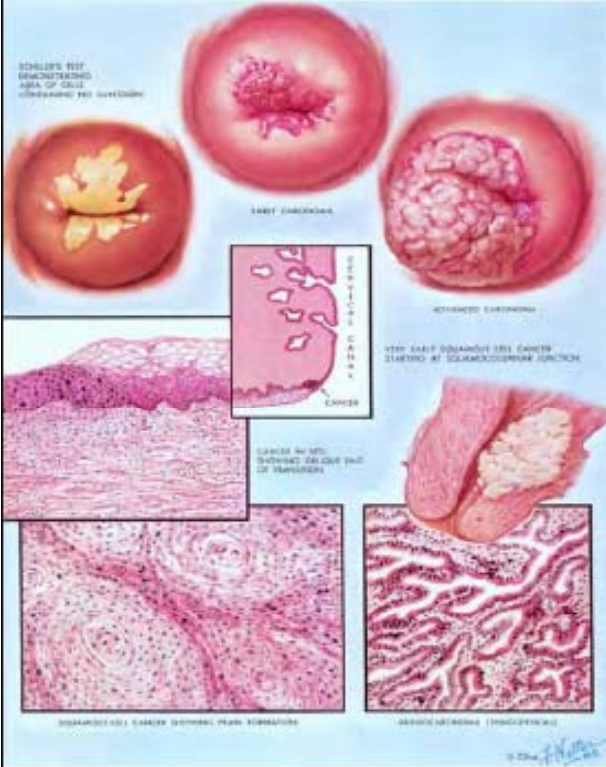


HPV: Virology, diagnosis and clinical significance :the digene hc2 hpv dna test

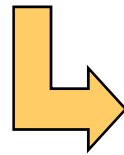


Cervical Cancer is the Very Rare Outcome of a Very Common Genital Infection! Why ?

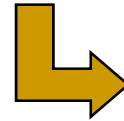


2 distinct types of viral gene expression !

acute infection, regulated gene expression pattern



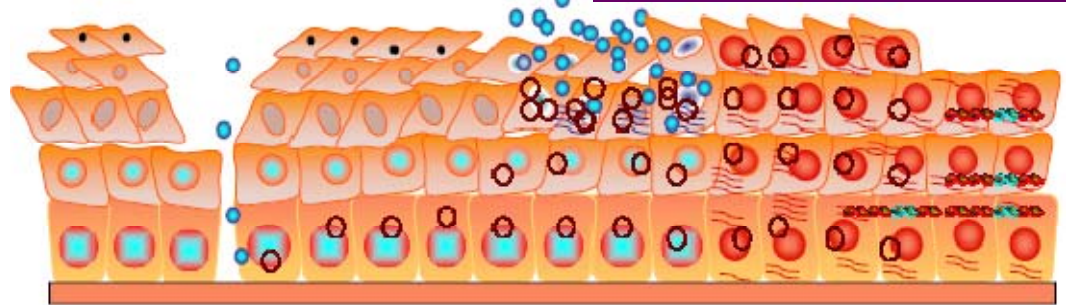
persistent infection, transforming gene expression



chromosomal instability



progression to high grade lesions



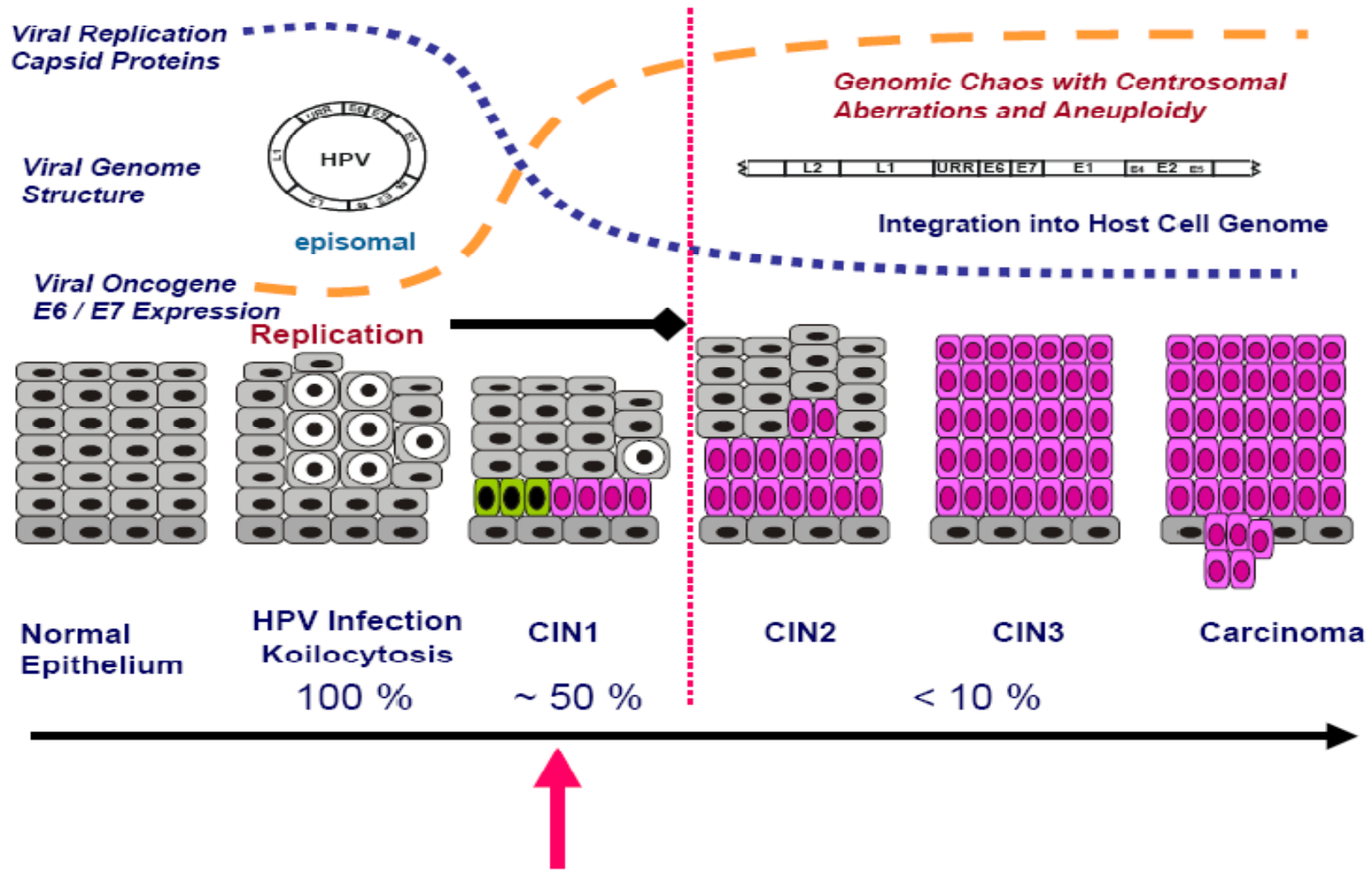
latent Infection > replicative infection > persistent & deregulated infection

normal

CIN 1

CIN 2 / 3

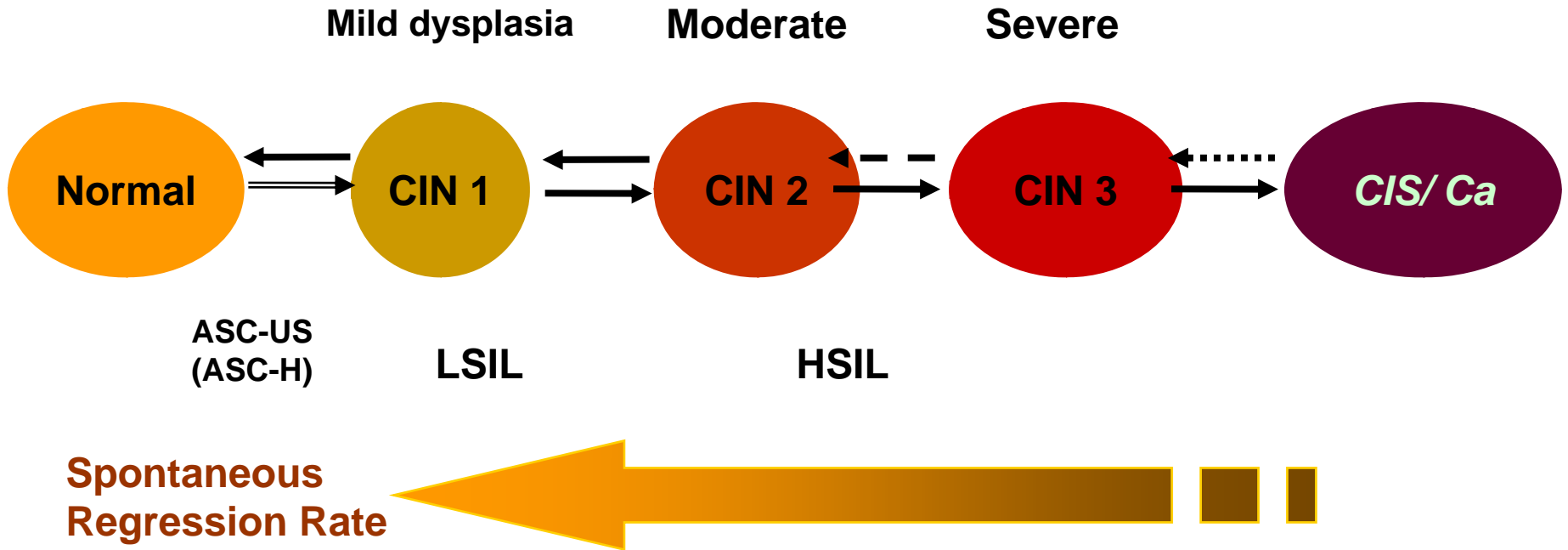
Transforming Infection:
 oncogenic functions were initiated by high level of E6 & E7 gene expression in proliferating basal cells !



deregulated methylation pattern of the viral genome induces the “transforming” infection !

Carcinogenesis

Natural History Initiation Promotion and Progression of ICC



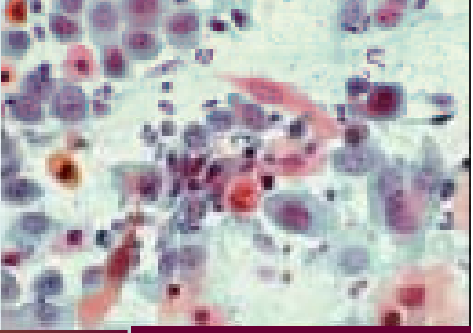
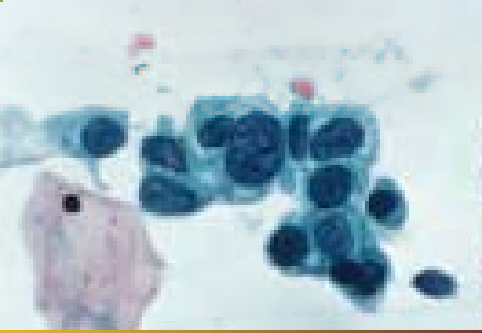
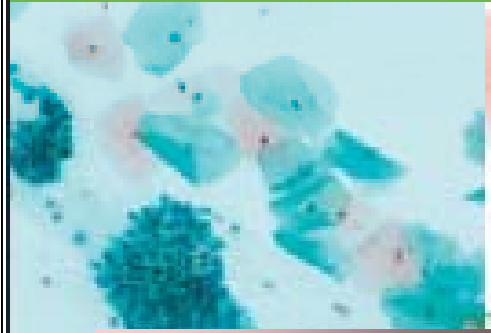
Spontaneous Regression Rate

Co-factors affecting natural history

- Host Immune status
- Nutrition, Smoking
- HPV 16-18
- Vit. A & B, Carotene
- STI (Chlamydia, Herpes, Bacterial Vaginosis)
- Parity, OCs

Transient infection

HPV viral persistence

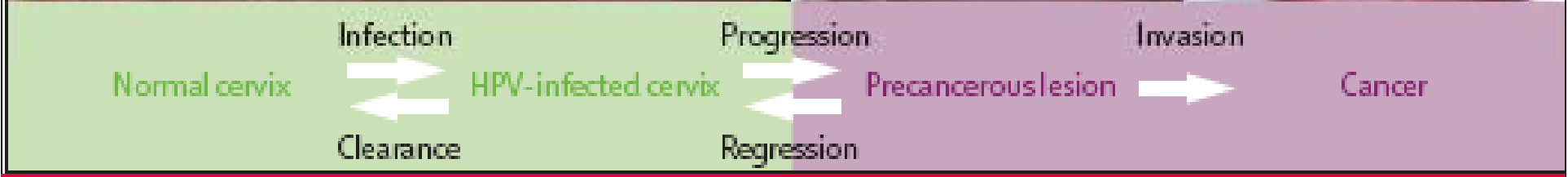


Viral Infection of metaplastic epithelium at cervical TZ

Viral Persistence

Progression to Pre-Ca

Invasive ca through basement membrane

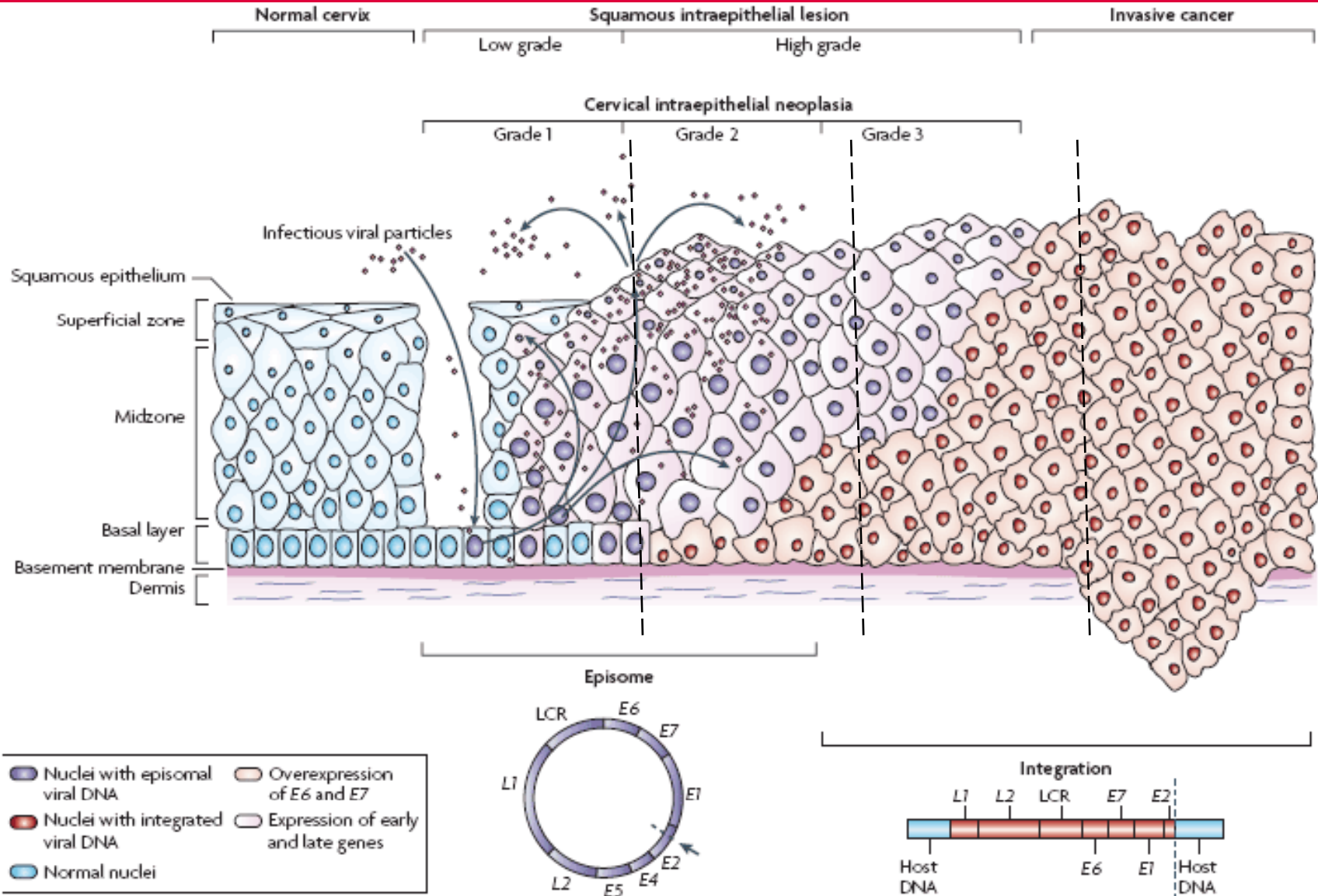


DDG1

Top row shows cytology, bottom row concurrent colposcopy. The major steps in cervical cancer development can be understood best in relation to age at first sexual intercourse as a proxy for age at first infection. The typical age of cervical HPV infection is similar to other sexually transmitted infections, with a large peak rapidly following average age of sexual initiation. This average age of HPV infection varies by culture, affecting average ages of subsequent stages. Incident HPV infection is best measured by molecular tests. Cross-sectionally, most HPV infections show no concurrent cytological abnormality. About 30% of infections produce concurrent cytopathology, usually non-classical (equivocal) changes. Most HPV infections clear within 2 years; the 10% that persist for 2 years are highly linked to precancer. Detection of precancers is delayed by their initially small size and the typically low sensitivity of screening methods. Precancers are usually detected around age 25–30 years (about 10 years after sexual debut) in regions with cytological screening. Adapted from Schiffman and Castle.

Dr. Dinesh Gupta, 3/24/2009

Cellular level changes



OBJECTIVE OF A SCREENING PROCEDURE

- 1. performed less frequently**
- 2. identify only those with clinical disease or who are at the highest risk, ideally 100% clinically sensitive;**
- 3. not identify any women who have no disease, nor at risk, 100% specific;**
- 4. cost-affordable/ cost-effective**
- 5. be a simple procedure for community field workers;**
- 6. simple logistics, preferably those already available**
- 7. not result into call-recall of screen-positive women**
- 8. not create excessive burden of referral patients on tertiary care hospitals**
- 9. not result into excessive human resource development and training,**
and finally,
- 10. result into quantifiable and measurable reduction in morbidity and mortality.**

Hybrid Capture 2- fits the bill



- DML2000
- Rotary shaker
- PC/V2 Software
- Plate Heater
- Plate Washer
- Vortex



Manual
Version 2

Digene HPV Test using Hybrid Capture[®] 2

- nucleic acid signal amplification, solution hybridization microplate assay providing same-day results
- Capable of detecting and differentiating high- and low-risk HPV types
 - High risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68)
 - Low risk (6, 11, 42, 43, 44)

Analytical Sensitivity

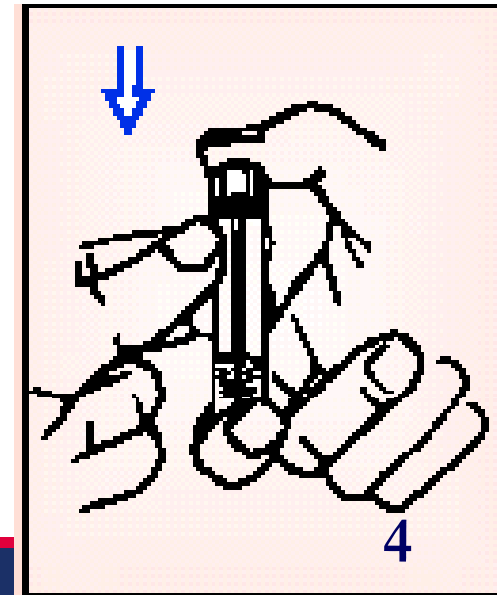
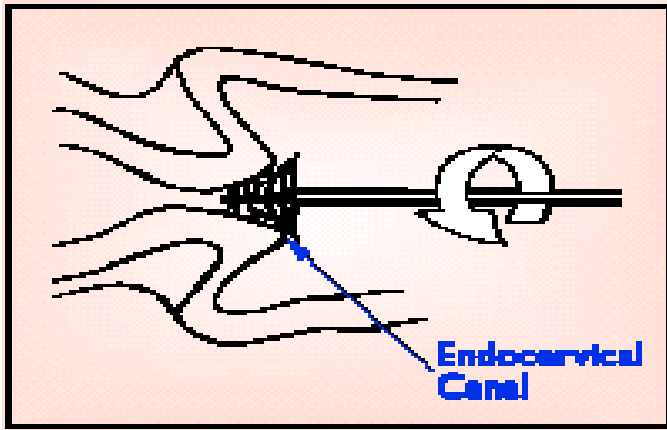
- 1 pg/mL or 5,000 genomes per assay

Clinical Sensitivity

- CIN++

Specimen Collection

Using the DNA Collection Device



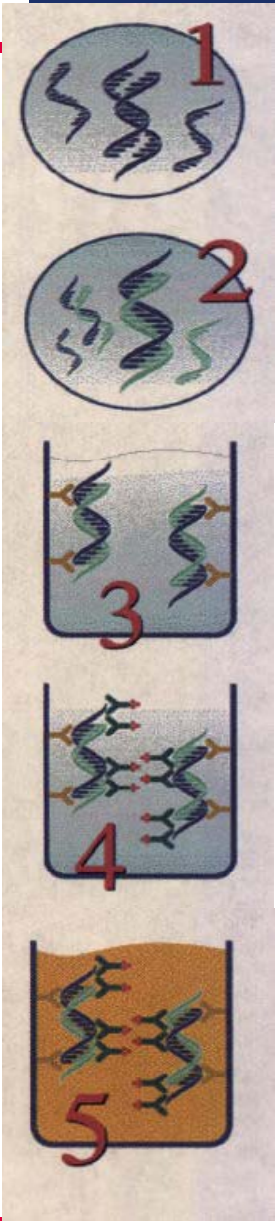
Specimen Collection and Handling

- **hc2 has been validated for the types of cervical specimens:**
 - **Specimens collected with the Digene Cervical Sampler (STM)**
 - **Biopsies collected in STM**
 - **Specimens collected using a broom-type collection device and placed in Cytoc PreservCyt Solution or SurePath Solution (LBC)**

Performance of Different Specimen Types

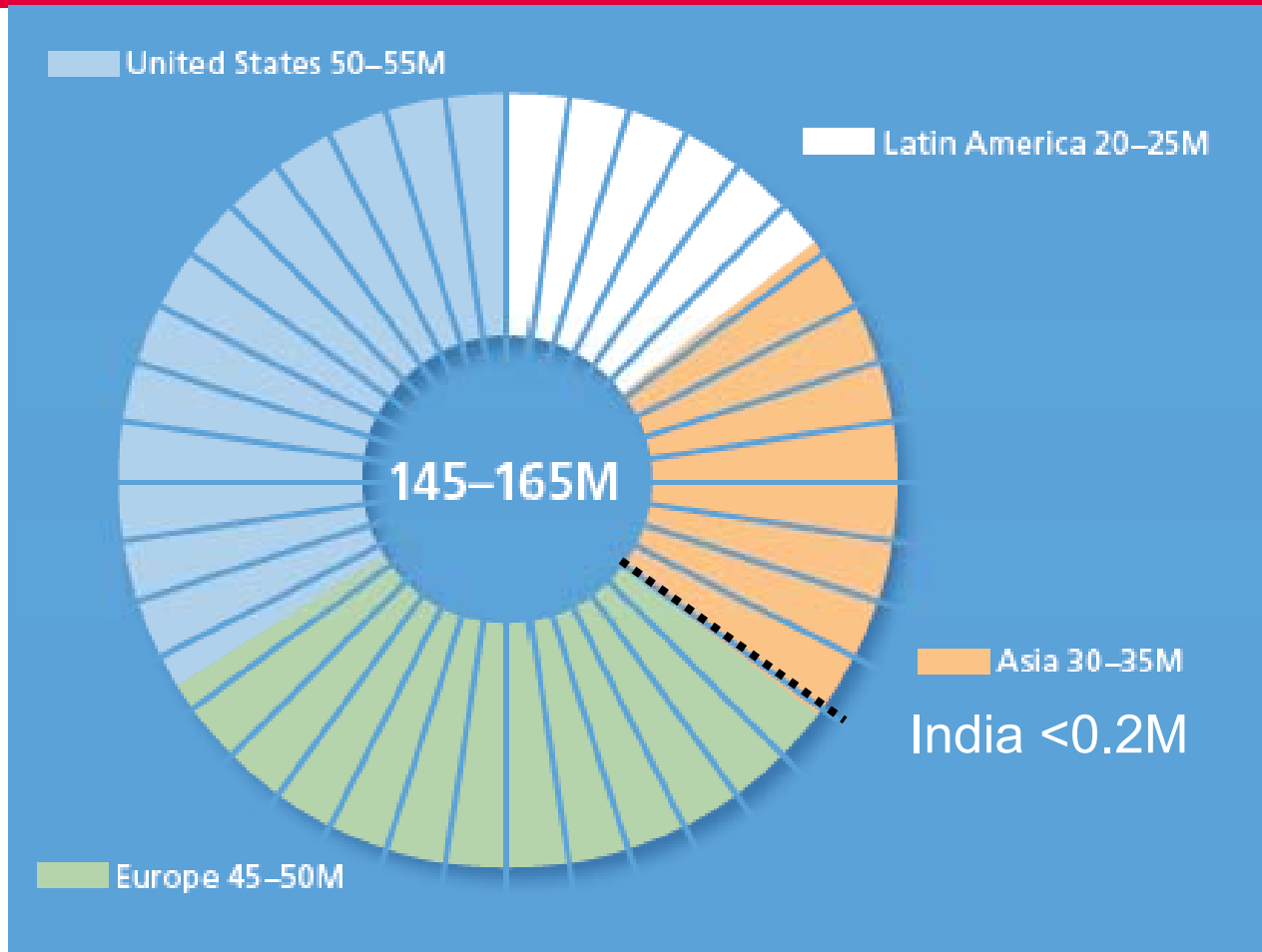
- **Comparing clinical specimens collected with Digene Cervical Sampler and corresponding PreservCyt specimens**
 - **89-91% overall agreement**

Hybrid Capture 2



- ② Denature HPV DNA with NaOH
- ② Hybridize with whole genomic RNA probes
- ③ Capture DNA:RNA hybrids with anti-hybrid antibody
- ④ Signal amplification with enzyme-linked anti-hybrid antibody
- ⑤ Substrate activation by bound antibody conjugated enzyme - READ luminescence

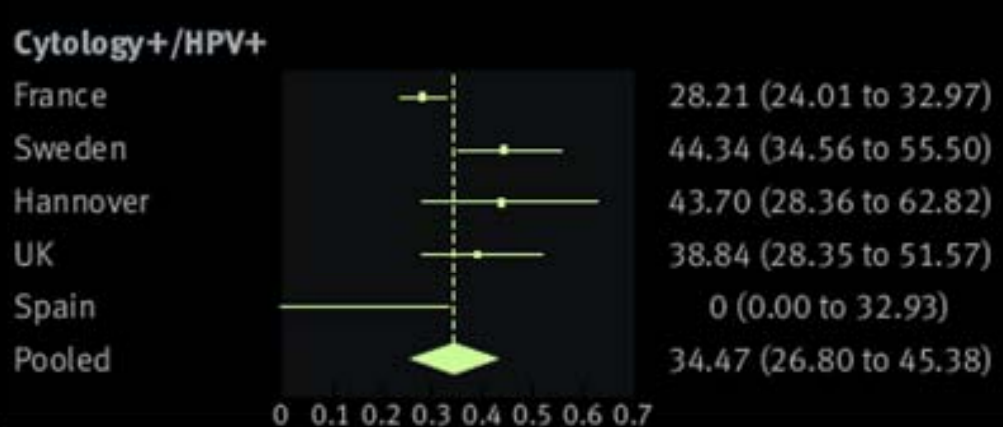
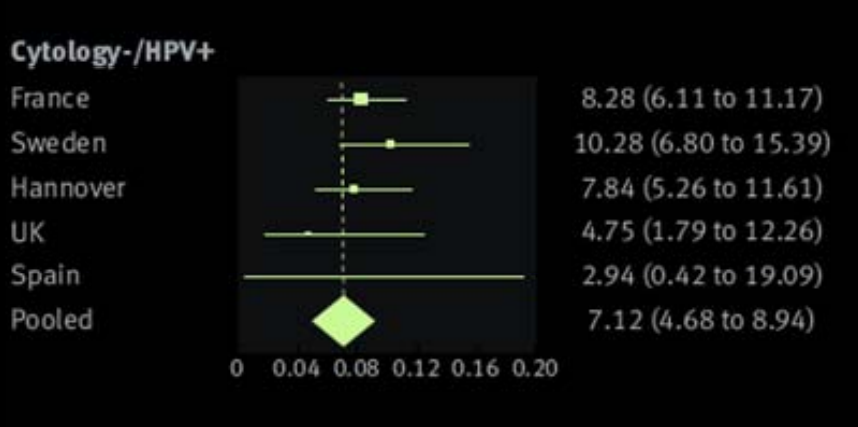
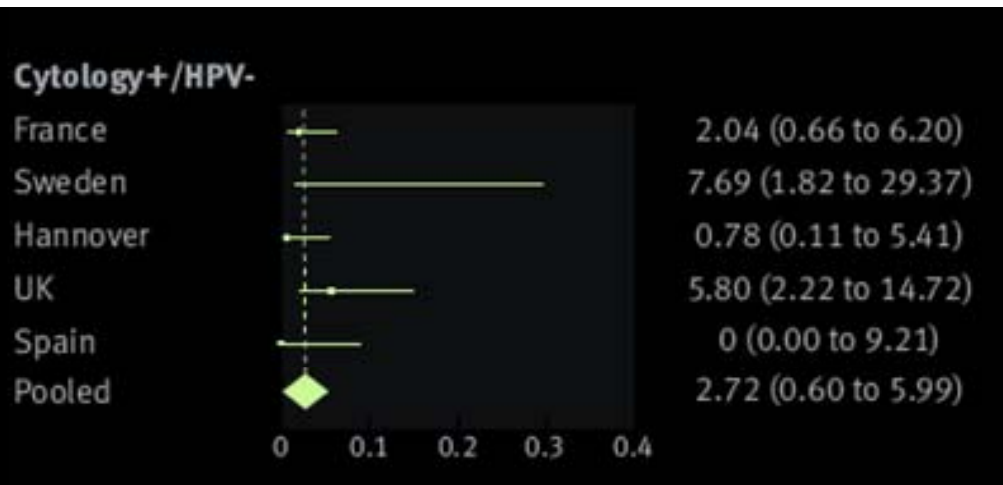
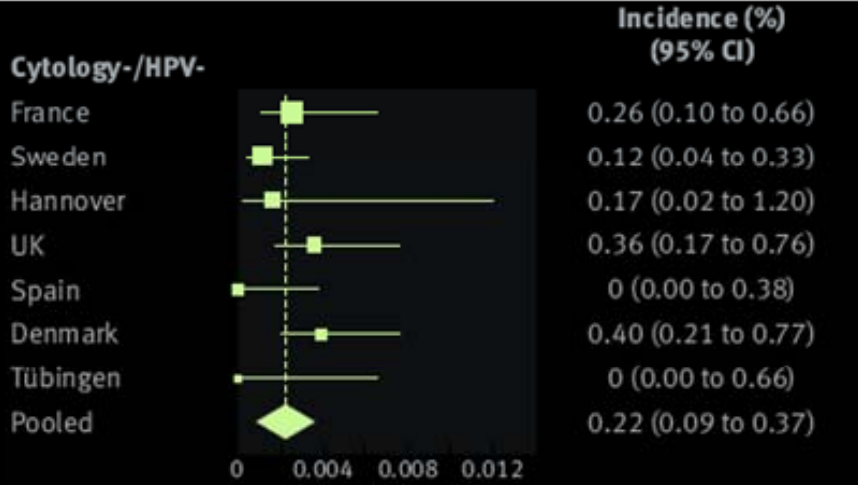
Global Pap Test suggests potential for HPV Testing: *Changing the standard of women's health-care*



It is estimated that more than half of all Pap Tests done are “eligible” for a HPV test. Recommended age (all 30+ according to ACOG guidelines) for the initiation of routine screening is a major criteria.

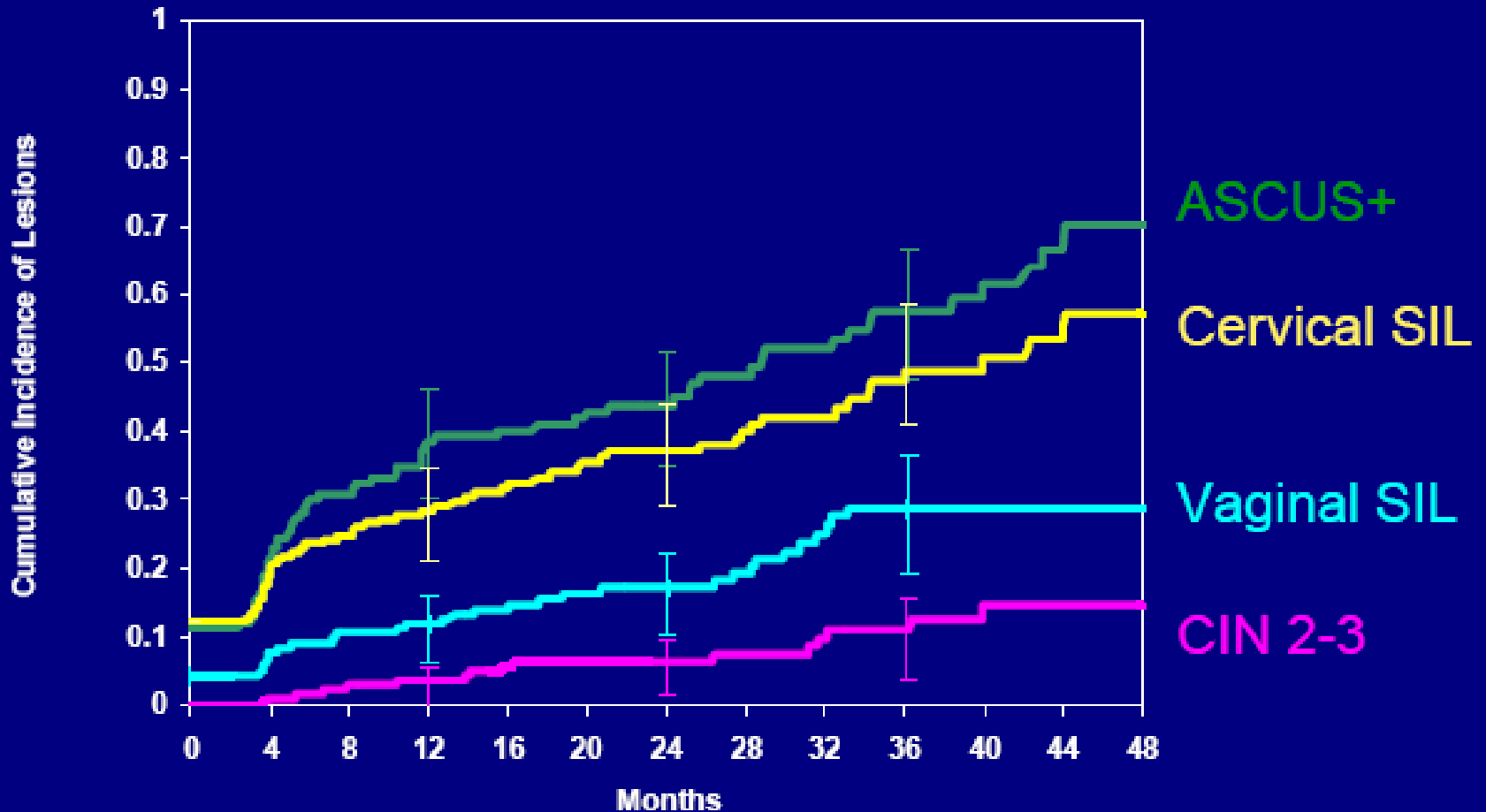


COMPARISON OF CUMULATIVE INCIDENCE RATE (95% CI) OF CIN3+ AT 60 MONTHS BY COUNTRY AND BY BASELINE TEST RESULTS



At six years of follow-up, the rate of CIN3+ was significantly lower among women negative for HPV (0.27%, 0.12% to 0.45%) than among women with negative cytology results (0.97%, 0.53% to 1.34%)

Cumulative Incidence of CIN 2/3, Vaginal SIL, Cervical SIL & ASCUS+ with HPV Positivity (n=197)



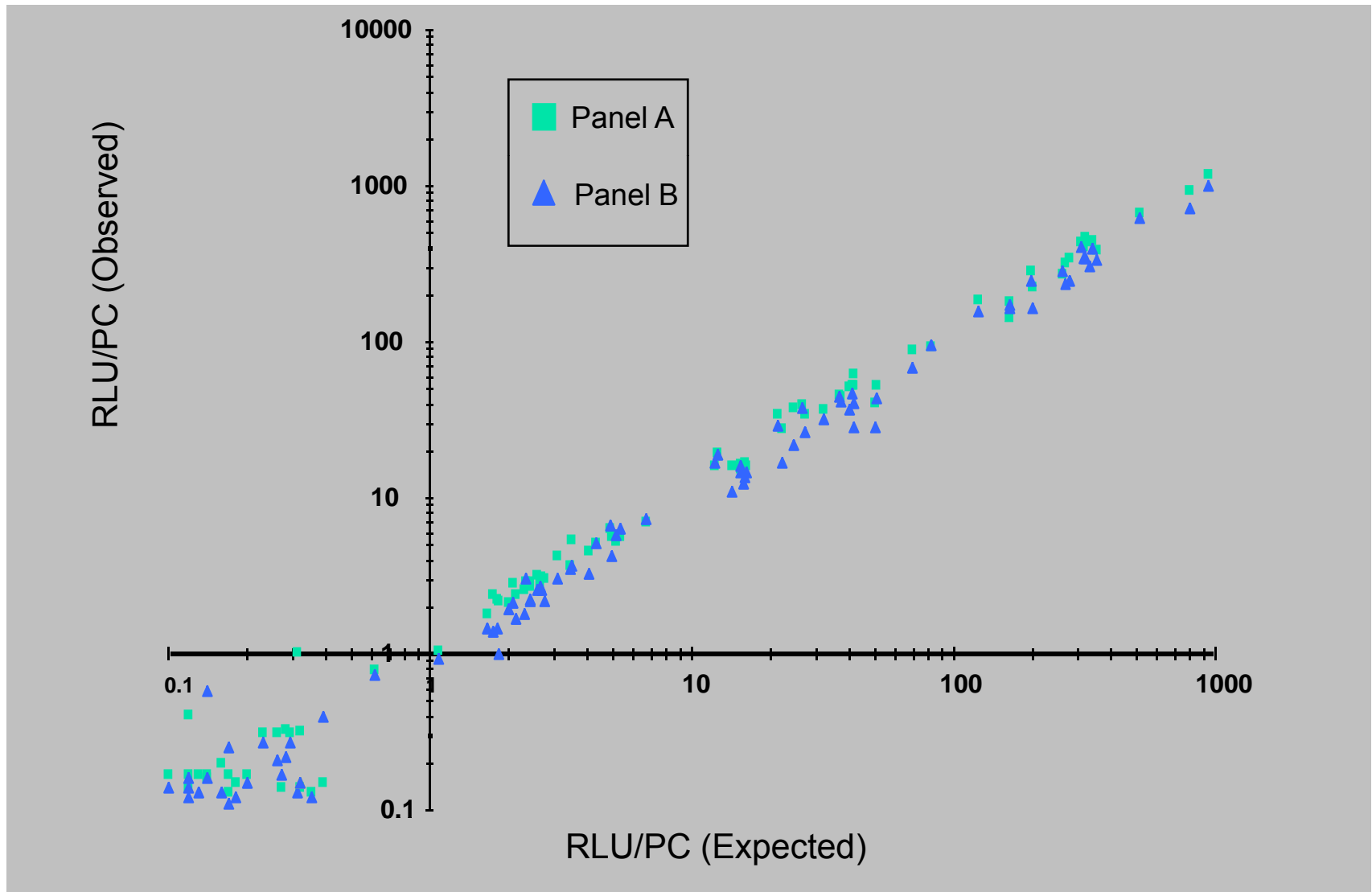
Winer R, Kiviat N, Hughes J, et. al. Journal of Infectious Diseases. 2005;191(5):731–8.

Development of CIN 2-3

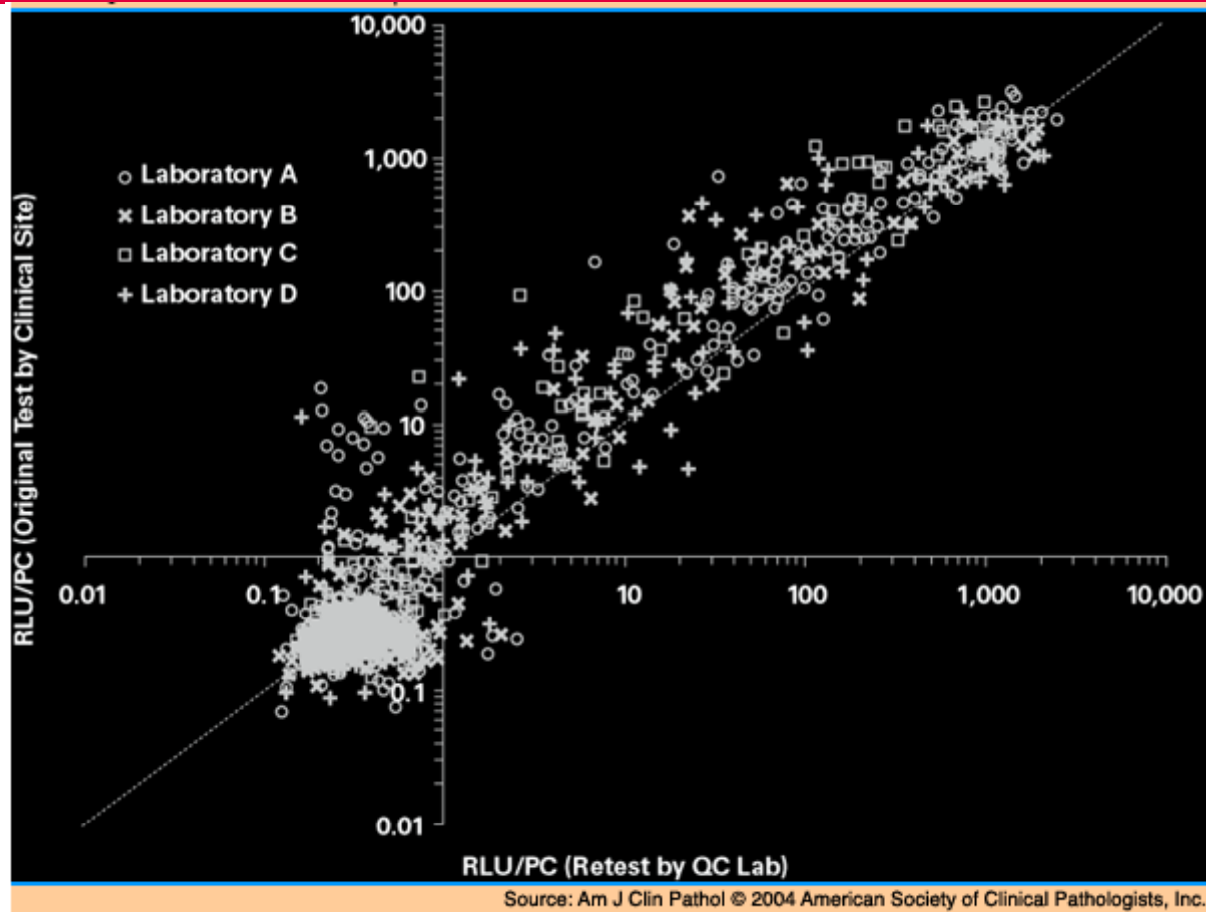
- The 36-month cumulative incidence of CIN 2-3 from time of incident HPV infection was 11.1% (95% CI: 6.5, 18.5).
- The median time from incident HPV detection to detection of CIN 2-3 was 16.3 months (IQR: 7.9, 31.2).
- All cases of CIN 3 were associated with HPV 16, and 60% of cases of CIN 2-3 were associated with either 16 or 18.



Hybrid Capture[®] 2 HPV DNA Test – Highly Reproducible



HC[®]2 HPV – Results from paired laboratories



Comparison of raw RLU/PC values for paired Hybrid Capture 2 test results by the clinical center laboratories and the human papillomavirus quality control (QC) laboratory (N = 1,072). The axes are shown at 1 RLU/PC, the positive cut point for Hybrid Capture 2. Overall Spearman $\rho = 0.82$ (95% confidence interval [CI], 0.80-0.84 [all test results]); Spearman for positive results (RLU/PC ≥ 1.0), $\rho = 0.94$ (95% CI, 0.93-0.95); $\kappa = 0.84$ (95% CI, 0.81-0.87). The dotted line indicates test equivalence. RLU/PC, signal strengths in relative light units compared with 1 pg/mL HPV type 16 DNA positive control samples (1.0 RLU/PC \sim 1 pg/mL)

BROAD INTERPRETATION OF –ve HPV DNA TEST RESULT

- Provides re-assurance of no current disease.
- **MAY GET PAP SMEAR DONE.** An equivocal (ASCUS) or Low Grade cytology report (Pap Test) - virtually at no risk of subsequent development of cervical disease (whose cervical cytology will probably return to **NORMAL** at the next examination).
- There is a high probability that a higher disease stage will not be found at colposcopy.
- **IF BOTH NEGATIVE , THE PROBABLE RISK OF DEVELOPING CERVICAL CANCER OVER NEXT 5-15 YRS IS MINIMAL (0.2%).**

BROAD INTERPRETATION OF +ve HPV DNA TEST RESULT

- Suggests current HPV Infection and is strong predictive of subsequent cervical SIL.

In women >30: indicates persistent infection with HR HPV. Further investigation are necessary. As long as a woman remains High Risk HPV positive, there is an increased risk of development and maintenance of CIN.

Women < 30's: Repeat HPV DNA test within 1 year. The HPV infections may be transient below this age. If the test repeats positive the woman should be investigated further for the presence and subsequent treatment of dysplasia. If HPV DNA cannot be detected after this time then the infection most probably has been resolved.

THESE WOMEN ARE AT HIGHER RISK (6-7% OR MORE) OF DEVELOPING CANCER CERVIX IF NOT TREATED.

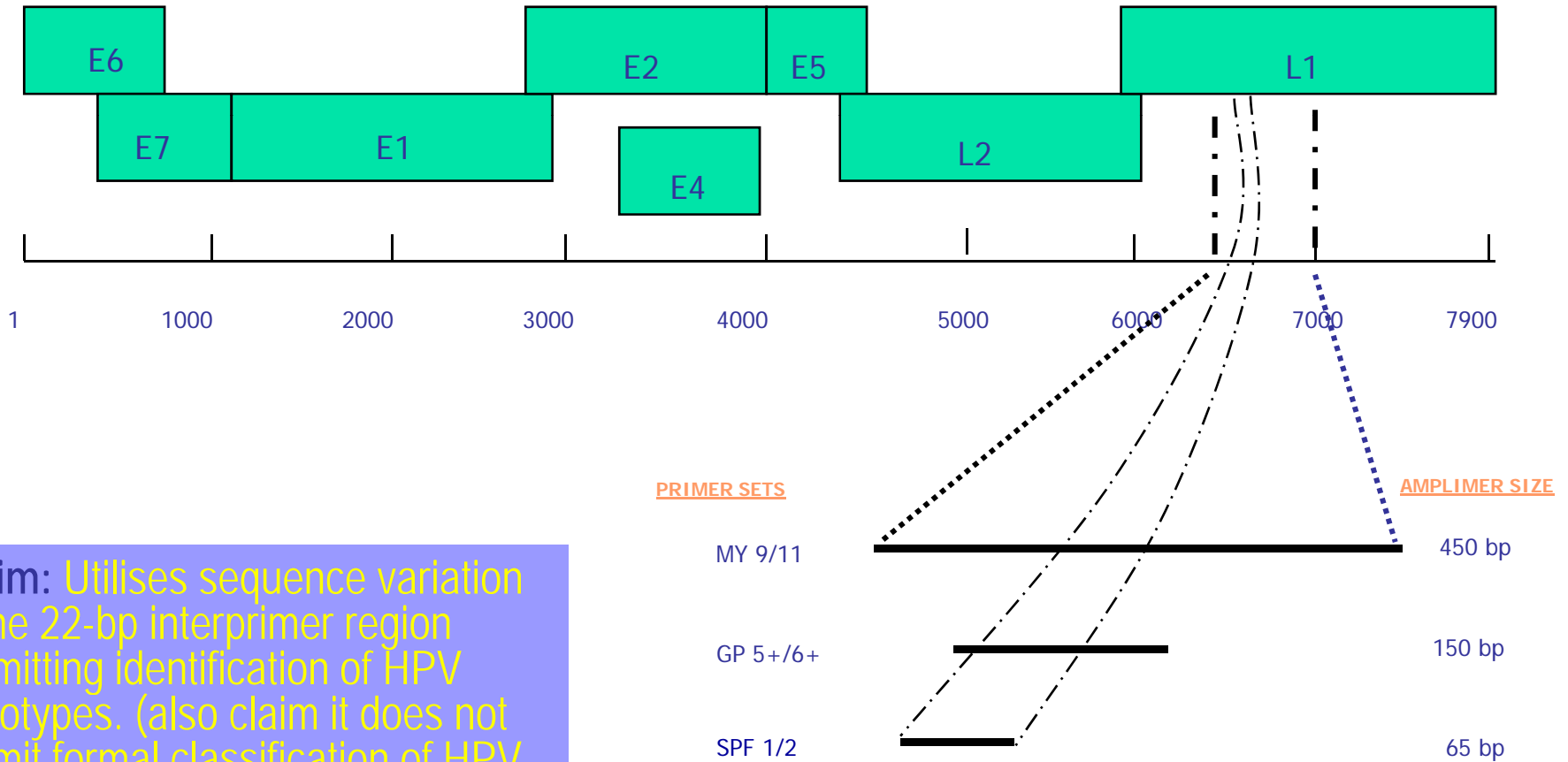
- COLPOSCOPY IS RECOMMENDED TO DETECT CIN .

INDICATIONS FOR HPV DNA TESTING

- ❖ ASCUS/ AGUS PAP SMEAR (inadequate/ inflammatory)
- ❖ POPULATION –BASED SCREENING PROGRAMS (ADJUNCT TO PAP TEST)
- ❖ RESOLVING INTER- OR INTRA TECHNIQUE DISPARITIES BETWEEN CYTOLOGY, COLPOSCOPY AND HISTOLOGY
- ❖ FOLLOW UP AFTER NORMAL COLPOSCOPY IN ABNORMAL PAP SMEAR
- ❖ FOLLOW UP AFTER TREATMENT AS A “TEST OF CURE”
- ❖ AS A PRIMARY SCREEN TEST

hc2 and PCR

HPV Genome: locations of general primer sets



Claim: Utilises sequence variation in the 22-bp interprimer region permitting identification of HPV genotypes. (also claim it does not permit formal classification of HPV genotypes. Ref: Bernhardt Kleter et al, J Clin Microbiol Aug. 1999, 2508-17)

PCR – a basic protocol:

L1 ORF

10 ul Isolated DNA + 10 mmole/l Tris-HCl pH 9.0
 50 mmole/l KCl + 2.5 mmole/l MgCl₂ + 0.1% triton
 0.01% Gelatin + 200 mmole/l DNTps + 20 pmole
 Of Forward and Reverse primers + 0.25 u Suoer Taq
 Made to 100 ul Rx volume

PCR

Preheating – 94°C; 1 min
 40 cycles of 94°C; 1 min
 45°C; 1 min
 72°C; 1 min
 Extension – 72°C
 Each expt with +ve and
 several -ve PCR controls

Detection of PCR prdts

Each PCR Amplimer – biotinylated &
 hybridised to HPV specific probe mix

10 ul

Dilute to 100 ul
 With hybri buffer
 (150mmole/l NaCl,
 15 mmole/l Na Citrate
 0.1% Tween 20) pH 7.0

Incubate
 42°C/ 30" in
 streptavidine
 coated microtitre
 plate

Wash
 3 times

100 ul Denatu soln,
 (100mmole/l NaOH)

Incubate 5" at RT
 Wash 3 times

Mix with Digoxigenin
 Labeled HPV probes

Incubate 42OC at 45"
 Wash 3 times

ELISA (enzyme binding)

Wash 5 times

ELISA (substrate binding)

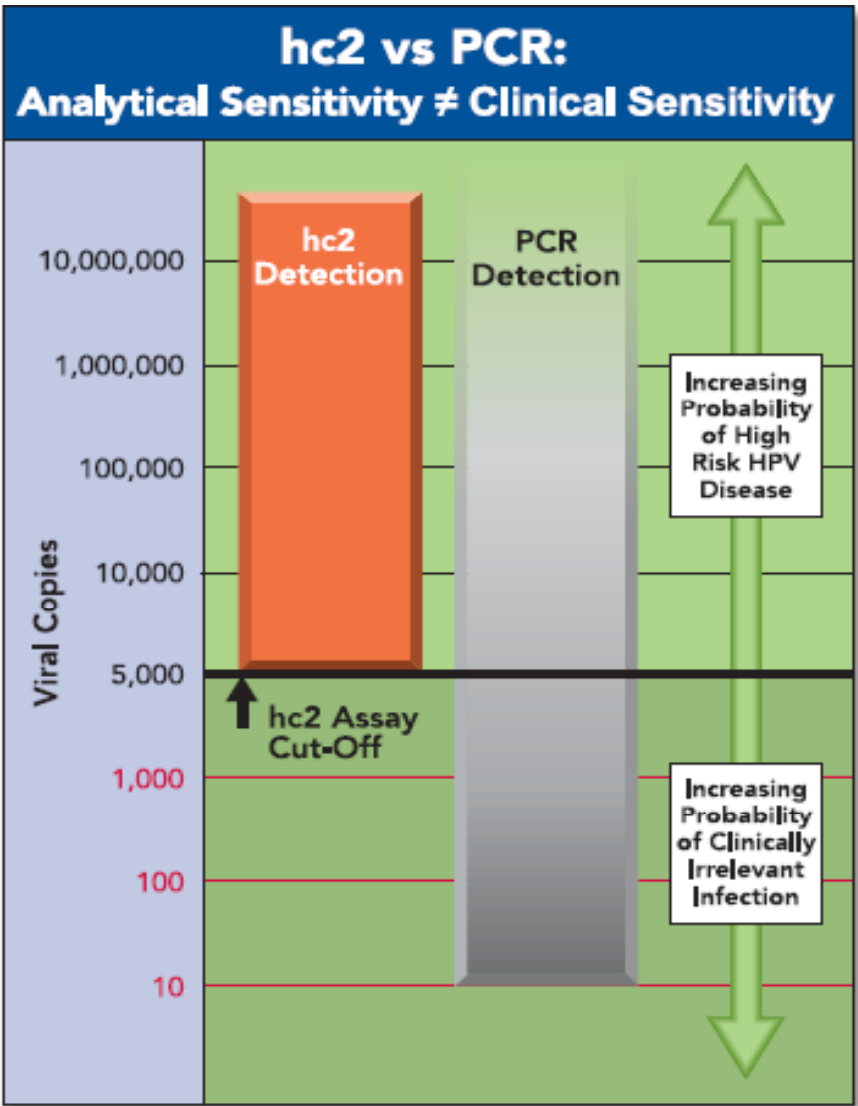
Wash 5, Stop Rx

OD at 450 nm
 OD > 2.5 times –ve control
 (each run -ve control; borderline
 and several +ve controls to be used)

Analytical versus Clinical Sensitivity

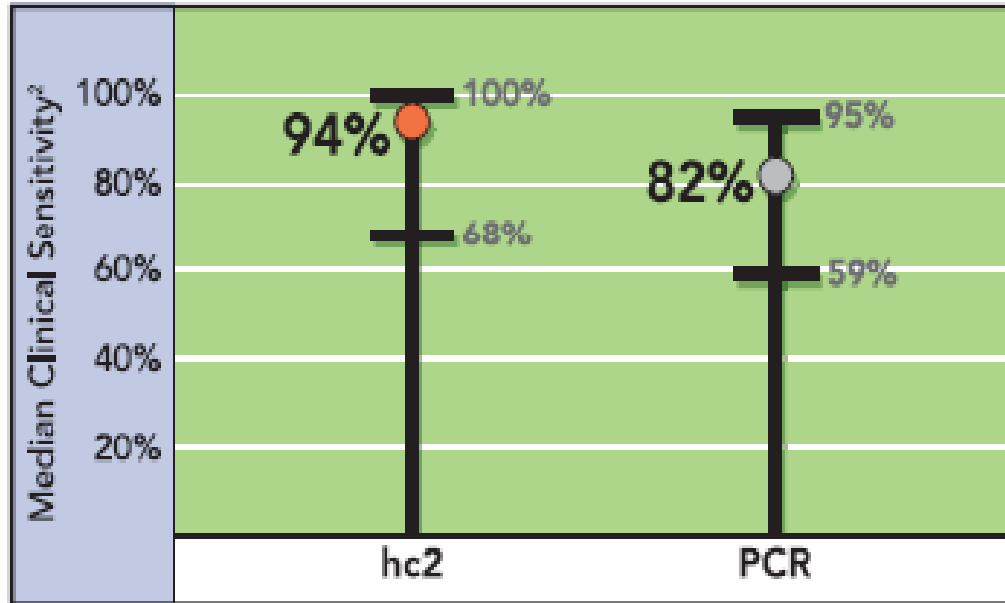
The Analytical Sensitivity of HC2 is 5,000 copies of HPV DNA

The Analytical Sensitivity of PCR methods can be <10 copies of HPV DNA



Adapted from Snijders et al. Journal of Pathology 2003; 201:1-6

Data from 16 published studies showed a median Clinical Sensitivity of 82% for PCR



* Schiffman et al, Am J Clin Path 2005; 124: 722-732
 Lorincz, HPV Testing by Hybrid Capture. Monsonog J(ed): Emerging Issues of HPV Infections: From Science to Practice. Basel, Karger, 2006: 54-62

“HC2 was more sensitive...than PCR for detecting 2-year cumulative CIN3 or cancer”*



Comparison hc[®] 2 v/s PCR

	hc2 HPV	PCR HPV
Clinical Performance	>= 96% Clinical Sensitivity >= 99% NPV	No true clinical evaluation data available. Analytical studies much smaller on population base. Only studies on research applications.
Standardized Method	YES	NO, Each lab has different protocols, different sets of primers.
Sample Prep	Not Required	Needed to purify and extract target DNA
Laboratory Requirements	Single work area	Minimum 2 work areas with strict uni-directional work flow
Throughput	up to 88 samples in 5 hours using modular hc2 system (176 samples in 8 hours)	Much lesser samples, run time > 7.5 hrs
Data used to support ACOG, ACS, & ASCCP Guidelines	YES	NO
High Risk Type Profile	Currently Accepted Clinically Relevant Types ⁵ 16,18,31,33,35,39,45,51,52,56,58,59,68	Currently Accepted Clinically Relevant Types ⁵ 16,18,31,33,35,39,45,51,52,56,58,59,68. Does not amplify all HPV high risk types equally.
Specimens (validated)	STM or UCM	Not standardized
Operator executed mixes	1	5
Complexity (scale 1 – 10) (1 = easy)	3	7



<p>CLINICAL SENSITIVITY AT DETECTING 5,000 COPIES OF HPV DNA PER ONE ML. BELOW THIS VIRAL DNA THRESHOLD LEVEL, MANY HPV INFECTIONS HAVE BEEN SEEN TO BE RESOLVED DUE TO BODY'S OWN IMMUNITY. HPV POSITIVITY ABOVE THIS THRESHOLD IS SEEN TO BE DEVELOPING INTO ABNORMAL PAP WITHIN 5 YEARS FOR ALMOST 1 IN 4 WOMEN AND INTO INVASIVE CANCER IN 1 WOMAN OUT OF 5.</p>	<p>ANALYTICAL TEST SENSITIVITY OF PCR MAY NOT BE CORRELATED WITH CLINICAL LESIONS ASSOCIATED WITH CIN CHANGES. PCR ASSAYS HAVE NO PROGNOSTIC VALUE OVER THE YEARS.</p> <p>LACK OF STANDARDIZATION CAUSES VARIABILITY IN CLINICAL RISK ASSESSMENT.</p>
<p>NO CHANCES OF HUMAN ERROR DURING SAMPLE PROCESSING</p>	<p>Requires DNA EXTRACTION</p> <p>REQUIRES EXTENSIVE EXPERTISE</p>
<p>SEMI AUTOMATED. SEMI QUANTITATIVE.</p>	<p>DIFFICULT HANDLING RT (REVERSE TRANSCRIPTASE) ENZYME</p>
<p>DETECTS 13 HPV HR SUBTYPES. IDENTIFIES FULL-LENGTH VIRAL DNA.</p>	<p>ONLY DETECTS 8 TO 9 HR SUBTYPES, AMPLIFY ONLY CONSERVED REGIONS (L1, L2, OR E6, E7).</p> <p>(BOSCH ET AL PAPER RE PREVELANCE OF THE TYPE IT IS MISSING 51/52/59 & 68)</p>

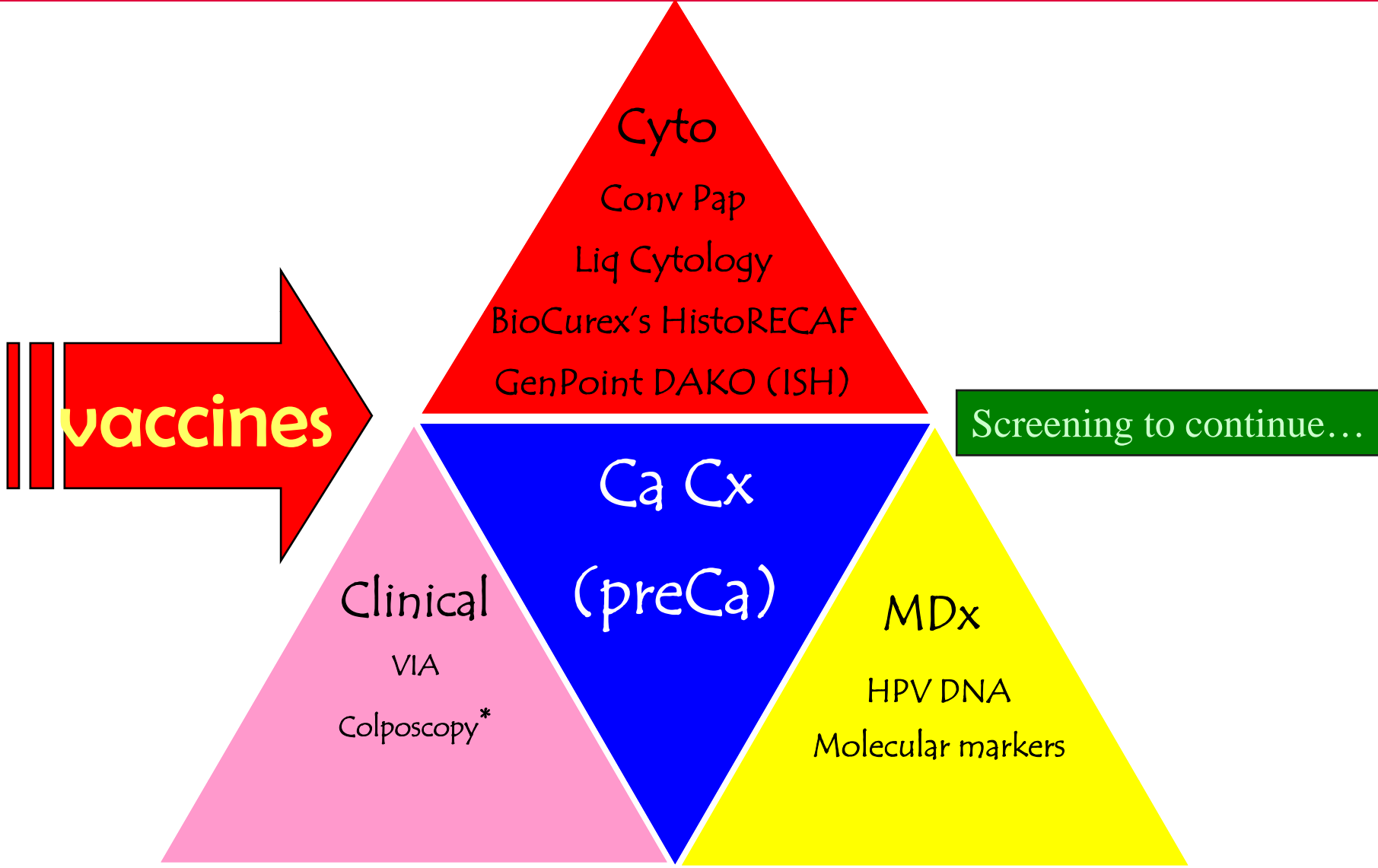
HPV Screening Studies

Location, Year	# Women	CIN 2/3 +	HPV DNA Test
London, 1999	1703	21	HC2
Newfoundland, 2000	2098	30	HC1 & HC2
Guanacaste, 2000	8554	138	HC2
Jena, 2000	4761	114	PCR
Cape Town, 2000	1365	47	HC2
Shanxi, 2001	1997	86	HC2
Reims, 2001	5671	71	HC2
Seattle, 2002	4075	137	HC2 & PCR
Morelos, 2002	7732	101	HC2
Portland, 2003	20810	171	HC2
Hannover, 2003	7592	27	HC2

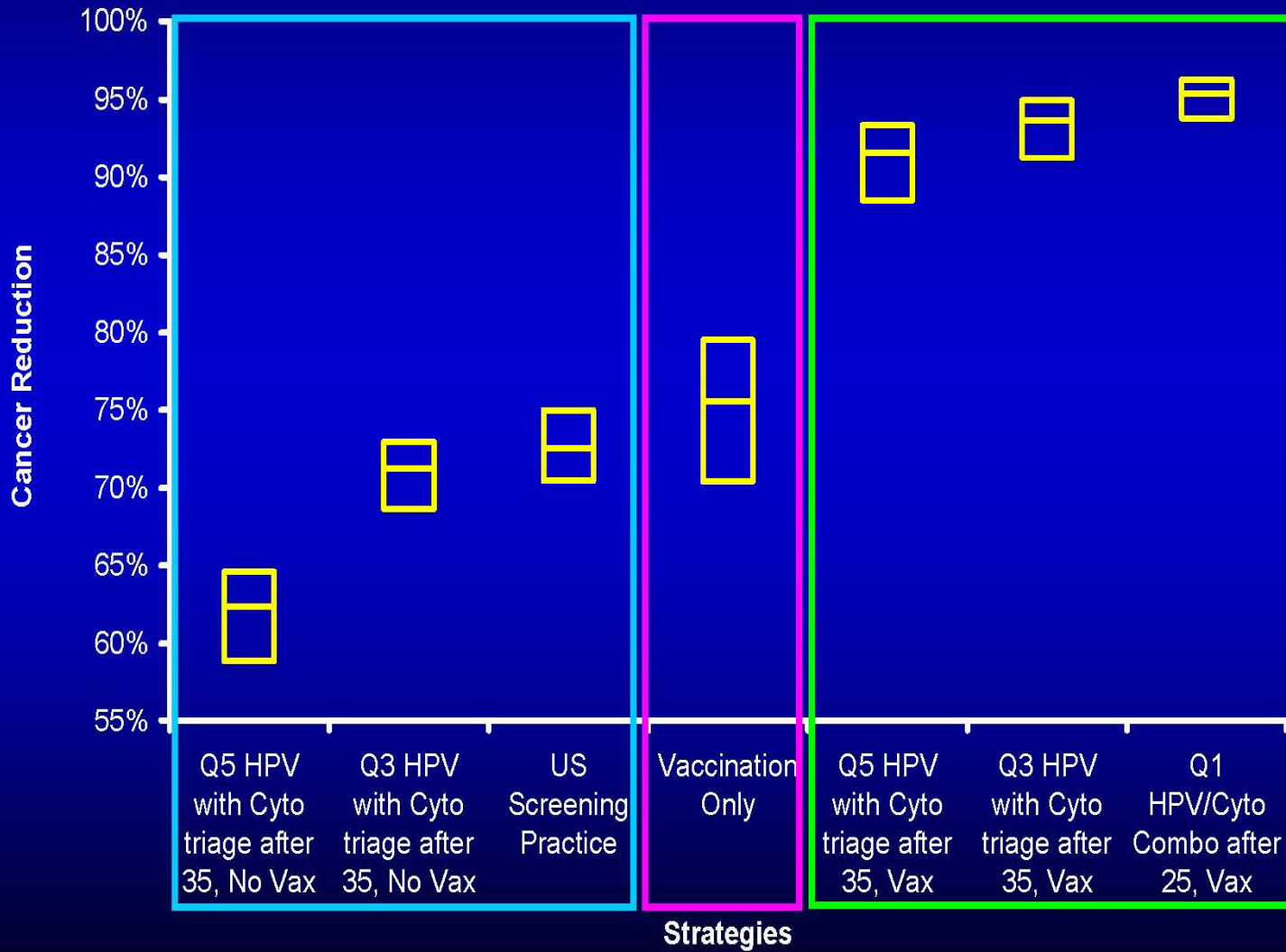
Most number with diverse cross sectional population : use HC2

Lorincz et al. 2003. Arch Pathol Lab Med 127:959

HPV & TOOLS TO CONTROL DISEASE



Results: Cancer Incidence Reduction, with/ without Vaccination



Goldie 2006



digene hpv test	PCR
<p>THE ONLY US FDA APPROVED, WELL CHARACTERIZED AND REPRODUCIBLE ASSAY</p>	<p>NOT APPROVED BY ANY AUTHORITY, FOR RESEARCH ONLY. RESULTS MAY VARY FROM LAB TO LAB, NOT REPRODUCIBLE.</p>
<p>LINEAR SIGNAL AMPLIFICATION. DOES NOT REQUIRE VIRAL DNA TO BE PURIFIED. BIOLOGICAL SPECIMEN TAKEN STRAIGHT FOR ASSAY. AMPLIFIED SIGNAL IS GENERATED BY THE BINDING OF FULL-LENGTH VIRAL DNA</p>	<p>TARGET AMPLIFICATION. REQUIRES TARGET DNA, PRIMERS AND OTHER REAGENTS TO BE HIGHLY PURIFIED, QUANTITY OF DNA ARTIFICIALLY INCREASED SEVERAL FOLD,. MOST ASSAYS USE L1 CONSENSUS PRIMERS THAT REPRESENT ONLY A MUCH SHORTENED PORTION OF THE VIRAL GENOME COMMON TO MOST ONCOGENIC HPVS.</p>
<p>ADAPTED TO IDENTIFY CLINICAL HIGH GRADE NEOPLASIA (CIN2/3 AND CANCER) THROUGH POPULATION BASED STUDIES (LANDMARK MULTICENTRIC TRIAL BY NCI/ KAISER PERMANENTE INVOLVING SEVERAL THOUSAND WOMEN).</p> <p>CLINICAL SENSITIVITY -ABOVE 98% AND WITH CONCURRENT PAP, 100%.</p> <p>NEGATIVE PREDICTIVE VALUE OF THE ASSAY ABOVE 99% AND WITH CONCURRENT PAP, 100%.</p> <p>EVIDENCE SUPPORTED BY OVER 300 POPULATION STUDIES, INCLUDING INDIA.</p>	<p>A CHANCE OF FALSE POSITIVITY/ FALSE NEGATIVITY DUE TO MUTATIONS IN THE DESIRED GENE SEQUENCE IS HIGH. AS PRIMERS ARE DESIGNED FOR SMALL TARGET (GENE SEQUENCE). STUDIES HAVE LIMITED TO IDENTIFY SEVERAL GENOTYPES OF HPV FROM RESEARCH STANDPOINT ONLY. MANY SUCH STUDIES IN THE LITERATURE DO COMPARE ITS PERFORMANCE WITH DIGENE HC2 TO IDENTIFY A CLINICAL APPLICATION.</p>

HPV

- family Papillomaviridae,
- small, non-enveloped DNA virus,
- tropism for epithelial tissue
- can induce hyperplasia, papillomatosis and verrucous lesions in the stratified squamous epithelium of skin and mucosa. (Scully et al 2002, Bouda et al 2000)
- over 100 types
- ~15 types cause cancers of anogenital, head & neck (Miller et al 2001, Mork et al 2001), oral mucosal (Shah et al, 1995; Vidal et al, 2004) or

Performances of consensus based PCR assays: variance

Clifford et al, BJC 2003

Primer Type	# of Studies	# of Cases	adjusted prevalence
MY09/11	31	4355	83.3%
GP5/6	6	506	77.8%
GP5+/6+	14	1681	90.1%
SPF10	3	275	97.2%
PUIM/2R	6	376	79.4%
LICI/C2	5	655	88.0%
Combination	9	1351	86.4%
Other	4	166	89.3%
TS-PCR only	7	693	74.7%

In conclusion ...

- ◆ Viral load determination is a PCR possibility but Hubbard states, “literature surveys indicate no clear utility for HPV viral load determination.”
- ◆ Genotyping is also a PCR possibility and Hubbard acknowledges that is only “reasonable to assume” that this information is important and “like viral load quantification... literature surveys indicate no clear utility for HPV genotyping information.”
- ◆ PCR can potentially perform multi-plex reactions. Hubbard offers this attribute, but does not discuss in detail. PCR is limited to detecting about 4 targets per reaction.
- ◆ Hubbard points out that although it is generally available for research use, use of PCR as an In Vitro Diagnostic is limited by legal and proprietary restrictions.
- ◆ Other PCR issues include: lack of standardization in homebrew assays, environmental contamination, and the need for stringent control procedures.

–Roger Hubbard, PhD. *Arch Pathol Lab Med- Vol 127, August 2003*

Intra / Inter Lab Reproducibility

HC2

“...the HC2 kit was used by 7 laboratories...the data showed high reproducibility in all 7 sites.”
Carozzi et al, Am J Clin Pathol 2005; 124(5):716-721

“Regarding HPV DNA detection HC II has shown no false positive results due to contamination and its inter-laboratory reproducibility reaches 98%.”
Bozzetti et al, Ann Epid 2000; 10(7):466

“It is important to note that HC2 demonstrated good to excellent inter-laboratory agreement...”
Castle et al, Am J Clin Path 2004; 122: 238-245

PCR

“PCR results were reproducible, as assessed by repeat analysis (96% agreement), by analysis of paired same-day specimens (89% agreement), and by inter-laboratory analysis (88% agreement).”
Kuypers et al, JCM 1993; 31(4):1003-1006

“Intra-laboratory agreement ranged from 86 to 98% for HPV DNA detection. The rate of intra-laboratory agreement excluding negative results for HPV typing ranged from 78 to 96%.”
Kornegay et al, JCM 2003; 41(3):1080-1086

“... the diagnostic reproducibility was...90.7% for generic HPV (any HPV type), and 76.9% for type-specific HPV's.”
Daniel et al, JCV 2000; 19:187-193



Clinical Validation

Author	Year	No. of Women
Lorincz	92	2,627
Cuzick	99	1,703
Bozzetti	00	977
Ratnam	00	2,098
Schiffman	00	8,554
Wright	00	1,365
Zielinski	01	278
Solomon	01	3,488
Belinson	01	1,997
Clavel	01	5,671
Pretorius	02	845

Author	Year	No. of Women
Kulasingam	02	4,075
Salmeron	03	7,732
Sherman	03	20,810
Petry	03	7,592
Kulmala	04	1,511
Castle	05	5,060 (ALTS 20k+)
Schiffman	05	10,000
Schiffman	05	3,363
Soderlund	05	239
Fetterman	05	123,909
Ronco	06	16,706

215,000+ women
Hundreds of thousands of samples

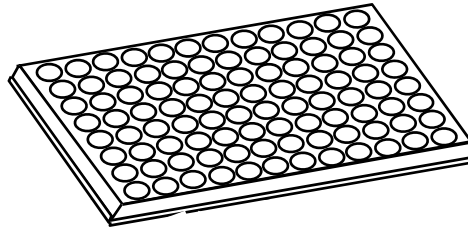
That is Clinical Validation



Cervical scrape or biopsy specimen



Detection in microtitre plate hybridization assay



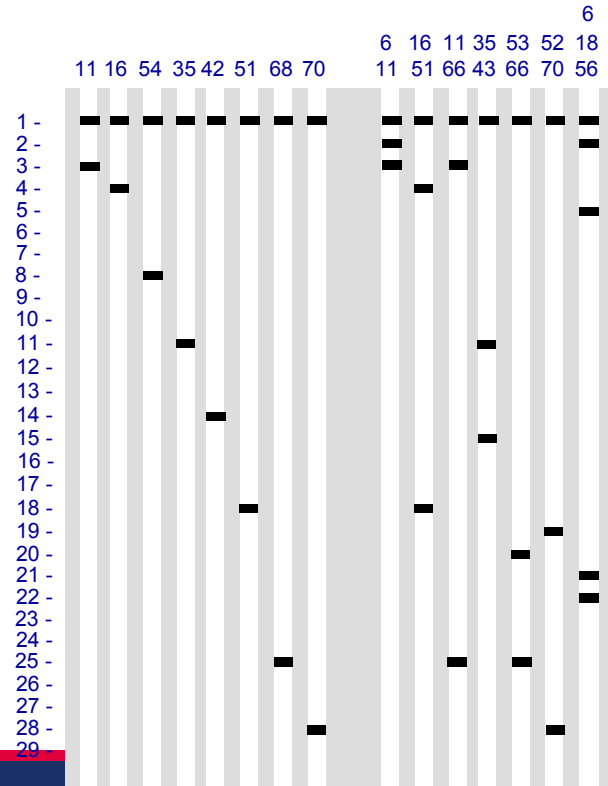
HPV-negative

HPV-positive

HPV genotypes

Probe

- 1 = Conjugate Control
- 2 = HPV 6
- 3 = HPV 11
- 4 = HPV 16
- 5 = HPV 18
- 6 = HPV 18
- 7 = HPV 31 / 40 / 58
- 8 = HPV c31
- 9 = HPV 33
- 10 = HPV 34
- 11 = HPV 35
- 12 = HPV 39
- 13 = HPV 40
- 14 = HPV 42
- 15 = HPV 43
- 16 = HPV 44
- 17 = HPV 45
- 18 = HPV 51
- 19 = HPV 52
- 20 = HPV 53
- 21 = HPV 56 / 74
- 22 = HPV c56
- 23 = HPV 58
- 24 = HPV 59
- 25 = HPV 66
- 26 = HPV 68 / 45
- 27 = HPV c68
- 28 = HPV 70
- 29 = HPV 74



SPF₁₀ PCR



DNA-EIA



LiPA

Suggested procedure

- Biotinylated PCR amplicons are captured onto streptavidin-coated microtitre plate and denatured
- Digoxigenin-labelled HPV type specific probes were hybridized to captured DNA strand under stringent conditions.
- Hybrids were detected using anti-digoxigenin HRP conjugate using tetramethylbenzene (TMB) as substrate

Primer Systems

Three important primer systems:

MY09, MY11 - L1 consensus primers, 450 bp product

GP5+, GP6+ - L1 general primers, 140 bp product

SPF₁₀ - L1 general primers, 65 bp product

Analytical sensitivity in clinical samples is approximately 100 HPV genome copies/ml for MY09, MY11 and GP5+, GP6+ and 10 copies/ml for SPF₁₀.