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CICLO XXVII

DEVELOPMENT AND VALIDATION OF *IN VITRO* DIAGNOSTIC DEVICES FOR HPV DETECTION AND GENOTYPING

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PREAMBLE

The subject of this dissertation is the work I carried out at AB ANALITICA s.r.l. (AB ANALITICA in this writing) in the context of a doctoral program based on higher apprenticeship, an innovative form of PhD aiming at reducing the distance between academia and business.

AB ANALITICA has over 20 years of experience in the development of devices for molecular diagnostics, their targets ranging from oncology relevant markers to markers for thrombophilia and coeliac disease. They also have a panel of devices for the detection of infective pathogens. In particular, their catalogue features several options for the detection of human papillomavirus (HPV), the etiological agent of cervical cancer.

At the beginning of my PhD, I worked on a project aiming at the enhancement of the performance of an already existing device for HPV detection, called REALQUALITY RI-HPV STAR. The objective of increasing sensitivity was fulfilled. However, doubts on the robustness of the improved prototype emerged and the project was abandoned. As the possibility to reach the desired level of sensitivity by modifying an already existing device had vanished, I moved to the design of two new assays for HPV detection. The validation tests on the new devices proved they performed well and, thus, will be soon commercialized under the names: REALQUALITY RQ-HPV HR MULTIPLEX and REALQUALITY RQ-HPV HR/LR MULTIPLEX.

To put into context the whole work it appears necessary to firstly introduce the biological target of these devices: in section 1, papillomaviruses are presented with a focus on their biology and the strict connection existing between persisting infections and cancer, especially that of the cervix. The characteristics of the devices I worked on and the precise requirements that had to be satisfied for their development are presented in section 2. In section 3, the experiments performed to set up the assays and assess their performances are reported. In section 4 methods employed throughout the all work are summarized. Discussion on the project outcomes and future perspectives follows in section 5.

1. INTRODUCTION

1.1. CLASSIFICATION OF PAPILLOMAVIRUSES

Papillomaviridae is a family of non enveloped viruses with circular double stranded DNA genomes. Due to these characteristics, it had originally been lumped with the polyomaviriuses in one family, the *Papovaviridae*. Once genomic data became available, it appeared clearly that the two virus groups share no major nucleotide or amino acid sequence similarities, and the International Committee on the Taxonomy of Viruses (ICTV) officially recognized them as two separate families (1).

The definition of a taxonomic hierarchy below the level of family represented a challenge as well. Usually taxonomic criteria used by the ICTV for species demarcation are based on multiple data such as, for example, natural host range, cell and tissue tropism, pathogenicity and cytopathology, mode of transmission, physiochemical and antigenic properties. The characterization of papillomaviruses (PVs) and their biological properties, though, has been hampered for a several reasons: an adequate tissue culture system (terminally differentiating epithelia) to propagate these viruses on a large scale was not available, neither was it possible to transmit them to laboratory animals. Moreover, in their natural hosts, these viruses do not elicit robust antibody responses, which impaired a classification based on "serotype" designations. Therefore, a classification of PVs based predominantly on nucleotide sequence similarities represented the only feasible alternative and served as the foundation for a nomenclature that was finally accepted by ICTV in 2003, despite representing an exception to conventional rules (2).

In the early phases of research on PV taxonomy, large amounts of viral particles were isolated from warts and lesions of *Epidermodysplasia verruciformis* patients. The existence of different HPV isolates, generally described as "types", was initially recognized by subjecting the viral DNA to restriction fragment analysis and pairwise southern blot hybridization under nonstringent conditions (3). Each laboratory started using its own nomenclature to name different types and this was

generating confusion in the field. To assure univocal designation, the participants to one of the first workshop on papillomaviruses, held at the University of South Alabama College of Medicine in 1978, proposed a uniform nomenclature for the PV types already described as well as a scheme for future designation (4). According to these criteria, the abbreviation designing a virus should have been formed by the first two letters of the English[1] name of the animal that was the natural host of the virus. For human and bovine papillomaviruses, HPV and BPV abbreviations, commonly used in literature at the time, were retained. The abbreviation had to be followed by a number providing priority to the publication date (e.g. HPV1, HPV2, etc.). A criterion to define a new PV type was defined as well: any new full length isolate showing less than 50% similarity[2] to any other known PV type. A subtype, i.e. an isolate with more than 50% similarity to another PV, would have been designated in alphabetical sequence in lower case (e.g. HPV1a, HPV1b, etc.).

Once PCR and sequencing technologies became available, the criterion for the definition of a new PV type was modified to the following: any full-length genome showing less than 90% similarity in the L1 sequence[3] to the closest related known PV type.

The need for a complete taxonomic classification, however, had not been fulfilled yet. In 1995 Chan et al. used a stretch of 291 conserved bp of the L1 ORF to generate a phylogenetic tree for PVs, which highlighted the existence of five "supergroups" (A-E) and several minor "groups" (e.g. A1, A2, etc.) (Fig.1) (6). Even though, at the time, the ICTV rejected the groupings proposed by Chan and colleagues, that classification was soon accepted and implemented by scientists in the field so that, for example, the expression "HPV16 belonging to A9" became common.

In 2003, Fauquet and colleagues (7) proposed a revision to ICTV taxonomic guidelines for species demarcation, suggesting taking into account also sequencing data. Later that year a group of scientist, comprising Fauquet himself,

[1] From 2010 the scientific name of the host species is used (e.g. *Felis domesticus* PV type 1 is FdPV1) (5).

[2] The degree of similarity should have been investigated preferentially by DNA:DNA hybridization followed by S1 nuclease digestion.

[3] L1 sequence is the most conserved sequence in HPV genome.

re-proposed a taxonomic classification of PVs based on sequence relatedness. Their phylogenetic tree, very similar to that of Chan and colleagues, was generated using the L1 sequences of 118 PVs (the 96 HPV types know at the time

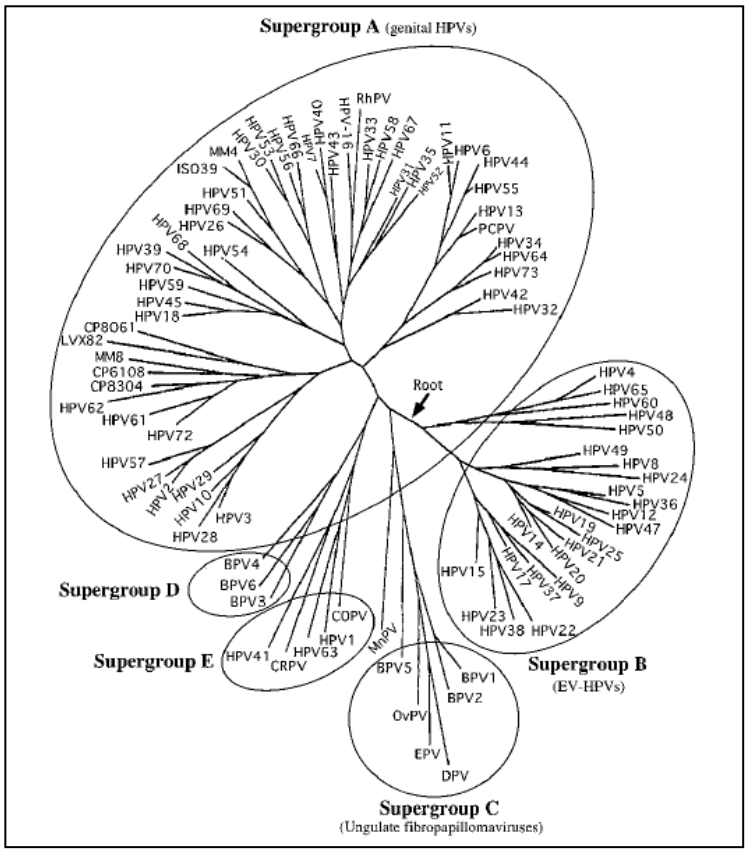


Fig. 1. Chan *et al.* phylogenetic tree of 92 PV types based on a weighted parsimony evaluation of the 291-bp L1 segment [from (6)].

plus 22 other animal PVs). In their proposal, though, the term "supergroup" was substituted by "genus" and the term "group" by "species" to align to the classification standards of ICTV. They also proposed to use Greek alphabet for genus and species (2). These amendments to the commonly used classification were favorably welcome by ICTV that finally accepted the proposed taxonomy and the deriving nomenclature. The proposal is discussed in details in de Villiers *et al.* (8). According to that taxonomy, different genera share less than 60% nucleotide sequence identity in the L1 ORF; species within a genus between 60% and 70%; types within a species between 71% and 89%; subtypes between 90% and 98%, and variants between 98 and 99%.

The existence of these levels as real natural taxa, and not only as groupings established by convention, could also be demonstrated by plotting frequencies against pairwise identity percentages obtained from the sequence comparisons of the L1 ORF of the same 118 PV types used for generating the phylogenetic tree (8). The deriving histogram clearly shows at least three major clusters corresponding to genus, species and type taxa (Fig. 2).

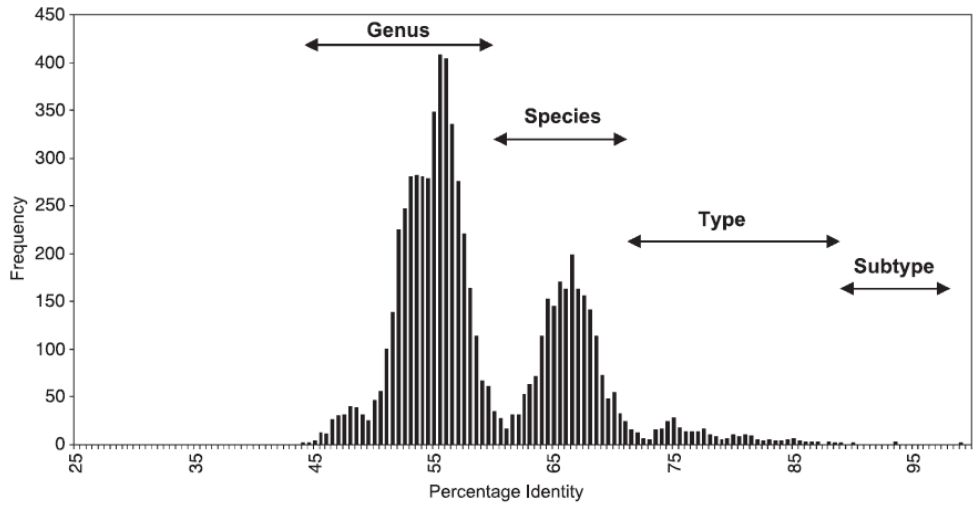


Fig. 2. Frequency distribution of pairwise identity percentages derived from the comparison of the L1 ORFs of 118 papillomavirus types [from (8)].

By now, nearly 200 different HPV types have been isoleted (2) and assigned to a species. High throughput sequencing of biological samples, though, revealed that there are many more of them. Viruses identified by metagenomic analysis are not officially recognized as new types yet, however, a debate on the subject is ongoing. In fig. 3 a phylogenetic tree generated using the sequences of 170 HPVs, single animal papillomaviruses and new HPVs identified using metagenomic sequencing (designated with the prefix “m” followed by a code) is presented. Medically relevant HPVs are comprised in the *alpha* (mucosotropic and cutanoetropic HPVs), *beta* (cutaneotropic HPVs) and *gamma* (cutaneotropic HPVs) genera (8).

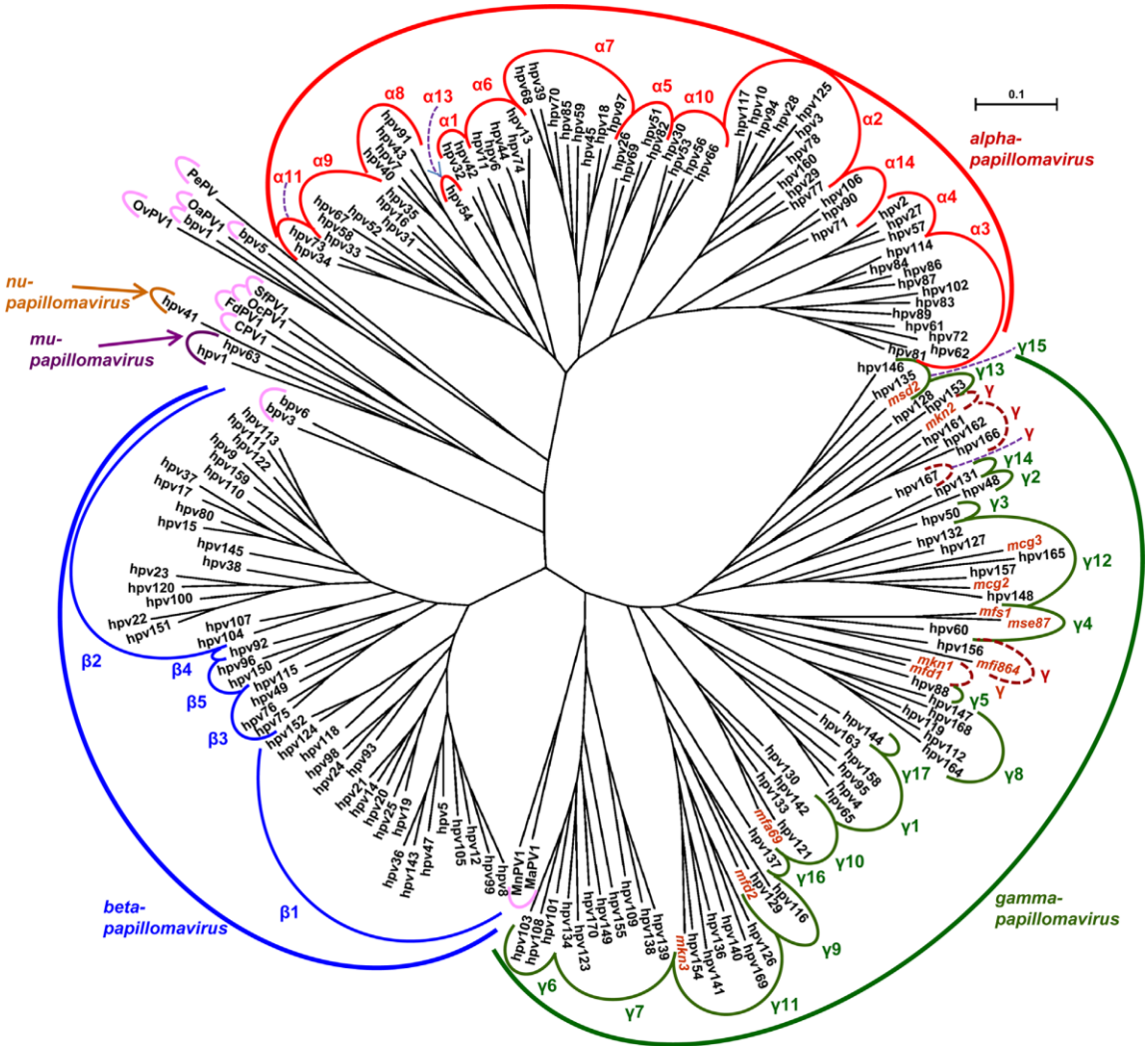


Fig.3. Phylogenetic tree based on the L1 ORF of 170 HPV types, single animal papillomaviruses and newly identified human papillomaviruses using metagenomic sequencing [from (2)]

1.2. HUMAN PAPILLOMAVIRUSES

1.2.1. STRUCTURE OF THE VIRION

Human papillomaviruses are small, non-enveloped, icosahedral DNA viruses that have a diameter of 52‑55 nm. The viral particles consist of a single double-stranded circular DNA molecule of about 8000 bp that is bound to cellular histones and contained in a protein capsid composed of 72 pentameric capsomers. The capsid is formed from two structural proteins − late 1 (L1, 55 kDa in size; 80% of total viral protein) and late 2 (L2; 70 kDa) (9).

1.2.2. HPV GENOME AND VIRAL PROTEINS

The genome of all HPV types comprises approximately eight open reading frames (ORF), which are transcribed as polycistronic messages from a single DNA strand. It can be divided into three functional parts: the early (E) region that encodes proteins (E1–E7) necessary for viral replication; the late (L) region that encodes the structural proteins (L1–L2) that are required for virion assembly; and a largely non-coding part that is referred to as the long control region (LCR) or the upstream regulatory region (UUR), which contains *cis* elements that are necessary for the replication and transcription of viral DNA (10) (Fig. 4). The early E1 protein possesses ATPase and helicase functions, binds to the origin as an hexamer and initiates viral DNA replication. E2 is the main regulator of virial gene transcription, binding viral promoters as a dimer. It is also implicated in replication as it recruits E1 to the origin. Despite its genomic location and its “E” designation, the E4 protein is expressed primarily at later stages. It interacts with the keratin cytoskeleton, via a leucine-rich motif at its N-terminus, and disrupts it, thanks to an hydrophobic sequence at its C terminus. This compromise the structural integrity of infected cells in the upper layers of warts and enable cells to rupture and release newly synthesized virus particles into the environment (9, 11). E5 is a membrane protein that localizes mainly at the endoplasmatic reticulum (ER) and the Golgi apparatus (GA). It can induce cell proliferation and inhibit

apostosis, activating grow factor receptors and other protein kinases. It also responsible for a mechanism of immune evasion as it interacts with the 16k subunit c of vacuolar ATPase, inducing alkalinization of the GA which impairs traffic of major histocompatibility complex (MHC) class I to the cell surface (12).

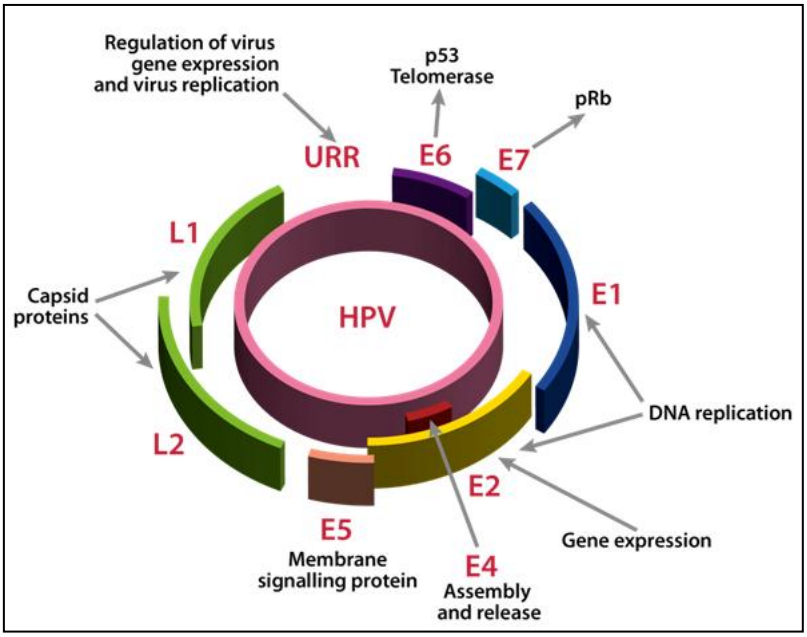


Fig. 4. Schematic presentation of the HPV genome showing the arrangement of the early genes (E), the capsid genes (L1 and L2) and the upstream regulatory region (URR) [Modified from (13)].

The best known property of the E6 protein is the ability to bind and degrade the tumour-suppressor protein p53 through the recruitment of the protein ligase, E6-associated protein (E6-AP). This results in inhibition of the transcriptional activity of p53 and the abrogation of p53-induced apoptosis. In addition, E6 binds to numerous other cellular proteins that can be divided into four broad classes: transcriptional co-activators, proteins involved in cell polarity and motility, tumour suppressors and inducers of apoptosis, and DNA replication and repair factors. E6 also induces the expression and activation of the telomerase (9). As E5 and E6, E7 induces unscheduled cell proliferation; it inactivates negative regulators of the cell cycle and tumour suppressors, primarily the retinoblastoma protein (pRb) (9). Both E6 and E7 expression is negatively regulated by E2 (11). L1 and L2 are the major and minor viral structural proteins respectively. They assemble in capsomers and capsids (9, 11).

1.2.3. LIFE CYCLE IN PRODUCTIVE INFECTION

HPVs establish productive infections only within stratified epithelia of the skin, the anogenital tract and the oral cavity. The viral life cycle is linked to the differentiation of the infected epithelial cells (Fig. 5). Virions penetrate the epithelium through microabrasions and infect epithelial stem cells that are located

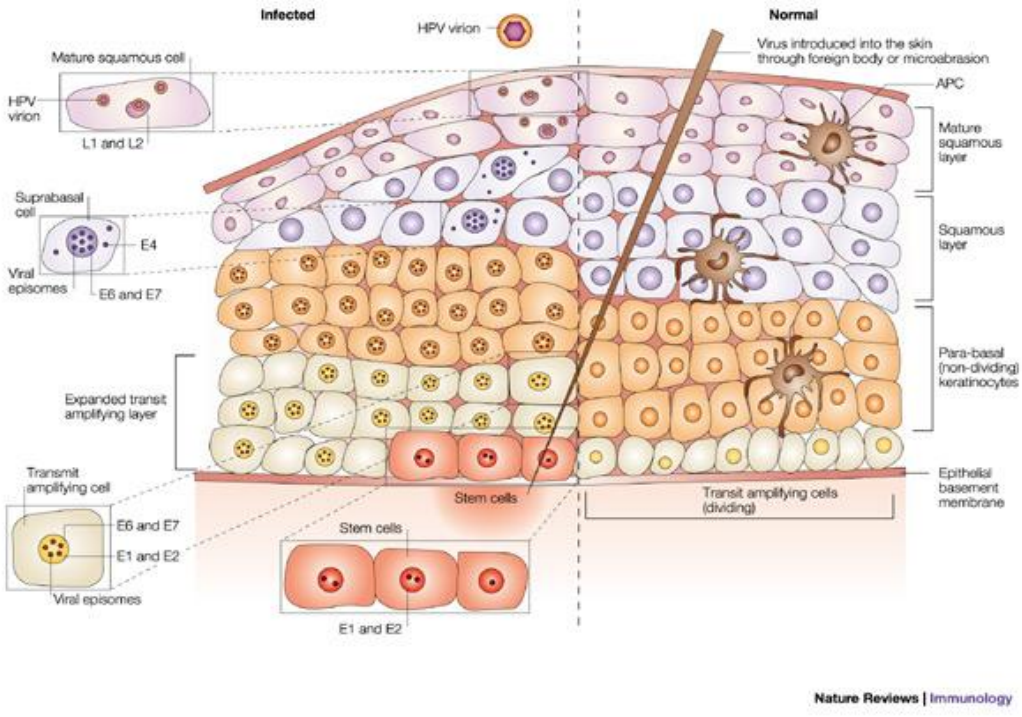


Fig. 5. Daughter cells of epithelial stem cells divide along the basement membrane and then mature vertically through the epithelium without further division (right side). After introduction of HPV into stem cells in the basal layer of the epithelium, expression of viral non-structural proteins occurs. Under the regulation of these proteins, the dividing-cell population expands vertically and epithelial cell differentiation is delayed and is less complete. Viral proteins are expressed sequentially with differentiation as shown, and mature virions are produced only in the most superficial layers of the epithelium. Intraepithelial antigen-presenting cells (APCs) are depleted in the HPV-infected epithelium [from (14)].

in the basal epithelial cell layer. In these cells and in their progeny, known as transit amplifying cells, HPVs replicate their DNA episomally, using two virus-encoded non-structural proteins, E1 and E2, and the cellular DNA-replication machinery. Expression of the viral E5, E6 and E7 non-structural proteins delays cell-cycle arrest and differentiation, which is normally observed as epithelial cells move up from the basement membrane to become mature keratinocytes. This

delay of cell-cycle arrest allows further viral episome replication using the host DNA-replication machinery in suprabasal epithelial cells, and produces the thickening of the skin (or wart) characteristic of some papillomavirus infections. When the differentiation of replicating epithelial cells to non-replicating mature keratinocytes eventually occurs, virus-encoded structural proteins, L1 and L2, are assembled in the cell nucleus to form mature virions. These are then released into the environment, taking advantage of the disintegration of epithelial cells occurring as a consequence of their natural turn-over at the superficial layers (14).

1.2.4. BENIGN MANIFESTATIONS

Human papillomaviruses can affect any area on the skin and mucous membranes. Infections with different strains are linked to a variety of skin manifestations ranging from common warts to malignancies (malignant manifestation are discussed in section 1.3 and 1.4). The current classification system for HPV, based on similarities in genomic sequences, generally correlates with the three clinical categories applied to HPV infections: 1) mucosal/anogenital, 2) cutaneous, and 3) linked to *Epidermodysplasia verruciformis*.

The main benign manifestations of mucosal/anogenital warts are cauliflower-like condylomata accuminata, which usually involve moist surfaces, and keratotic and smooth papular warts, generally found on dry surfaces. They are often asymptomatic, but occasionally may cause itching, bleeding after intercourse, urinary obstruction, burning, and pain. The majority of clinically manifest anogenital warts are caused by HPV genotypes 6 or 11.

The most commonly seen cutaneous HPV induced lesion is the common wart or verruca vulgaris, which can occur anywhere on the skin surface. It is usually caused by HPV types 1, 2, 4, 27, and 57.

Epidermodysplasia verruciformis is a rare hereditary skin disorder characterized by abnormal susceptibility to human papillomavirus. The uncontrolled HPV infection results in the growth of numerous scaly papules, particularly on hands and feet, but also on head and neck. Lesions in *Epidermodysplasia verruciformis* patients usually contain HPV types 5 or 8 (15).

1.3. HUMAN PAPILLOMAVIRUS AND CANCER

1.3.1. A BRIEF HISTORICAL ACCOUNT

In 1842 an Italian physician, Rigoni-Stern, analyzed death certificates of women in Verona during the period 1760–1839 and observed a high frequency of cervical cancer in married women, widows and sex workers, but their rare occurrence in virgins and nuns (16). He concluded that the development of this type of cancer should be related to sexual contacts. The rapid development of bacteriology in the second part of the 19th century resulted in early attempts to link cervical cancer to sexually transmitted infectious events, without reproducible data until the end of the 1960s. At this time, first reports appeared incriminating Herpes simplex type 2 as the etiological agent of cervical cancer (17). Although, initially, a number of confirmatory data have been published, a large scale prospective study performed in former Czechoslovakia failed to confirm these results (18). In the 1970s a German virologist, Harald zur Hausen, initiated a series of studies on the possible role of human papillomaviruses in cervical cancer (19, 20). Eventually, his hypothesis was confirmed, both biologically and epidemiologically (21, 22). In 2008, zur Hausen received the Nobel Prize in Physiology or Medicine for his discovery (23). Investigation on the carcinogenic potential of human papillomaviruses, however, were not relegated to the cervix. Even though with less striking results, the causal link between HPV and cancers affecting other districts was also confirmed (24, 25).

1.3.2. EPIDEMIOLOGICAL EVIDENCE FOR THE CAUSAL LINK BETWEEN HPV AND CERVICAL CANCER

In 1995 the International Agency for Research on Cancer (IARC) assembled a large series of cases of invasive cervical cancer investigated with a standard protocol. About 1000 women with histologically verified invasive cervical cancer were recruited from 22 countries around the world. Frozen biopsies from the tumors were analyzed in a central laboratory for the detection of HPV-DNA, using strict control for the presence of malignant cells in sections adjacent to the

sections used for PCR-based assays. After reanalysis of the initially HPV-negative cases, HPV-DNA was detected in 99.7% of the tumors, leading to the conclusion that HPV is a necessary cause of cervical cancer (26).

1.3.3. EPIDEMIOLOGICAL EVIDENCE FOR THE CAUSAL LINK BETWEEN HPV AND OTHER ANOGENITAL CANCERS

The number of studies on the role of HPV in other genital cancers is limited, and in most, the search for HPV-DNA has been done for a few HPV types and in fixed tissue. The available epidemiological studies indicate that cancers of the vagina and of the anus resemble cancer of the cervix with respect to the role of HPV. In both, HPV-DNA is detected in the great majority of tumors and their precursor lesions. 64-91 % of vaginal cancers and 82-100 % of VAIN-3 (VAginal Intraepithelial Neoplasia, grade 3) lesions are HPV-DNA positive. In anal cancers, HPV-DNA is detected in 88–94 %. Also cancers of the vulva and of the penis have been associated to HPV. The majority (60–90%) of tumors diagnosed in young individuals are positive for HPV and their pre-neoplastic lesions are also strongly associated with HPV. In older subjects, the tumors are rarely (less than 10%) associated with HPV (10).

1.3.4. EPIDEMIOLOGICAL EVIDENCE FOR THE CAUSAL LINK BETWEEN HPV AND NON-ANOGENITAL CANCERS

HEAD AND NECK SQUAMOUS CELL CARCINOMA (HNSCC)

A recent metanalysis of thirty-nine publications reporting on the prevalence of HPV infection in head and neck cancers in European populations produced the following results: the pooled prevalence of HPV, as determined by the presence of viral DNA, in 3,649 head and neck cancers patients was 40.0%. Estimates by type of cancer indicated that the prevalence of HPV infections was highest in tonsillar cancer (66.4%). The systematic review also identified two eligible studies reporting on cancers of Waldeyer's ring (a broader anatomical category including various tonsillar and tonsil-like tissues), the pooled prevalence of HPV for this

group was estimated to be 32.9%. By contrast, HPV prevalence estimates were lowest for pharyngeal cancer (15.3%) and tongue cancer (25.7%) (27) (Fig.6).

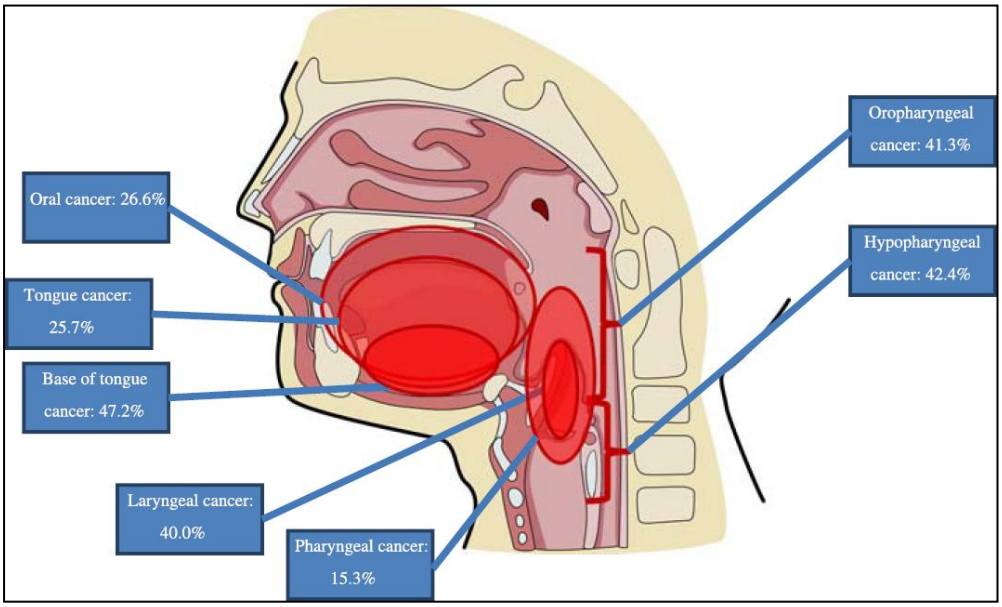


Fig. 6. Graphical presentation of the prevalence of HPV in HNSCC by cancer site [from (27)].

NON-MELANOMA SKIN CANCER (NMSC)

Apart from genital types, the HPV family includes many cutaneous types that belong to the so-called *Epidermodysplasia verruciformis* (EV)-HPV types. These types are potentially involved in the development of non-melanoma skin cancer (NMSC), which includes SCC and basal cell carcinoma. HPV-DNA has been identified in a substantial proportion (30–50%) of NMSCs in immunocompetent populations; this proportion increases up to 90% in immunosuppressed organ transplant recipients (10).

OCULAR SURFACE SQUAMOUS NEOPLASIA (OSSN)

The relationship between human papillomavirus and OSSN is rather controversial with variable results. The prevalence of HPV in OSSN ranges from 0% to 100% depending on geographical region. Most African studies report prevalence of 75–85%. On average, considering studies from different regions of the world, 33.8% of OSSN lesions are HPV positive. There may be a true geographical variation in the prevalence of HPV in OSSN (28).

1.4. CERVICAL CANCER

1.4.1. INCIDENCE AND MORTALITY

Cervical cancer is the fourth most common cancer in women worldwide (Fig. 7) and the second in less developed countries (Fig.8) (29). Screening programs, where available, have helped to reduce the associated mortality considerably.

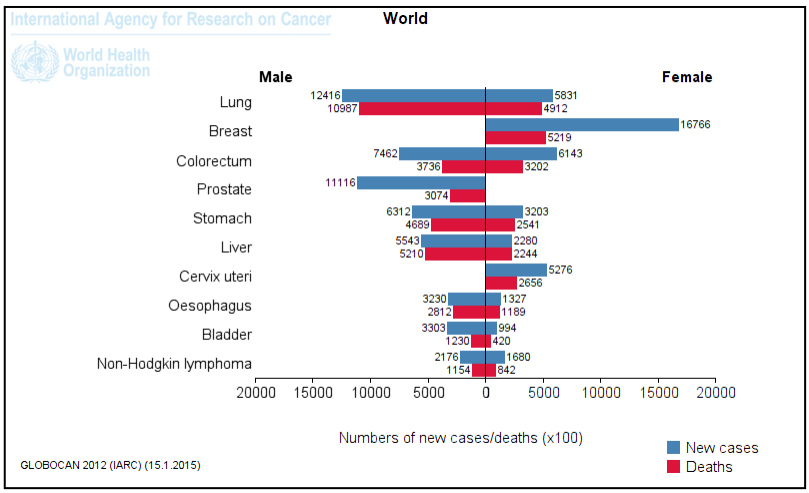


Fig. 7. Incidence (blue bars) and mortality (red bars) for the ten most common cancers in males and females in the world [from (29)].

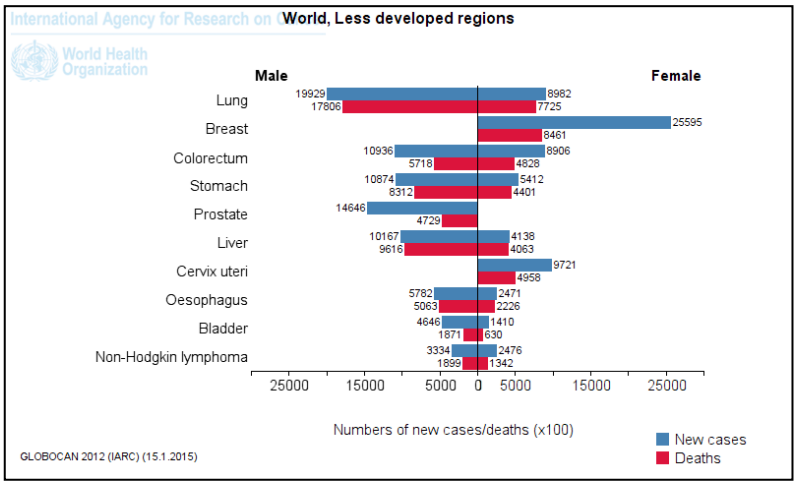


Fig. 8. Incidence (blue bars) and mortality (red bars) for the ten most common cancers in males and females in less developed countries [from (29)].

1.4.2. HIGH RISK HPV TYPES

Cervical cancer is one of the few human cancers to be entirely attributable to infection with a virus, the human papillomavirus. Not all HPVs, however, are able to induce cervical cancer. Nearly 200 different types of HPV have been identified so far; only 40 (all belonging to the *alpha* genus), though, are able to infect the mucosal epithelium of the cervix. Of these, less than twenty have been actually correlated to cervical cancer development. There is no univocal agreement on which are the HPV genotypes capable of inducing cervical cancer, generally called high risk (HR) HPV types. In Table 1, a classification of 62 HPV genotypes, based on the risk of inducing cervical cancer, according to four different sources (30-33), is provided. Twelve HPV types are unanimously recognized as high risk types by all sources: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59.

GENOTYPE	Munoz *et al.* 2003	IARC 2012	Sorbo *et al.* 2010	Cuschieri *et al.*2011
HPV 3			Low risk	
HPV 6	Low risk	Low risk	Low risk	Low risk
HPV 10			Low risk	
HPV 11	Low risk	Low risk	Low risk	Low risk
HPV 13			Low risk	
HPV 16	High risk	High risk	High risk	High risk
HPV 18	High risk	High risk	High risk	High risk
HPV 26	Probable High risk	Possible High risk	Probable High risk	Probable High risk
HPV 28			Low risk	
HPV 29			Low risk	
HPV 30		Possible High risk	Probable High risk	
HPV 31	High risk	High risk	High risk	High risk
HPV 32			Low risk	
HPV 33	High risk	High risk	High risk	High risk
HPV 34		Possible High risk	High risk	
HPV 35	High risk	High risk	Probable High risk	High risk
HPV 39	High risk	High risk	High risk	High risk
HPV 40	Low risk		Low risk	Low risk
HPV 41			Probable High risk	
HPV 42	Low risk		Low risk	Low risk
HPV 43	Low risk		Low risk	
HPV 44	Low risk		Low risk	
HPV 45	High risk	High risk	High risk	High risk
HPV 51	High risk	High risk	Probable High risk	High risk
HPV 52	High risk	High risk	Probable High risk	High risk
HPV 53	Probable High risk	Possible High risk	Probable High risk	Probable High risk
HPV 54	Low risk		Low risk	Low risk
HPV 55			Low risk	Low risk
HPV 56	High risk	High risk	High risk	High risk

GENOTYPE	Munoz *et al.* 2003	IARC 2012	Sorbo *et al.* 2010	Cuschieri *et al.*2011
HPV 58	High risk	High risk	Probable High risk	High risk
HPV 59	High risk	High risk	High risk	High risk
HPV 60			Probable High risk	
HPV 61	Low risk		Low risk	Low risk
HPV 62			Low risk	Low risk
HPV 64			High risk	Probable High risk
HPV 66	Probable High risk	Possible High risk	Probable High risk	Probable High risk
HPV 67		Possible High risk	High risk	Probable High risk
HPV 68	High risk	Probable High risk	High risk	High risk
HPV 69		Possible High risk	Probable High risk	Probable High risk
HPV 70	Low risk	Possible High risk	Probable High risk	Probable High risk
HPV 71			Low risk	Low risk
HPV 72	Low risk		Low risk	Low risk
HPV 73	High risk	Possible High risk	High risk	Probable High risk
HPV 74			Low risk	
HPV 78			Low risk	
HPV 81	Low risk		Low risk	Low risk
HPV 82	High risk	Possible High risk	High risk	Probable High risk
HPV 83			Low risk	Low risk
HPV 84			Low risk	Low risk
HPV 85		Possible High risk	High risk	Probable High risk
HPV 86			Low risk	
HPV 87			Low risk	
HPV 89	Low risk		Low risk	
HPV 90			Low risk	
HPV 91			Low risk	
HPV 92			Probable High risk	
HPV 94			Low risk	
HPV 96			Probable High risk	
HPV 97		Possible High risk	High risk	
HPV 98			Probable High risk	
HPV 99			Probable High risk	
HPV 100			Probable High risk	

Table 1. Classification of 62 HPV genotypes, based on the risk of inducing cancer, according to four different sources (Munoz *et al*. 2003 (30); IARC 2012 (31); Sorbo *et al*. 2010 (32); Cuschieri *et al.* 2011 (33)).

In 2009 the World Health Organization (WHO) released a manual for laboratories that perform HPV genotyping. In this manual, it is recommended that genotyping assays should "detect, at a minimum, the fourteen most common high risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) and the two low risk (LR) HPV types targeted by a current HPV vaccine (6 and 11)" (34). From this instructions, it is possible to infer that the WHO regards as high risk types also HPV 66 and HPV 68.

Independently from the total number of high risk genotype considered as such, the major role played by two HPV types, HPV 16 and 18, in cervical cancer development cannot be questioned. These two genotypes, together, are responsible for the 70% of all cervical cancers (Fig. 9) (35).

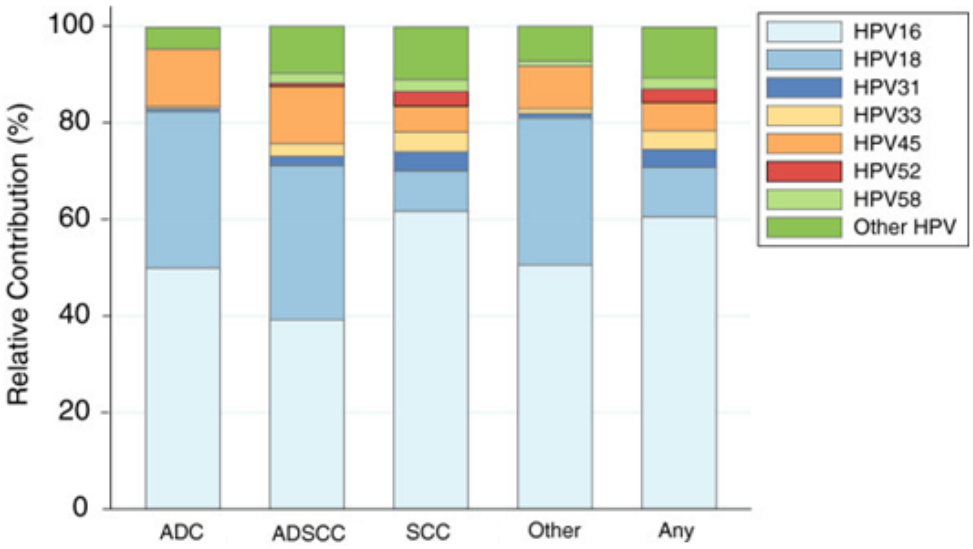


Fig. 9. Relative contribution of HPV 16/18/31/33/45/52/58 in cervical cancer. "SCC": Squamous cell carcinoma; "ADC": Adenocarcinoma; "ADSCC": Adenosquamous cell carcinoma; "Other": Includes undifferentiated, neuroendocrine, basal adenoid and cystic adenoid carcinomas [Modified from (35)].

1.4.3. PATHOGENESIS: CARCINOGENIC MECHANISM OF HIGH RISK HPV TYPES

One of the key events of HPV-induced carcinogenesis, and a prerogative of high risk types (36), is viral genome integration into a host chromosome. HPV genome integration often occurs near common fragile sites of the human genome (37), but there are no apparent hot spots for integration and no evidence for insertional mutagenesis (38). Integration follows a more specific pattern with respect to the HPV genome. Expression of the viral E6 and E7 genes is consistently maintained, whereas other portions of the viral DNA are deleted or their expression is disturbed. Loss of expression of the HPV E2 transcriptional repressor is significant, as it results in upregulated HPV E6 and E7 expression. There is also evidence for increased E6/E7 mRNA stability after integration (39). Cells that express E6/E7 from integrated HPV sequences have a selective growth advantage

over cells with episomal HPV genomes (40). The concept that loss of E2 repressor function may be critical for malignant progression is supported by experiments showing that re-expression of E2 in cervical cancer cell lines causes growth suppression. These experiments clearly demonstrate that continued E6/E7 expression in cervical cancers is necessary for the maintenance of the transformed phenotype (41).

E7 proteins interact with the retinoblastoma tumor suppressor protein pRB and others "pocket proteins", such as p107 and p130, through a conserved LXCXE sequence at its N-terminus. Pocket proteins regulate the activities of the transcription factors of E2F family that control multiple cell cycle transitions as well as other cellular activities (42). High risk HPV E7 proteins interact with pocket proteins more efficiently than E7 proteins encoded by low-risk mucosal HPVs (43). Moreover, they have the unique ability to destabilize pocket proteins through a proteasome-dependent mechanism (44). In addition, E7 has other cellular targets that are relevant to cellular transformation, for example, it can override the growth-inhibitory activities of cyclin-dependent kinase inhibitors, such as $p21^{CIP1}$ (45) and $p27^{KIP1}$ (46).

Induction of unscheduled proliferation in cells that presumably lack environmental mitogen stimulation results in conflicting growth signals. This situation, usually, triggers a cellular defense mechanism, the "trophic sentinel response", that eliminates such deviant cells from the proliferative pool through cell type-specific abortive processes, including cell death, differentiation, and senescence. This was originally discovered in transgenic mouse models in which E7 expression caused aberrant proliferation, which, ultimately, resulted in cell death. This process was found to be p53 dependent (47). E6 proteins eliminate the trophic sentinel response triggered by E7 expression, through inactivation of p53. E6 do not directly associate with p53, but form a complex with the cellular E6-AP protein. E6-AP is the founding member of the homology to E6 C terminus (HECT) family of E3 ubiquitin ligases. E6 proteins recruits E6-AP to induce ubiquitination and rapid proteasomal degradation of p53 (48). High risk HPV E6 proteins degrade p53 more effectively than E6 proteins encoded by low risk types. These proteins also have some unique structural features such as, for example, a PDZ binding motif (PBM) on the extreme C-terminus (Fig. 10). Among the

cellular PDZ domain-containing targets of E6 are Discs Large (DLG1) and Scribble, both component of the cell polarity control machinery. These two proteins are compelling candidates for a role in HPV-induced malignancy, with loss of expression or mis-localization being a common occurrence in many tumor types (49). Finally E6 induces the expression of the telomerase catalytic subunit hTERT which causes life span extension and facilitates immortalization. In Fig. 11 the main steps required for HPV induced carcinogenesis are summarized (50).

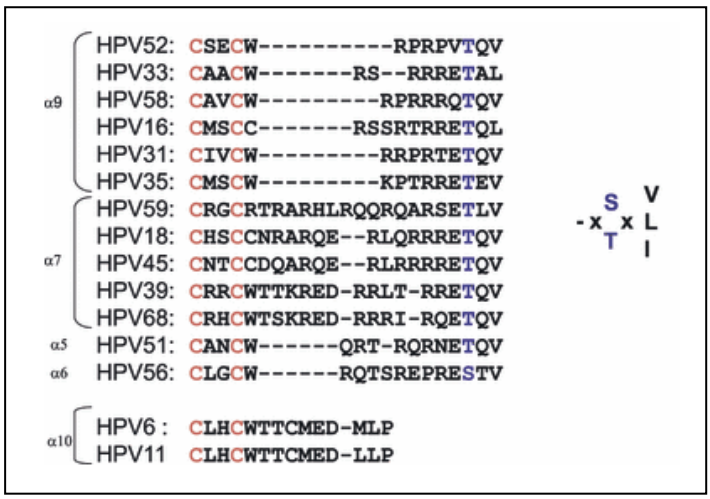


Fig. 10. Sequence alignments of the C-terminal PBM-containing region (last four amino acids) of E6 oncoproteins derived from 13 high risk HPV types. Conserved residues are in red and blue. The PBM is not present in the low risk types HPV 6 and 11. Shown on right is the consensus sequence of a class I PBM [Modified from (49)].

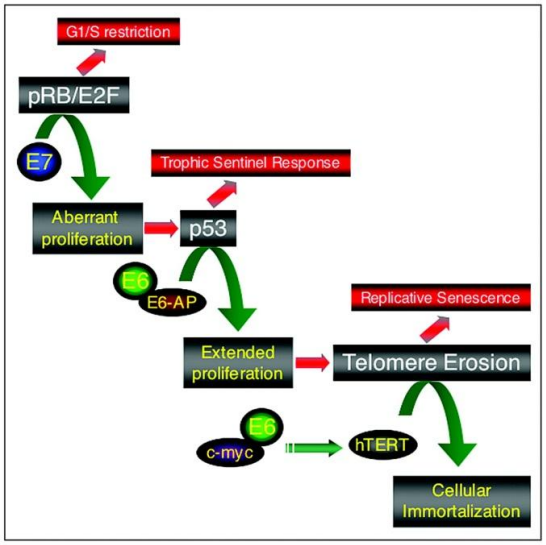


Fig. 11. Schematic outline of the critical steps of HPV-induced carcinogenesis: inactivation of pRB and p53 tumor suppressor pathways, and expression of telomerase catalytic subunit hTERT [From (50)].

1.4.4. PATHOGENESIS: PROGRESSION FROM PRECANCEROUS LESIONS TO CANCER

The cervix is the lower fibromuscular portion of the uterus. Ectocervix is the most readily visible portion of the cervix; endocervix is largely invisible and lies proximal to the external os (Fig. 12). Ectocervix is covered by a pink stratified

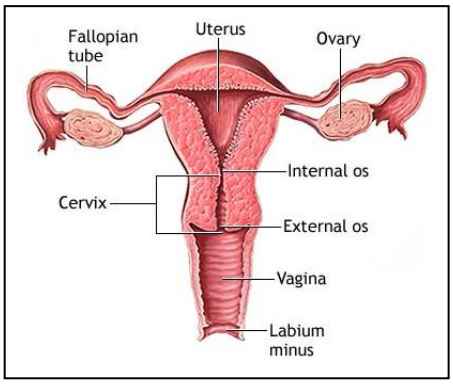


Fig. 12 Cartoon representing the female reproductive system [From (51)].

squamous epithelium, consisting of multiple layers of cells, whereas a reddish columnar epithelium consisting of a single layer of cells lines the endocervix. Upon entering puberty, due to hormonal influence, and during pregnancy, the columnar epithelium extends outwards over the ectocervix. Eventually the exposed columnar epithelium can undergo physiological metaplasia and revert to tougher metaplastic squamous epithelium. This zone of unstable epithelium is called the transformation zone and is particularly vulnerable. Generally, HPV virions penetrate the epithelium of the transformation zone through microabrasions and infect epithelial stem cells that are located in the basal epithelial cell layer. Infection with all mucosal HPV types, when persistent, can lead to the development of epithelial lesions. Only high risk HPV types, though, induce precancerous and cancerous lesions. According to the 2001 Bethesda system (52), when analyzed through cytology, epithelial lesions can be categorized, from less to more severe, as described in table 2:

BETHESDA SYSTEM	FEATURE
ASCUS (Atipical Squamous Cell of Undetermined Significance)	Atypia
ASCH (Atipical Squamous Cell cannot exclude HSIL)	Atypia
LSIL (Low grade Squamous Intraepithelial Lesion)	Mild dysplasia and dyskaryosis
HSIL (High grade Squamous Intraepithelial Lesion)	Moderate to severe dysplasia and dyskaryosis

Table 2. The Bethesda system.

Histological specimens, such as punch biopsy or excision specimen, are categorized according to the CIN (Cervical Intraepithelial Neoplasia) grading system as described in table 3 (53):

CIN GRADING SYTEM	FEATURE
CIN 1 (Cervical Intraepithelial Neoplasia, grade 1)	Good maturation with minimal nuclear abnormalities and few mitotic figures. Undifferentiated cells are confined to the deeper layers (lower third) of the epithelium. Mitotic figures are present, but not very numerous. Cytopathic changes due to HPV infection may be observed in the full thickness of the epithelium.
CIN 2 (Cervical Intraepithelial Neoplasia, grade 2)	Dysplastic cellular changes mostly restricted to the lower half or the lower two-thirds of the epithelium, with more marked nuclear abnormalities than in CIN 1. Mitotic figures may be seen throughout the lower half of the epithelium
CIN 3 (Cervical Intraepithelial Neoplasia, grade 3)	Differentiation and stratification may be totally absent or present only in the superficial quarter of the epithelium with numerous mitotic figures. Nuclear abnormalities extend throughout the thickness of the epithelium.

Table 3. The CIN grading system.

The LSIL category of the Bethesda systems corresponds to CIN 1 in the CIN grading system, whereas the HSIL category corresponds to CIN 2-3. Only HSIL/CIN 2-3 lesions are considered precancerous lesions.

During cancer progression, the pattern of viral gene expression changes. In CIN 1, the order of events is generally similar to that seen in productive lesions (see paragraph 1.2.3). In CIN 2 and CIN 3, however, the onset of late events is retarded, and, although the order of events remains the same, the production of infectious virions becomes restricted to small areas close to the epithelial surface. Integration of HPV sequences into the host cell genome accompanies these changes leading to the loss of E1 and E2 replication proteins and further upregulation of the expression of E6 and E7. In cervical cancer, the productive

stages of the virus life cycle are no longer supported and viral episomes are usually lost (Fig. 13) (54).

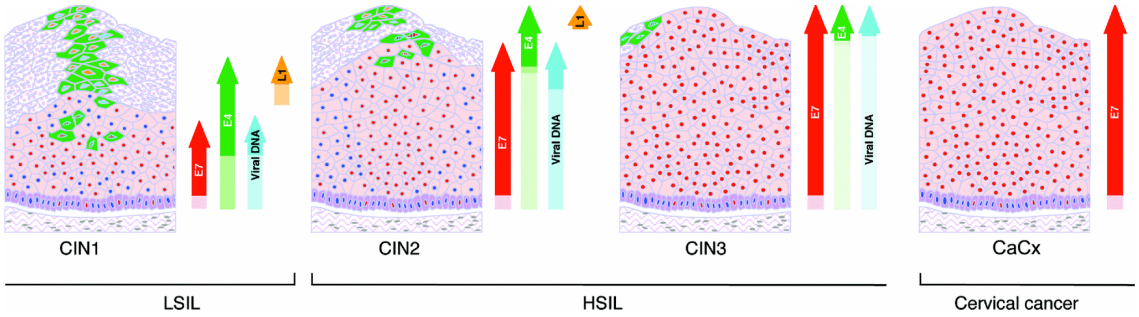


Fig. 13. Cartoon showing viral gene expression changes during progression from LSIL / CIN 1 to cervical cancer (CaCx) [From (54)].

It is important to state, though, that this process requires a long time to reach completion (~10 years) and is not unidirectional. Most low and high grade lesions regress spontaneously to normal epithelium. A comprehensive review of the literature highlighted that regression to normal epithelium occurs in nearly 60% of CIN 1 patients, whereas persistence, progression to CIN 3 and progression to invasion occur in around 30%, 10% and 1% of patients respectively. The corresponding approximations for CIN 2 are 40%, 40%, 20%, and 5%. Finally, CIN 3 regresses in 33% of cases and progresses to invasive cancer in about 12% of cases. It is evident from the above figures that the probability for an atypical epithelium to become invasive increases with the severity of the atypia, but does not occur in every case. Even the higher degrees of atypia regress in a significant proportion of cases (55).

1.4.5. CERVICAL CANCER PREVENTION: SCREENING PROGRAMS

Organized, population-based screening programs, were available, have successfully decreased cervical cancer incidence and mortality. This reduction is due to 1) an increase in the detection of invasive cancer at early stages, when the 5-year survival rate is higher than 90%; and 2) the detection and treatment of preinvasive lesions, which reduces the overall incidence of invasive cancer.

The Papanicolaou test (Pap-test) is traditionally employed for cervical cancer screening. This test is performed by opening the vaginal canal with a speculum

and collecting cells from the external os and the endocervix with a brush or a spatula. Collected cells can, then, be smeared directly on a microscope glass or be dispersed in a preservative medium for liquid based cytology (LBC). In case LBC is to be performed, the sample is sent to the laboratory where it is spun and treated to remove obscuring material, such as mucus or blood. Then, a thin layer of cells is deposited onto a slide. The slide is finally examined in the usual way under the microscope by a cytologist. The sample is categorized according to the Bethesda system (see paragraph 1.4.3). In case no anomalies are observed, the woman is referred to a normal screening schedule, which means she will be invited to repeat the Pap-test after three years. American (56) and European (57, 58) screening guidelines provide gynecologist with proper management algorithms for all the other possible cytological outcome (ASCUS, ASCH, LSIL, HSIL). Without going into details, ASCH and HSIL positive patients are always referred to colposcopy. LSIL positive patients are referred to colposcopy according to American guideline, whereas both colposcopy and repeating the Pap-test are acceptable strategies according to European guidelines. For ASCUS positive patients, three options are offered by both guidelines: 1) high risk HPV test triage, 2) Pap-test repetition or 3) direct colposcopy.

Beside its use in triage of women with equivocal cytological results, HPV testing is employed in the follow-up of women treated for CIN to predict success or failure of treatment. Furthermore, in recent years, it has been proposed as a tool for primary screening, alone or in combination with cytology (co-testing). American screening guidelines already recommend HPV testing, in the form of co-testing, for women older than 30 years as a preferable alternative to cytology alone. For co-testing, the recommended screening interval, in case of negative results, is five year instead of three. European guidelines have not defined a procedure for HPV testing implementation in primary screening settings yet. However, pilot studies in EU countries have been encouraged. Results from this studies have provided national governments with sufficient data to define their own screening strategies until a unifying European strategy is released.

In table 4, is a summary of the recommendations on the use of HPV test as a primary screening tool extracted from five different documents (59). One of these documents corresponds to the above mentioned “American guidelines”, which is a

multi-societal document compiled by members of the ACS (American Cancer Society), the ASCCP (American Society for Colposcopy and Cervical Pathology) and the ASCPS (American Society for Clinical Pathology Screening). In USA other guidelines have been released by the USPSTF (U.S. Preventive Service Task Force) and their content is reported in table 4 as well. The other three reported documents are 1) the recommendations of the Canadian Task Force on Preventive Health Care, 2) the Italian Healt Technology Assessment (HTA) report and 3) the Dutch Health Council recommendations. Only the Canadian document excludes the possibility to employ HPV testing in primary screening. USA guidelines recommend its use in the form of co-testing and European documents suggest to use HPV testing for primary screening and cervical cytology only for triage of HPV positive patients.

			Screening target population			Target age for HPV primary test				Management of abnormal results			
Country	Year	Agency	Start	Stop	HPV for primary screening recommended	Start	Stop	Screening interval for HPV negative	Cytology and HPV combination strategy	HPV+ cyto+	HPV+ cyto-	HPV- cyto+	HPV typing recommended
USA	2012	USPSTF[17]	21	65	yes	30	65	5yy	Cotesting (a)	Colposcopy	1 year HPV + cytology	?	-
USA	2012	ACS AS-CCP ASCP[16]	21	65	yes	30	65	5yy	Cotesting (a)	Colposcopy	1 year HPV + cytology	ASC-US -> 5yy; L-SIL -> ?;(c) H-SIL -> colpo	HPV 16 and/ or 18 may be referred to colposcopy even if cyto -
Canada	2013	Canadian Task Force[15]			no			-	-	-	-	-	-
Italy	2012	Ministry of Health[19]	25	64	yes	30-35	64	5yy	Triage (b)	Colposcopy	1 year HPV	-	Only in research
Netherlands	2011	Health Council[14]	30	60	yes	30	60	30-40 - >5yy >40 - >10yy	Triage (b)	Colposcopy	1 year HPV + cytology	-	-

Table 4. Summary of the recommendations on the use of HPV testing as a primary screening tool extracted from five different documents (USPSTF guidelines; ACS/ASCCP/ASCP guidelines; Canadian Task Force on Preventive Health Care recommendations; Italian HTA report; Duch Health Council recommendations). a) Both cytology and HPV test performed simultaneously as primary screening test; b) Cytology performed sequentially only in case of HPV positive results; c) Non-management for HPV negative L-SIL was reported on the multi-societal guidelines in 2012. According to the recommendation by the ASCCP published in 2013, these women would be referred to 1-year control with HPV testing and cytology [Modified from (59)].

1.4.6. CERVICAL CANCER PREVENTION: VACCINATION

Vaccination programs against HPV are being widely implemented, with young adolescent girls being the primary target group for most programs. Two prophylactic vaccines are now available: bivalent (bHPV) vaccine targets the two HPV types associated to the highest risks of developing cervical cancer (i.e. HPV 16 and 18); quadrivalent (qHPV) vaccine targets HPV 16 and 18 together with the two most common genital low risk HPV types, both responsible for condilomata (benign warts) in the genital district (i.e. HPV 6 and 11). Both vaccines continue to be immunogenic and well tolerated up to 9 years following vaccination. All randomized controlled clinical trials for the bHPV and the qHPV vaccines provide evidence of an excellent safety profile (60).

1.4.7. HPV MOLECULAR DETECTION

At the beginning of the HPV molecular detection era, only cumbersome methods, like Southern Blot and in situ hybridization, existed. Nowadays, instead, multiple different technologies, easier to perform and amenable to automation, are available. As previously discussed (see paragraph 1.4.4) HPV detection is eventually being implemented also in the context of screening settings (first level testing), alone or in combination with conventional cytology. In such a context genotyping information are not required, provided that the test is specific for high risk genotypes. Genotyping tests are generally used in second level analyses (e.g. follow-up of women with abnormal screening results who are negative at colposcopy/biopsy, follow-up of women after CIN treatment, etc.).

Ideally a screening test should be able to identify all CIN 2+ (CIN 2, CIN 3 and cancer) patients. As high risk HPV DNA can be present also in low grade lesions, the analytical sensitivity of a screening tests based on HPV DNA detection should not be excessively high, in order to avoid referral to colposcopy or further examination when not needed. The sensitivity of genotyping tests (second level analysis), instead, has to be as high as possible (for details on the sensitivity requirements for an HPV genotyping test see paragraph. 2.1).

Tests generally employed in clinical settings target HPV DNA; however, assays for the detection of HPV RNA and proteins have been developed as well.

DNA DETECTION METHODS

DNA detection methods generally rely either on signal amplification technologies or on target amplification technologies (i.e. PCR).

Among the first group of assays, the most wildly used test is the digene high risk HPV HC2 DNA Test (QIAGEN; HC2 in this writing). This is a non-radioactive signal amplification method based on the hybridization of the target HPV DNA to labeled RNA probes in solution. This test detects 13 HR-HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58,59 and 68), providing no information on the specific genotype present in the sample. It was the first device to receive FDA approval and is frequently used in primary screening.

As for PCR assays, some of them employ degenerated or consensus primers, such as MY09/11, GP 5+/6+ and SPF10, to detect most HPV types in aggregate. Subsequent restriction fragment length polimorfism (RFLP) or reverse line blot (RLB) steps can provide genotyping information. Commercial devices based on degenerated/consensus PCR and RLB are the AMPLIQUALITY HPV-TYPE EXPRESS (AB ANALITICA), the LINEAR ARRAY HPV Genotyping test (ROCHE) and the INNO-LiPA HPV Genotyping Extra (Innogenetics). These assays can be employed for second level analyses. Very similar to the above mentioned products, is one of the devices subject of this dissertation: the REALQUALITY RI-HPV STAR device (AB ANALITICA; for details see paragraph 2.3.1). High risk HPV specific devices based on Real-time PCR are available as well. They usually exploit all four channels of the Real-time equipment and provide specific genotyping information for HPV types 16 and 18, while concurrently detecting other high risk HPV types in a pooled result. Examples are the cobas HPV Test (ROCHE) and the Real Time High Risk HPV (Abbot) devices (11). These assays are quite versatile and can be used for both first level and second level assessments. The REALQUALITY RQ-HPV HR MULTIPLEX and the REALQUALITY RQ-HPV HR/LR MULTIPLEX devices, subjects of this dissertation, are based on the same technology.

RNA AND PROTEINS DETECTION METHODS

So far no guidelines have been issued for the routine application of RNA or protein detection methods, thus, at the moment, they should be used only as additional tools for clinical decisions. The most widely used commercial method for RNA detection is NucliSENS HPV easyQ. It uses isothermal NASBA to detect the E6/E7 transcripts of five high risk HPV types (HPV 16, 18, 31, 33, 45). Protein based methods, usually, do not detect viral proteins, but rather p16INK4A, a host surrogate for HPV E7 (e.g. CINtec p16 Histology from VENTANA) (11).

HPV NOVEL BIOMARKERS

Ideal biomarkers should be able to pinpoint only the low and high grade lesions that will progress to cancer beyond any doubt.

Methods for the detection of HPV genome integration are being developed. Epigenetics represents another hot area of research with groups detecting promoter CpG island hypermethylation either in L1 or E2 HPV genes (11).

2. PROJECT OVERVIEW

2.1. SHORT SUMMARY

In the first part of my PhD I worked on the new version of a product for HPV detection, recently developed at AB ANALITICA: the REALQUALITY RI-HPV STAR device. My task was to bring the original device to a higher level of analytical sensitivity for certain HPV genotypes as required by the World Health Organization (WHO). The objective of increasing sensitivity was fulfilled. However, doubts on the robustness of the improved prototype emerged and the project was abandoned. As the possibility to reach the desired level of sensitivity by modifying an already existing device had vanished, I moved to the design of two new assays for HPV detection. The validation tests on the new devices proved they performed well and, thus, will be soon commercialized, under the names: REALQUALITY RQ-HPV HR MULTIPLEX and REALQUALITY RQ-HPV HR/LR MULTIPLEX.

2.2. REQUIREMENTS FOR THE DEVICES TO BE DEVELOPED

In 2009 the World Health Organization (WHO) released a manual for laboratories that perform HPV genotyping. In this manual, it is recommended that genotyping assays should detect, at a minimum, the fourteen most common high risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) and the two LR HPV types targeted by a current HPV vaccine (6 and 11). Moreover, the WHO HPV LabNet has agreed that a laboratory that performs HPV DNA detection and typing - and, thus, also the assay used - should be considered proficient if it is able to detect 50 International Unit (IU) / 5 µl of DNA for HPV16 and HPV18, and 500 genome equivalents (GE) / 5 µl of DNA for the other HPV types (34).
For the sake of simplicity, in the following sections of this writing, the IU unit of measure will be substituted by GE. An International Unit is an arbitrary unit assigned to International Standards (ISs). ISs for HPV 16 and 18 DNA were established by the WHO ECBS (Expert Committee on Biological Standardization)

in 2008 and were assigned an IU content corresponding to their exact GE content (34). Compliance with the WHO requirements for HPV genotyping has been a pivotal aspect of my PhD work at AB ANALITICA.

2.3. RATIONALE BEHIND THE DESIGN OF THE ASSAYS

2.3.1. THE NEW VERSION OF THE REALQUALITY RI-HPV STAR DEVICE

The original REALQUALITY RI-HPV STAR device allows to ascertain the presence of HPV genome in clinical cervical samples via Real-time PCR and melting curve analysis with intercalating dye technology. It uses consensus primers to amplify in aggregate most HPV types. For each samples it requires to perform two separate reactions, one for the pathogen target and the other for the internal control gene which is the *HBB* (hemoglobin, beta) gene. The device works on the StepOnePlus Real-Time PCR System (Applied Biosystems; "ABI StepOnePlus" in this writing). In Fig. 14, is a melting curve output obtained by amplifying with the HPV reaction mix three samples, i.e. an HPV negative sample, an HPV positive samples, and the HPV positive control (PC) provided with the device. Both the positive sample and the PC generate an HPV specific melting peak around 78°C. The negative sample also presents a peak, around 68°C, which corresponds to primer dimers, a PCR byproduct. In order to allow the

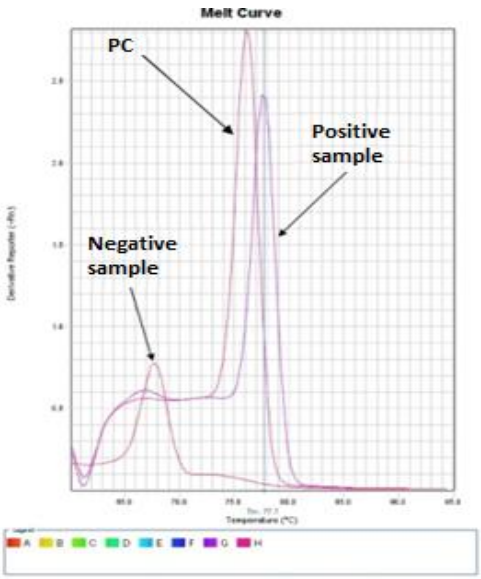


Fig. 14. Melting curve output for two samples, one negative and one positive, and the HPV positive control amplified with the HPV mix (ABI StepOnePlus, software v.2.2.2.

final user to distinguish between primer dimers and HPV specific peaks a cut-off temperature is provided.

When developed in 2010, the REALQUALITY RI-HPV STAR product was meant as a screening device[4], also allowing the possibility to directly genotype positive samples when associated to another product from AB ANALITICA: the AMPLIQUALITY HPV-TYPE device (for details see paragraph 4.1.11). The possibility to perform screening and genotyping all in once could have represented a strong marketing feature, as, the only screening device with FDA approval, at the time, the HC2 test (for details see paragraph 1.4.5) could not provide any genotyping information. However, penetrating the screening related segment of the market resulted more complicated than expected. Instead, the product was warmly welcomed by laboratories performing second level analysis, especially those dealing with a high number of samples, as the possibility to perform genotyping only on the subset of samples actually positive for HPV had the advantage to speed up the process considerably.

The primers used in the PCR reaction of the original REALQUALITY RI-HPV STAR are the GP5+/GP6+ primers. As mentioned above, these are consensus primers, targeting the L1 region of HPV genome, which allow the detection of most HPV types in aggregate. The sensitivity they provide, though, is not equal for all types (62). A study conducted at AB ANALITICA, comparing the REALQUALITY RI-HPV STAR assay with the HC2 assay, reveled that the first has lower sensitivity for the following high risk HPV types: HPV 31, HPV52 and HPV 58. It also struggles with the detection of the possible high risk type HPV 53. This is due to some mismatches the GP5+/GP6+ primers present when aligned to these genotypes (Fig. 15). Being the device mostly used as a second level test, it was considered appropriate to try to enhance its analytical sensitivity and the WHO requirements were taken as a reference. The new version of the device had to be based on the same technology and work on the same Real-time instrument. To achieve higher analytical sensitivity, the set up of the assay had to be modified

[4] The device is not high risk HPV specific, as required for screening tests; however, direct genotyping of positive samples assures discrimination between high and low risk HPV type positive samples. Also, being based on a PCR reaction with GP5+/GP6+ primers, it should have been considered adequate as a first level test according to the landmark paper written by Meijer *et al.*(61).

considerably. The occasion was used to also improve an ergonomic aspect and amplify the target pathogen and the internal control gene in a single reaction, so as to halve the amount of work for the final user.

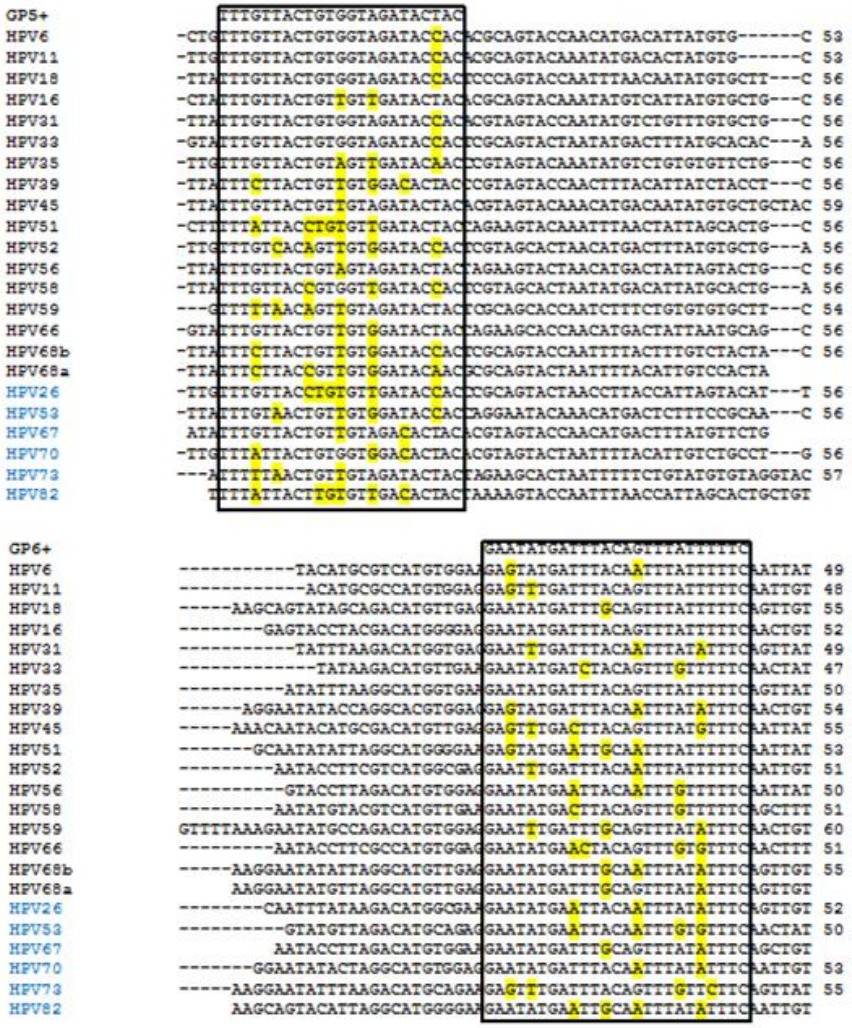


Fig. 15. Sequences of 23 HPV genotypes aligned against the GP5+/GP6+ primers. Mismatches are highlighted in yellow.

2.3.2. THE REALQUALITY RQ-HPV HR MULTIPLEX AND THE REALQUALITY RQ-HPV HR/LR MULTIPLEX DEVICES

As the possibility to reach the required level of sensitivity by modifying an already existing device had vanished, the design of a completely new product for HPV detection became necessary. In that occasion, it was decided to abandon the RLB technology, which can result cumbersome and time consuming for the final user, and rely only on Real-time PCR, which, on the contrary, is a very popular technology in the field of diagnostics, as it is simple and does not require too much hand-on time for the final user. This decision, obviously, influenced the design of the new assay radically, as, for example, it was no more possible to detect and genotype a very high number of different HPVs. Keeping that in mind, it was decided to primarily focus on those HPV types that should be detected by a genotyping assay in compliance with WHO requirements (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68). A possible strategy to detect and genotype all of them would have been that of designing a multiplex reaction with specific primers and rely on melting temperatures to discriminate among different types. This time, though, melting curve analysis was not considered as an option due to the problems experienced with the previous project. Instead, Real-time PCR with target specific primers and TaqMan fluorescent probes was employed.

Another significant difference with respect to the previous project is that the target region of the new devices is E6/E7 instead of L1. This choice was due to both commercial and practical reasons. A key event in HPV-induced carcinogenesis is the integration of HPV genome in one of the host chromosomes, which can, in some rare cases, lead to the loss of L1, but not of E6/E7. Since residual integrated HPV DNA can, ultimately, be the only form of viral DNA present in infected cells, devices based on L1 detection might miss a small, but yet significant, portion of infections, whereas E6/E7 targeting devices will not (63). Targeting E6/E7, thus, represents a strong marketing feature, especially considering that devices from most competitors target L1 (11). On the practical side, being L1 sequence the most conserved in HPV genome, the design of type specific primers and probes in that region would, probably, have been much more difficult. Finally, two separate assays, both based on a four fluorophore reaction, were

designed. The correspondence between targets and fluorophores for both the assays is summarized in table 5 and graphically represented in Fig. 16. The first assay (assay 1 in this writing) was designed to detect all the high risk types from the WHO list, while also providing partial genotyping information, relative to HPV 16 and 18 positivity. Being both HPV 16 and 18 universally recognized as the most dangerous types, such a solution appeared to be the most convenient. The *HBB* gene was chosen as internal control gene. The second assay (assay 2 in this writing) was design to provide specific genotyping information for the two low risk types from the WHO list, HPV 6 and 11, while concurrently detecting other possible high risk HPV types (according to IARC classification (31)) in a pooled result. The possible high risk genotypes are not in the WHO list; However, they were added in order to offer a complete panel for HPV investigation. Again the *HBB* gene was chosen as the internal control gene.

Assay	JOE	CY5	FAM	ROX
1	**HPV 16** (higher risk)	**HPV 18** (higher risk)	**HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68** (high risk)	*HBB* (internal control)
2	**HPV 6** (low risk)	**HPV 11** (low risk)	HPV **26, 53, 67, 70, 73, 82** (probabile high risk)	*HBB* (internal control)

Table 5. The correspondence between target genotypes and fluorophores for the newly designed assay is reported.

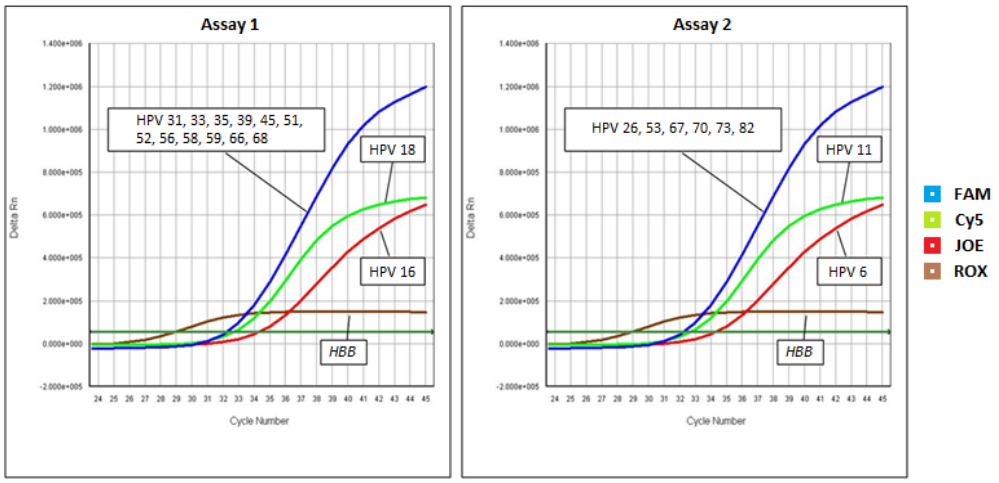


Fig. 16. Graphic representation of the correspondence between targets and fluorophores for the first and the second assay.

Assay 1 was meant as an independent product and will be soon commercialized under the name REALQUALITY RQ-HPV HR MULTIPLEX. Assay 2, if associated to assay 1, provides the final user with a complete panel for HPV investigation. For this reason, assay 1 and assay 2, togheter, will be also proposed as an alternative product, under the name REALQUALITY RQ-HPV HR/LR MULTIPLEX. The assays were meant to work on both the 7500 Fast Dx Real-Time PCR System (Applied Biosystems; "ABI 7500 Fast Dx" in this writing) and the Dx Real-Time System (BIO-RAD; "Biorad Dx" in this writing).

3. RESULTS

3.1. SECTION OVERVIEW

This section is divided in two parts: in the first part, I describe the efforts made to enhance the performance of a product already available from AB ANALITICA's catalogue, the REALQUALITY RI-HPV STAR device; in the second part, the work for the development of two new assays for HPV detection is presented. The first of the two assays (assay 1) was meant as an independent product and will be soon commercialized under the name REALQUALITY RQ-HPV HR MULTIPLEX. The second assay (assay 2) will be proposed, only in combination with the first assay, as an alternative product, under the name REALQUALITY RQ-HPV HR/LR MULTIPLEX.
The rationale behind the design of all assays and their characteristics have been summarized in paragraph. 2.3.

3.2. PART I: DEVELOPMENT OF THE NEW VERSION OF THE REALQUALITY RI-HPV STAR DEVICE

3.2.1. SETTING THE REACTION UP

3.2.1.1 Selecting primers for HPV detection

Primers used in the PCR reaction of the original REALQUALITI RI-HPV STAR device are called GP5+ and GP6+. They are consensus primer targeting L1 region in HPV genome and allowing the detection of most HPV types in aggregate. The sensitivity they provide, though, is not equal for all the genotypes detected. A study conducted at AB ANALITICA, comparing the REALQUALITY RI-HPV STAR assay with a gold standard assay for HPV detection (HC2) reveled it has lower sensitivity for the following high risk HPV types: HPV 31, HPV 52 and HPV 58. It also struggles to detect the possible high risk type HPV 53. To

enhance the assay sensitivity for these and other genotypes, new primers had to be added to the original pair. Only primers producing amplicons compatible with the AMPLIQUALITY HPV-TYPE genotyping system, however, could be selected. This meant that mainly degenerated versions of the original primer pair were considered. A study of the literature helped to identify two candidate primer sets (A and B) (64, 65). To ascertain which one worked better, end-point PCR was performed followed by both gel electrophoresis and reverse line blot with the AMPLIQUALITY HPV-TYPE device. Similarly good results on the critical genotypes were obtained on RLB strip (Fig. 17). On agarose gel, though, the superiority of set B emerged clearly as only HPV specific bands (~ 150 bp) were visible. Instead, primer set A produced multiple bands from both HPV positives and negative samples (Fig. 17). Initially, set A PCR was performed using the thermal profile reported in the reference paper. This paper suggested to use an annealing temperature (T_A) of 42°C for the first 5 cycles and then of 64°C for other 45 cycles. Higher T_As for the first cycles were tested, however, aspecific bands could not be eliminated (Fig. 18). The presence of aspecific products would have definitely represented a problem for melting curve analysis, thus, only primer set B could be used for the following tests in Real-time PCR.

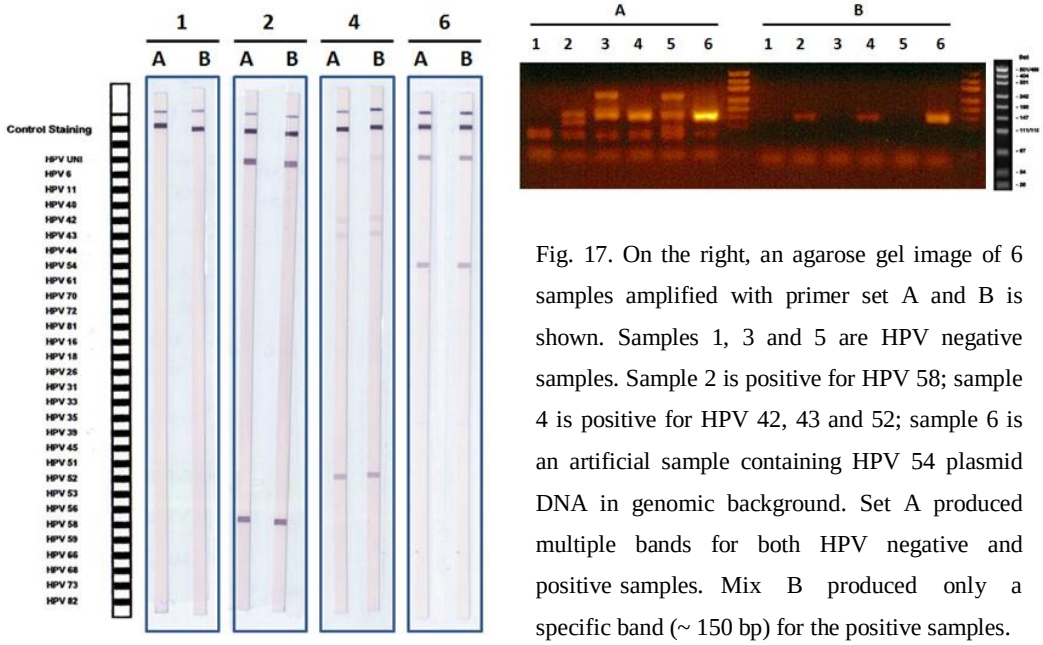


Fig. 17. On the right, an agarose gel image of 6 samples amplified with primer set A and B is shown. Samples 1, 3 and 5 are HPV negative samples. Sample 2 is positive for HPV 58; sample 4 is positive for HPV 42, 43 and 52; sample 6 is an artificial sample containing HPV 54 plasmid DNA in genomic background. Set A produced multiple bands for both HPV negative and positive samples. Mix B produced only a specific band (~ 150 bp) for the positive samples. On the left, the RLB strips for samples 1, 2, 4 and 6 are shown. Both sets A and B produced the anticipated result.

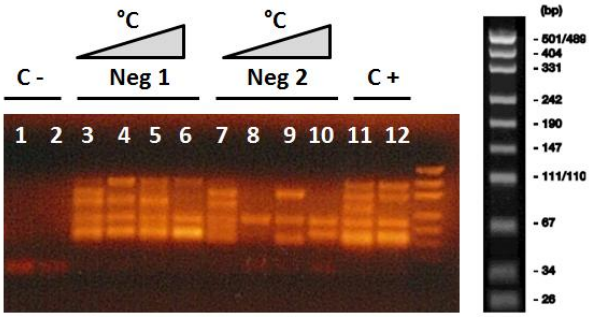


Fig. 18. An agarose gel image of two HPV negative sample, Neg 1 (wells 3, 4, 5, 6) and Neg 2 (wells 7, 8, 9, 10), amplified with primer set A in gradient PCR (T_A = 42-43-44-45 °C) is shown. No-temple-controls (C-) were loaded in well 1 (T_A = 42°C) and 2 (T_A = 45°C). Positive controls (C+; Plasmid DNA carrying the L1 region of HPV 54 diluted in genomic background) were loaded in well 11 (T_A = 42°C) and 12 (T_A = 45°C). At all T_As aspecific bands are visible in both the HPV negative samples and the positive controls.

3.2.1.2. Selecting the internal control gene

When analyzing a sample for a target that is not necessary present, it is advisable to simultaneously amplify an internal control (IC), i.e. a sequence we are sure to be present in our sample. Usually housekeeping genes or corresponding mRNAs are selected for the scope. The IC allows verifying whether the sample is suitable for amplification, has been extracted properly and does not contain inhibitory substances. With the previous setting, the IC and the pathogen target were amplified in two separate reactions. In the new version of the device the IC and the target pathogen had to be amplified in multiplex so as to halve the amount of work for the final user. However, this choice required the melting temperature of the IC to be sufficiently far from the range of melting temperatures covered by different HPVs[5] in order to avoid confusion during result interpretation.

In the original assay, the IC gene was the *HBB* (hemoglobin, beta) gene. Other devices in AB ANALITICA's catalogue, though, use the *TST* (thiosulfate sulfurtransferase) gene as IC. These two genes were both considered as candidate ICs and tested to verify which one could have served the purpose better. In Fig. 19 the melting curves obtained from a sample amplified with two multiplex mixes

[5] Melting temperature of different HPV types amplified with degenerated primers are slightly different from one another as slightly different is the nucleotide composition of the corresponding amplicons, thus, they cover a range of temperatures.

are shown. One mix contained primers for HPV and *HBB* (blue curve), whereas the other contained primers for HPV and *TST* (red curve). For both the conditions three peaks are visible on the graph: the first two peaks correspond to primer dimers and HPV respectively and overlaps almost perfectly. The third peak corresponds to the internal control gene. *HBB* peaks is detected around 82°C (blue curve), whereas *TST* peak appears around 86°C (red curve). Being the efficiency of HPV amplification equal[6] for both conditions, the only criterion took into account for choosing the IC gene was that of the distance from the HPV peak. Under this criterion, the *TST* gene represented a better choice. Moreover, another advantage was that the RLB strip of the AMPLIQUALITY HPV-TYPE device already had a *TST* specific band, whereas no *HBB* specific bands were present.

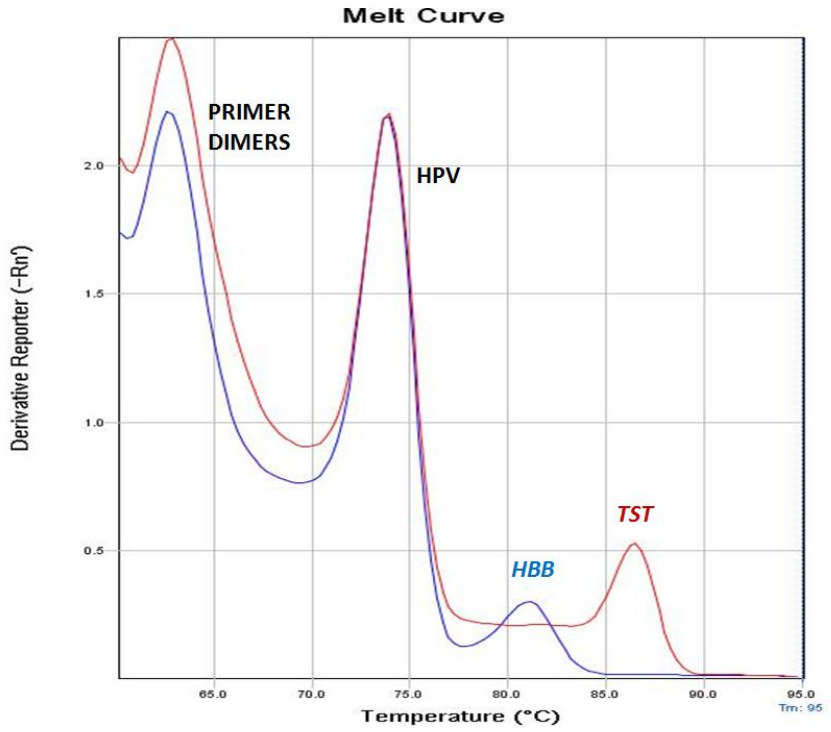


Fig. 19. Melting plot obtained from a sample amplified with two multiplex mixes. One mix contained primers for HPV and *HBB* (blue curve), whereas the other contained primers for HPV and *TST* (red curve). The first two peaks correspond to primer dimers and HPV respectively; the third peak corresponds to the IC (ABI StepOnePlus, Software v. 2.2.2).

[6] Inferred from HPV peak heights.

3.2.1.3. Selecting the intercalating dye

The old mastermix was not adequate to sustain a PCR reaction with multiple primers and was substituted with one specific for multiplex reactions. The new mastermix, however, did not contain an intercalating dye which had to be added in order to allow melting curve analysis. Two different intercalating dyes (dye 1 and 2), were tested in order to understand which one worked better. When tested the same samples, dye 1 gave stronger signals than dye 2 (Fig. 20) and was selected.

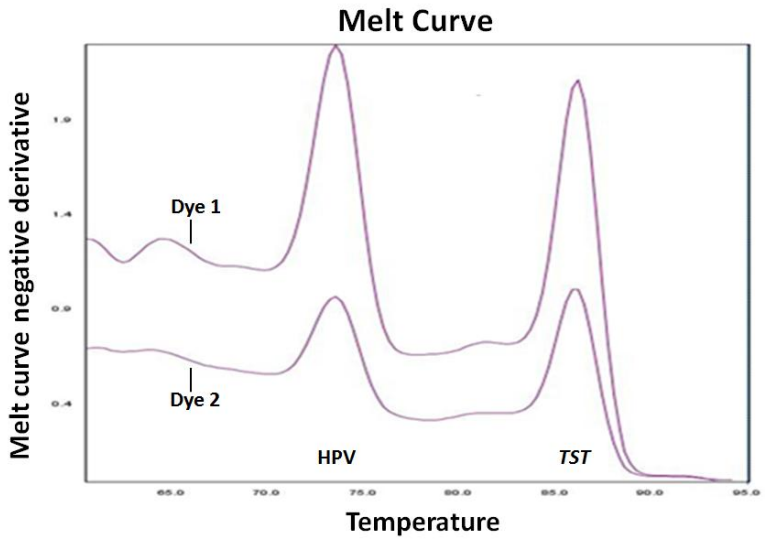


Fig. 20. Melting Curves obtained amplifying the same artificial sample with two different intercalating dyes. The sample contained a plasmid carrying the L1 sequence of HPV 16 (5 genome equivalent (GE) / µl) diluted in human genomic backgroud to mime a clinical sample. The signal from dye 1 is stronger than that from dye 2 (ABI 7500 Fast Dx, Software v. 1.4).

3.2.1.4. Setting the conditions for the multiplex reaction

The mastermix and the intercalating dye were used according to manufacturer instructions. The only thing that had to be set was primer concentration. Without going into the details, it is worth saying that, being the pathogen target (HPV) and the internal control (*TST*) amplified in multiplex, it was necessary to make sure that the two targets were not competing in a way that would have favored IC

amplification against pathogen target. This was assured by using primers for the IC at a concentration lower than that used for the pathogen target.

3.2.1.5. Defining a range for HPV positive call after Real-time PCR

The new assay was meant to work on the ABI StepOnePlus instrument just as the original REALQUALITY RI-HPV STAR device. On that instrument, a typical melting curve output for a sample positive for a single HPV type was a plot showing three melting peaks, the first peak corresponding to primer dimers, the second to HPV and the third to *TST*.

Generally the primer dimer peak was around 65°C, the *TST* peak around 86°C and the HPV specific peaks somewhere in the middle. To make sure that the final users would have been able to distinguish HPV positive from negative samples, it was necessary to define a precise range for HPV positivity. In order to understand which genotypes generated melting peaks at the lowest and highest temperatures, 50 clinical samples positive for different HPV types, in single and multiple infections, were tested. The analyzed genotypes were the following: HPV 6, 11, 16, 18, 31, 33, 35, 39, 42, 43, 45, 51, 52, 56, 58, 59, 66, 68, 73, 81, 82. The lowest

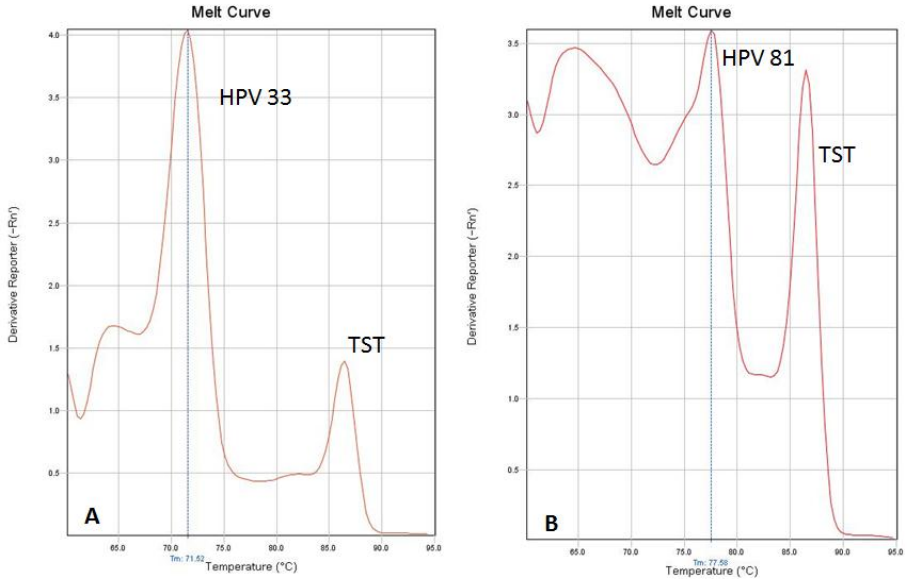


Fig. 21. A. The melting curve for a clinical sample positive for HPV 33 is shown (melting temperature = 71.52°C). B. The melting curve for a clinical sample positive for HPV 81 is shown (melting temperature = 77.58°C) (ABI StepOnePlus, software v. 2.2.2).

melting temperatures were generated by HPV 33 positive samples (range 71.52 – 71.67 °C; Fig. 21 A), whereas the highest corresponded to HPV 81 positive samples (range 77.06 – 77.58 °C; Fig. 21 B). The range for the positive call was, therefore, provisionally set between 71°C and 80°C, i.e. ~ 0.5 °C below the lowest recorded melting temperature and ~ 2.5°C above the highest.

3.2.2. PRELIMINARY DATA ON PERFORMANCE

3.2.2.1. Dye 1 stability after freezing and thawing

Generally, for all the reagents that need to be stored at – 20°C, AB ANALITICA guarantees that they remain stable for up to three freeze and thaw cycles. The mastermix chosen for the new assay had already been used in other kits and had resulted stable, whereas the chosen intercalating dye had never been tested before. For this reason, before proceeding, it was necessary to make sure that the dye, diluted in the mastermix, would have tolerated repeated cycles of freezing and thawing with no significant changes in its performance. To check that, I produced a stock solution of dye 1 added mastermix. I also produce two artificial samples by diluting plasmids carrying the L1 sequence of HPV 16 and 18 in genomic DNA (conc. 50 genome equivalents (GE) / µl) to mime clinical samples. The mix containing the dye was tested on the artificial samples on the day of its production to set a reference and then stored at -20 °C. After a week it was thawed and tested again. The procedure was repeated for the two following weeks. The artificial samples were aliquoted on the day of their production and stored at -20°C until use, so that a fresh aliquot would have been available for each of the tests. In this way, any change in the test results would have been due to degradation of dye 1 and not due to degradation of plasmids. No relevant changes in performance were observed as the height of the melting peaks for HPV and *TST* remained constant from the first to the last test (Fig. 22). Dye 1 was, therefore, considered adequate for setting up the reaction for the new assay.

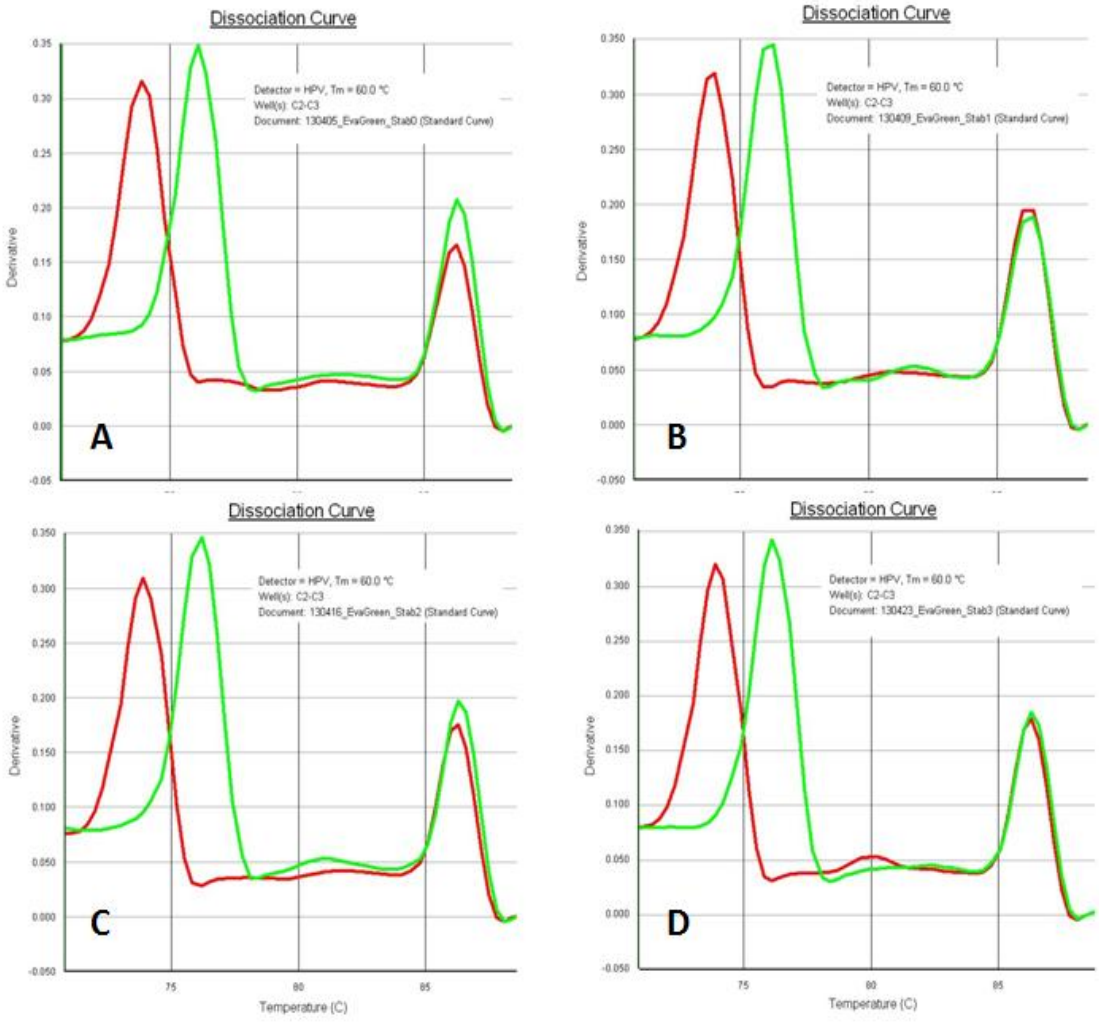
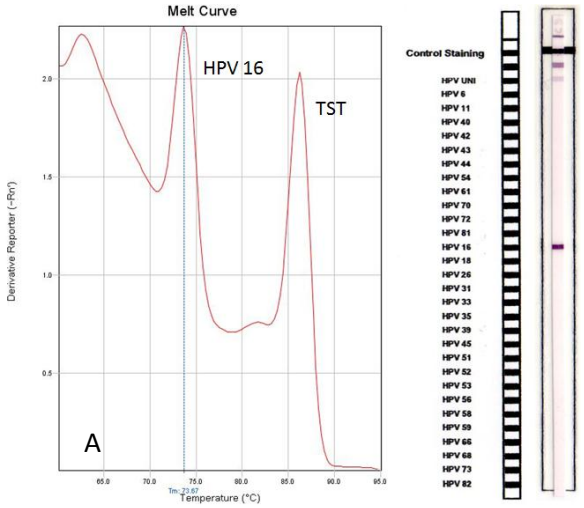


Fig. 22. The result of a test on the stability of dye 1 after 3 freeze and thaw cycles is presented. Red curves were obtained from the amplification of a sample containing HPV 16 plasmids in genomic background (conc. 50 GE/µl), green curves were obtained from the amplification of a sample containing HPV 18 plasmids in genomic background (conc. 50 GE/µl). The result of the run performed at time 0 (day of production of the reaction mix containig dye 1) is reported in A. The result of the run performed at time 1 (one week after the reaction mix was produced; one freeze and thaw cycle) is reported in B; The result of the run performed at time 2 (two weeks after the reaction mix was produced; two freeze and thaw cycles) is reported in C; The result of the run performed at time 3 (three weeks after the reaction mix was produced; three freeze and thaw cycles) is reported in D. No changes in the performance of the dye 1 containing mix were observed (ABI 7500 Fast Dx, software v. 1.4).

3.2.2.2. Preliminary tests on the analytical sensitivity of the newly designed Real-time PCR reaction in combination with the AMPLIQUALITY HPV-TYPE assay

The WHO recommends that genotyping assays should detect, at a minimum, the fourteen most common high risk HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) and the two low risk HPV types targeted by a current HPV vaccine (HPV 6 and 11). Moreover, the WHO HPV LabNet has agreed that a laboratory that performs HPV DNA detection and typing – and, thus, also the assay used - should be considered proficient if it is able to detect 50 genome equivalents (GE) / 5 µl of HPV16 and HPV18 DNA, and 500 GE / 5 µl of the other HPV types. The newly designed assay, as most commercial assays, requires exactly 5 µL of DNA for each single reaction, thus, the limit concentrations can also be expressed as GE/reaction or GE/rx.

To check whether the newly designed assay was in compliance with the WHO requirements I tested, on the ABI StepOnePlus instrument, all the HPV plasmids available, at the time, at AB ANALITICA and then genotyped them with the AMPLIQUALITY HPV-TYPE device (for details on this product see paragraph 4.1.11). The genotypes from the WHO list I tested were the following: HPV 16, 18, 31, 33, 39, 51, 52, 59, 66, 68 and 6. I also checked the assay sensitivity for other possible high risk genotypes, i.e. HPV 53 and 73, as I had the respective plasmid at disposal. The plasmids were diluted in genomic background to mime clinical samples. The assay succeeded in detecting all tested genotypes at the required limit concentration (Fig. 23).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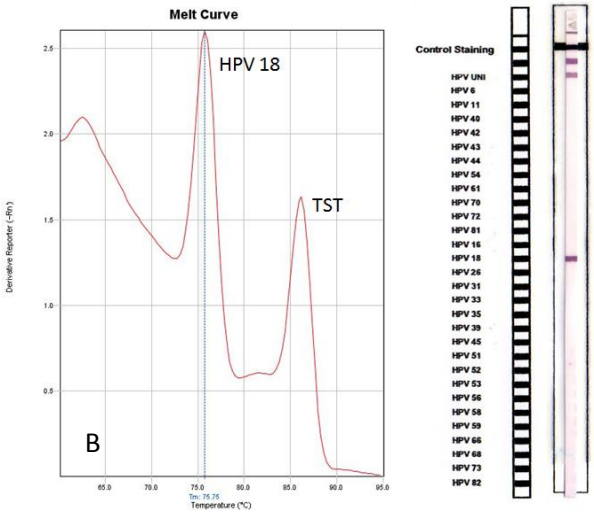
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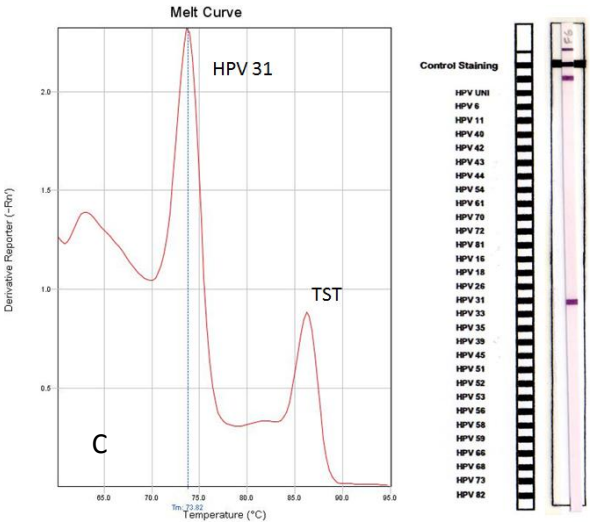
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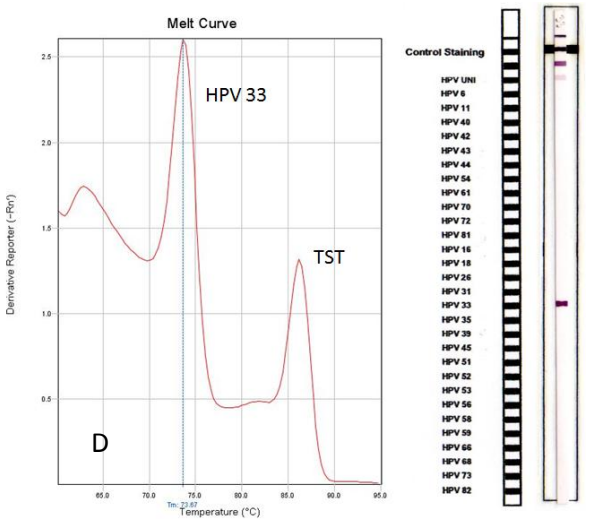
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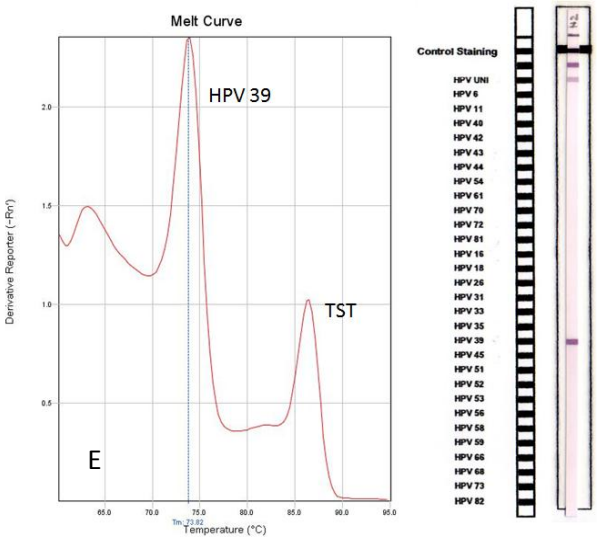
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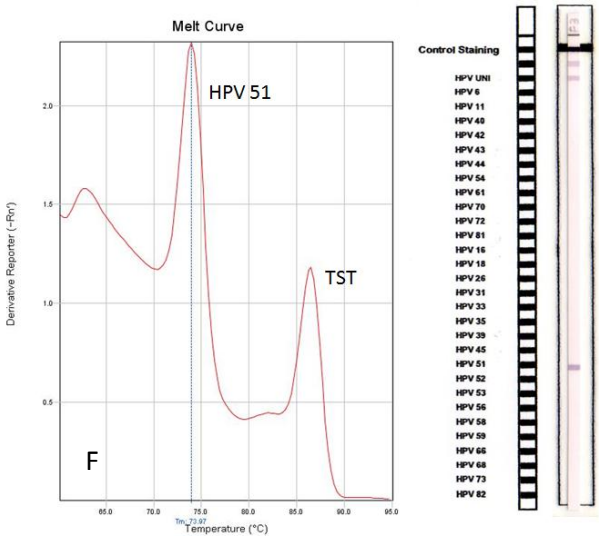
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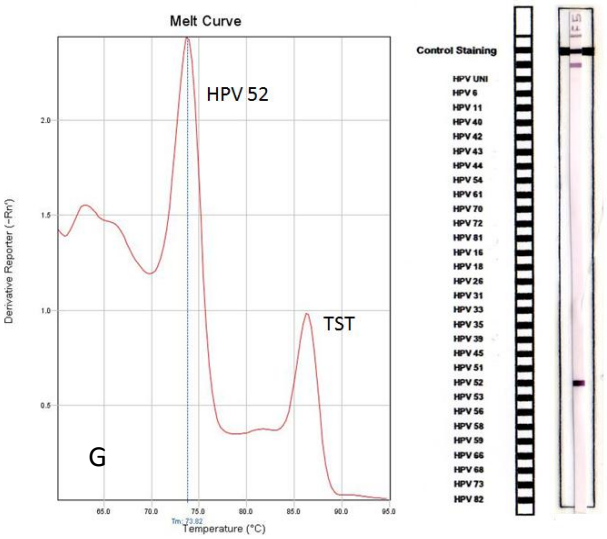
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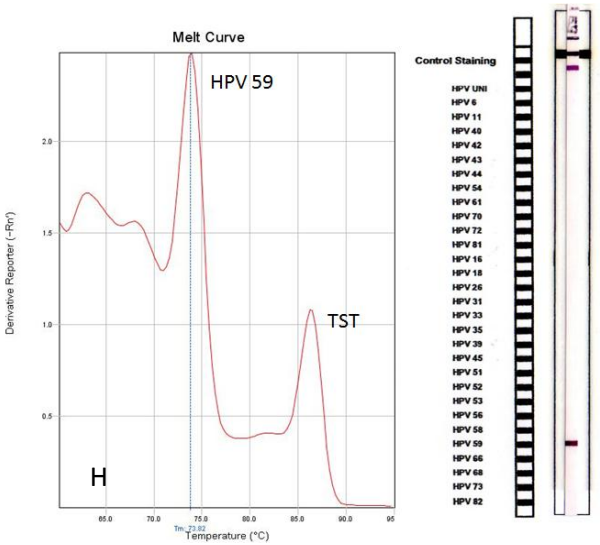
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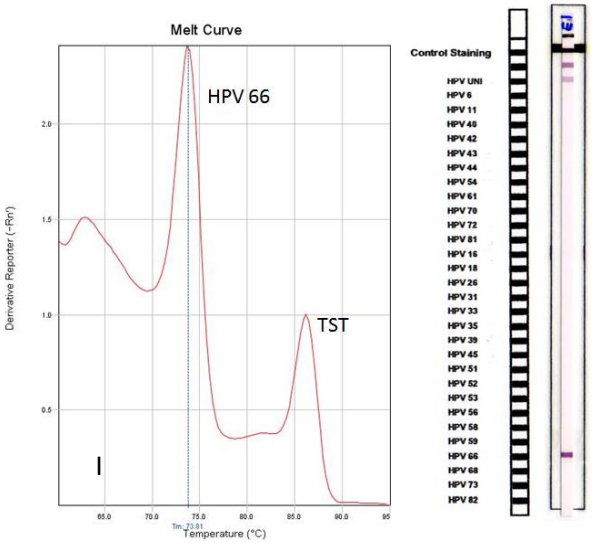
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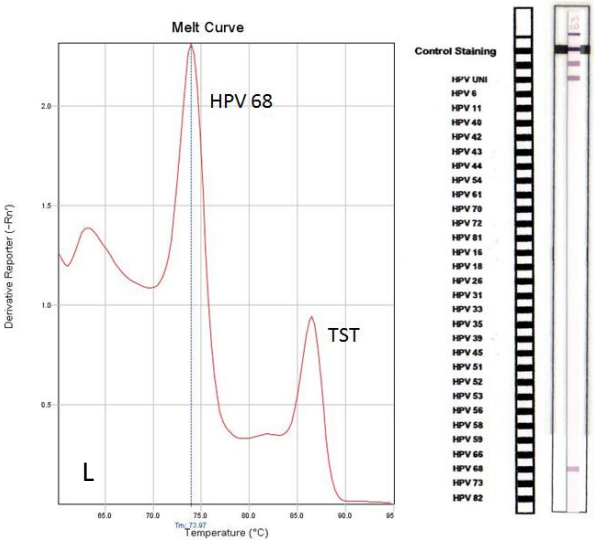
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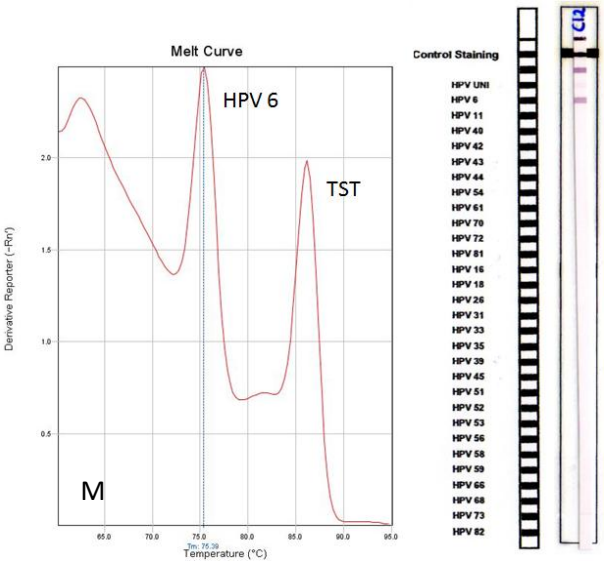
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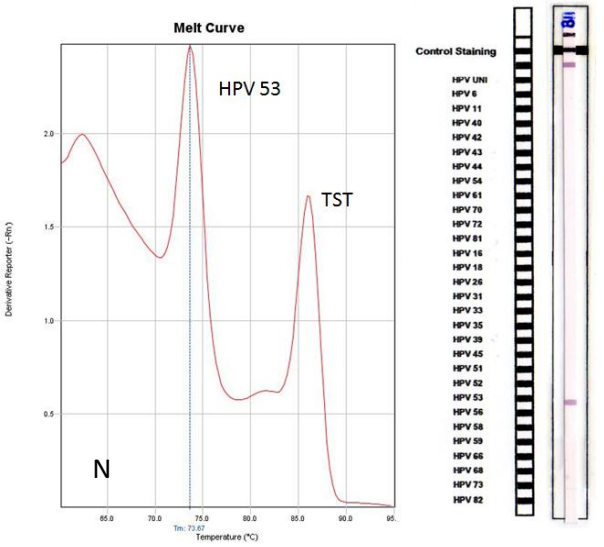
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HPV 82

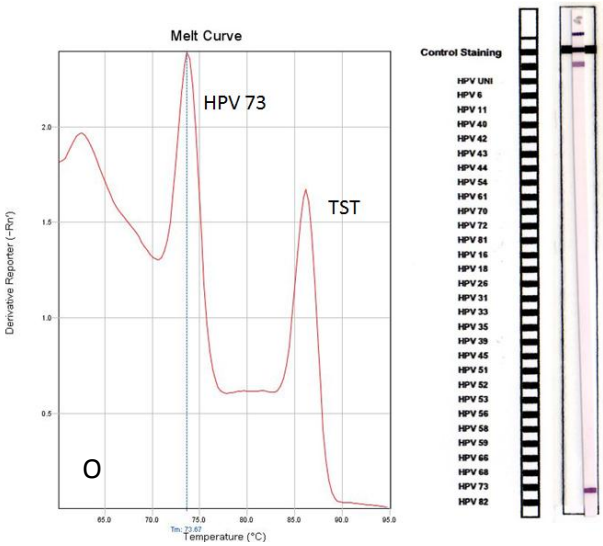

Fig. 23. Melting temperature peaks and reverse line blot (RLB) results for 13 artificial samples positive for different HPV genotypes. For each genotype three bands are visible on the RLB strip: the *TST* band (below the black line at the top of the strip box), the universal HPV band (below the *TST* band; it might appear pale in case most HPV PCR product hybridized to the specific HPV type band) and the specific HPV type band. A. HPV 16 plasmid in genomic background (conc. 50 genome equivalents (GE) / rx). B. HPV 18 plasmid in genomic background (conc. 50 GE/rx). C. HPV 31 plasmid in genomic background (conc. 500 GE/rx). D. HPV 33 plasmid in genomic background (conc. 500 GE/rx). E. HPV 39 plasmid in genomic background (conc 500 GE/rx). F. HPV 51 plasmid in genomic background (conc. 500 GE/rx). G. HPV 52 plasmid in genomic background (conc. 500 GE/rx). H. HPV 59 plasmid in genomic background (conc. 500 GE/rx). I. HPV 66 plasmid in genomic background (conc. 500 GE/rx). L. HPV 68 plasmid in genomic background (conc. 500 GE/rx). M. HPV 6 plasmid in genomic background (conc. 500 GE/rx). N. HPV 53 plasmid in genomic background (conc. 500 GE/rx). O. HPV 73 plasmid in genomic background (conc. 500 GE/rx) (ABI StepOnePlus, software v. 2.2.2).

3.2.2.3. Preliminary tests on the clinical specificity of the Real-time PCR reaction

To assess the clinical specificity of the newly designed assay, 150 HPV negative clinical samples previously tested with a reference method (AMPLIQUALITY HPV-TYPE EXPRESS v 2.0, AB ANALITICA) were analyzed on the ABI StepOnePlus instrument. Accordingly to the provisionally defined range for HPV positivity (71 – 80 °C), seven samples resulted positive after Real-time PCR. These samples were subjected to RLB with the AMPLIQUALITY HPV-TYPE device to check whether they were actually positive for HPV. In all cases the RLB gave a negative result, meaning that neither a HPV type specific band nor the HPV universal band were visible on the strips. To further confirm this result the PCR products for the critical samples were run on agarose gel and again no HPV specific bands were observed, thus the samples were regarded as false positives, reasonably caused by the amplification of aspecific PCR products. In table 6 the

melting temperatures recorded by the Real-time PCR instrument for those seven false positive samples are reported.

SAMPLE	MELTING TEMPERATURE
1	74.78
2	71.08
3	71.25
4	71.40
5	72.28
6	78.56
7	78.70

Table 6. The melting temperature recorded for seven HPV negative samples which resulted positive after Real-time PCR are reported.

Considering these seven false positive sample the clinical specificity of the Real-time PCR assay would have been 95.3%. However, it has to be noted that the higher limit of the HPV positivity range could have been lowered to 78°C, maintaining it 0.4 °C above the highest HPV specific melting temperature ever recorded on previous tests (see paragraph 3.1.1.5). According to this new range (71 – 78 °C) the specificity would jump to 96.7 % as samples 6 and 7 would no more be considered HPV positive.

3.2.2.4. Doubts on the robustness of the newly designed Real-time PCR assay and end of the project

The specificity value obtained testing 150 HPV negative samples could have been considered acceptable. However, the analysis of the melting curves for those samples highlighted a phenomenon that had to be taken into serious consideration: in 22 samples (15 % of all analyzed samples) the melting peaks corresponding to primer dimers presented a shoulder invading the lower limit of HPV positivity range. The Real-time PCR instrument never recognized those shoulders as actual peaks, nevertheless, they were clearly visible by eye (Fig. 24). First of all, such an output could have generated confusion in the final user. Secondly, it was not possible to foresee whether the problem could have exacerbated under standard production conditions, which require frequent changes in the reagent batches used, and whether, at a certain point, the Real-time PCR instrument would have started to recognize those shoulders as real peaks. I tested different reaction

conditions in order to solve the problem, mainly acting on primer concentration and on the thermal profile, however no relevant improvements were accomplished. On that premise, abandoning the project was considered the only feasible possibility, as the risk of putting on the market a product generating an uncontrollable number of false positive results was not acceptable.

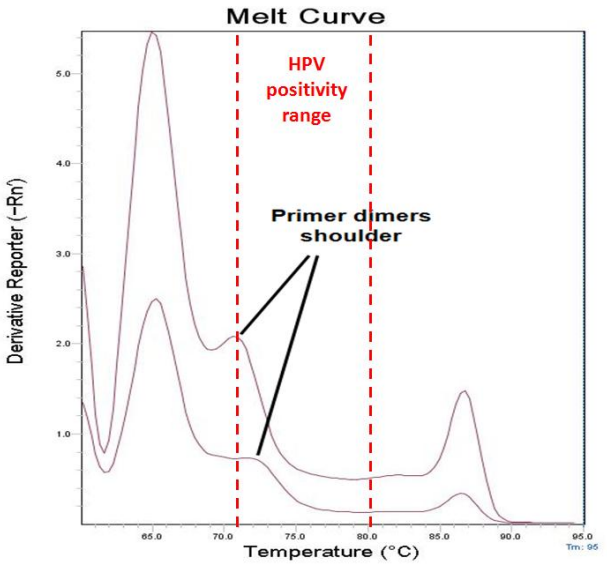

Fig. 24. Melting curves, obtained from the amplification of two negative samples, presenting primer dimer shoulders invading the range of HPV positivity (ABI StepOnePlus, software v. 2.2.2).

3.3. PART II: DEVELOPMENT AND VALIDATION OF THE REALQUALITY RQ-HPV HR MULTIPLEX AND THE REALQUALITY RQ-HPV HR/LR MULTIPLEX DEVICES

3.3.1. SETTING UP THE REACTIONS FOR ASSAY 1 AND ASSAY 2[7]

3.3.1.1. Designing specific primers and probes

Primes from the previous project had been selected from literature; they were consensus degenerated primers targeting L1, the most conserved region in HPV genome and historically preferred, especially when desiring to amplify most HPV types in aggregate. This time, primers had to be target specific, thus they were designed in a different region, called E6/E7. This region, beside being less conserved among HPVs, presents another significant advantage: it is never lost during HPV infections evolving to cancer.

Many aspects had to be considered before selecting primers and probes. A phylogenetic tree was used as a guide to predict which types could have been, more likely, the cause of aspecific PCR products.

Also, before starting the acutal design, several papers providing primer and probe sequences targeting the HPV E6/E7 region were consulted. Genotype specific primers form the literature tended to be concentrated in delimited portions of the E6/E7 sequence suggesting they might have been favorable for some reason. When possible, primers and probe were designed in those regions.

Once selected a candidate primer or a probe, analytical specificity was evaluated *in silico* by BLAST analysis. Primers and probes that resulted specific for their target could be ordered.

[7] Assay 1 corresponds to the REALQUALITY RQ-HPV HR MULTIPLEX device, whereas assay 1 and 2, together, correspond to the REALQUALITY RQ-HPV HR/LR MULTIPLEX device (for details on assay design see paragraph 2.3.2).

3.3.1.2 Testing primers and probes on samples

Primers and probes were tested in simplex against three kind of samples:
1) cervical samples positive for the target HPV type;
2) cervical samples positive for possibly cross-reactive genotypes[8];
3) HPV negative cervical samples (to check cross-reactivity with human genome).
In Fig. 25 an amplification plot is shown; it was obtained while testing HPV 52 primers and probe on three samples, previously tested with the reference method AMPLIQUALITY HPV-TYPE EXPRESS v 3.0 (AB ANALITICA). Two were samples positive for HPV 52 and produced sigmoid amplification curves; one was a sample positive for HPV 33, which gave no amplification curve. The reaction, therefore, was correctly amplifying the target genotype while giving no aspecific signals in presence of the possibly cross-reactive HPV 33 genotype.
For some genotypes, different primer pairs were ordered and compared to select the one performing better, before ordering the respective probe. Comparison was done via Real-time PCR with intercalating dye followed by gel electrophoresis. In Fig. 26, is a gel picture showing the PCR products obtained testing two different primer pairs for HPV 58 in parallel on the same samples. The top line and the bottom line were loaded with the PCR products obtained with primer pair 1 and 2 respectively. Columns 1-2 correspond to sample positive for HPV 58, columns 3-5 to samples positives for HPV 33, columns 6-7 to samples negative for HPV and column 8 to the no-template-control (C-). Both primer pairs produced a specific band (~100 bp) in presence of HPV 58 genome (top line and bottom line, columns 1-2). Only primer pair 2, though, resulted specific as it gave no amplification products in presence of HPV 33 or human genomic DNA (bottom line, columns 3-7). Primer pair 1, instead, was amplifying also human genomic DNA as a band, slightly higher than that specific for HPV 58, is visible in the samples positive for HPV 33 (which also contain genomic DNA) as well as in those negative for HPV (top line, columns 3-7). Primer pair 2 for HPV 58 was selected to set up the reaction for assay 1.

[8] Non-target genotypes presenting only e few mismatches with the primers of interest when analyzed with BLAST (for details on BLAST analysis see paragraph 4.2.10.)

Once tested in simplex, primers and probes had to be tested in multiplex as well. Assay 1 and assay 2 mixes, however, were not assembled all in once, but rather by adding primer pairs and the respective probes two by two. Each time, the partial mixes were tested on known HPV positive and negative clinical samples in order to check whether the newly added oligos could interfere with the others.

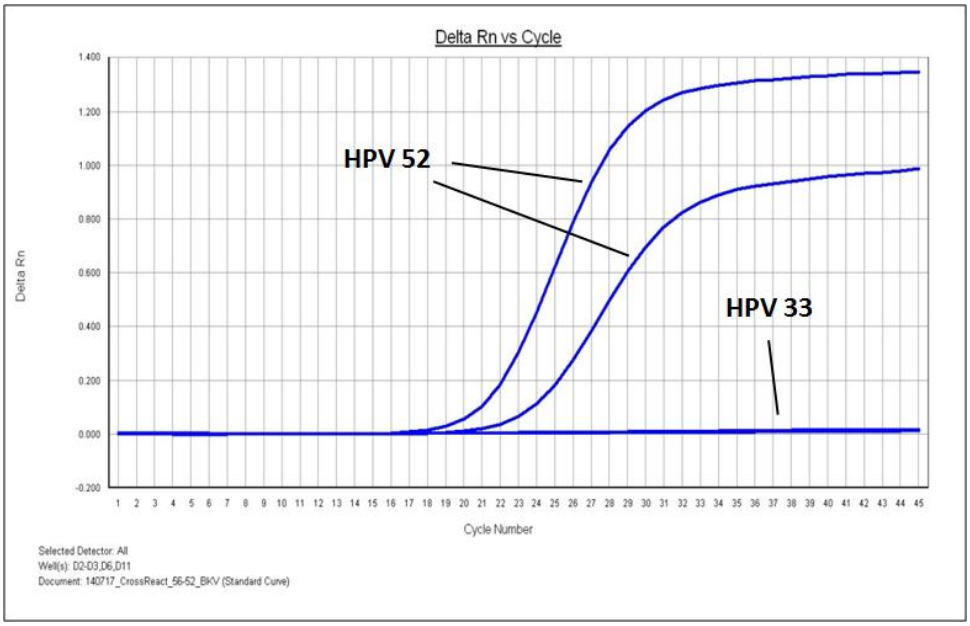

Fig. 25. An amplification plot is shown. It was obtained while testing HPV 52 primers and probe on three samples, previously tested with the reference method AMPLIQUALITY HPV-TYPE EXPRESS v 3.0 (AB ANALITICA). Two were samples positive for HPV 52 and produced sigmoid amplification curves, one was a sample positive for HPV 33 which gave no amplification curve (ABI 7500 Fast Dx, software v. 1.4).

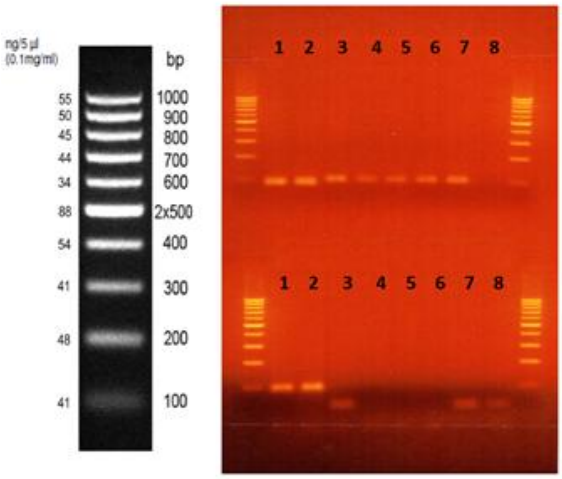

Fig. 26. Gel picture showing the PCR products obtained testing two different primer pairs for HPV 58 in parallel on the same samples. The top line and the bottom line were loaded with the PCR products obtained with primer pair 1 and 2 respectively. Columns 1-2 correspond to sample positive for HPV 58, columns 3-5 to samples positives for HPV 33, columns 6-7 to samples negative for HPV and column 8 to the negative control (C-). Only primes pair 2 is specific for HPV 58.

3.3.1.3. Setting the conditions for the multiplex reactions

The mastermix chosen for the assays already contained all the elements needed for amplification. The only thing that had to be set was primer and probe concentration. Without going into the details, they were set in order to favor target pathogen (HPV) amplification against internal control (*HBB*) amplification.
Florescence saturation phenomena had also to be taken into account. In Fig. 27, the amplification curves obtained by amplifying 30 samples with assay 1 on the Biorad Dx instrument are shown in "no baseline subtraction mode". The signals measured in FAM channel are represented in blue, whereas those measured in JOE, Cy5 and ROX are in green, purple and orange respectively. It is clearly visible that the FAM baseline fluorescence is much higher than that of the other fluorophore and close to the recording limit of the machine. This is due to the fact that, in assay 1, FAM labeled probes are 12, whereas JOE, Cy5 and ROX probes are one for each of the fluorophore. The situation is similar in assay 2, even though FAM probes are only six.

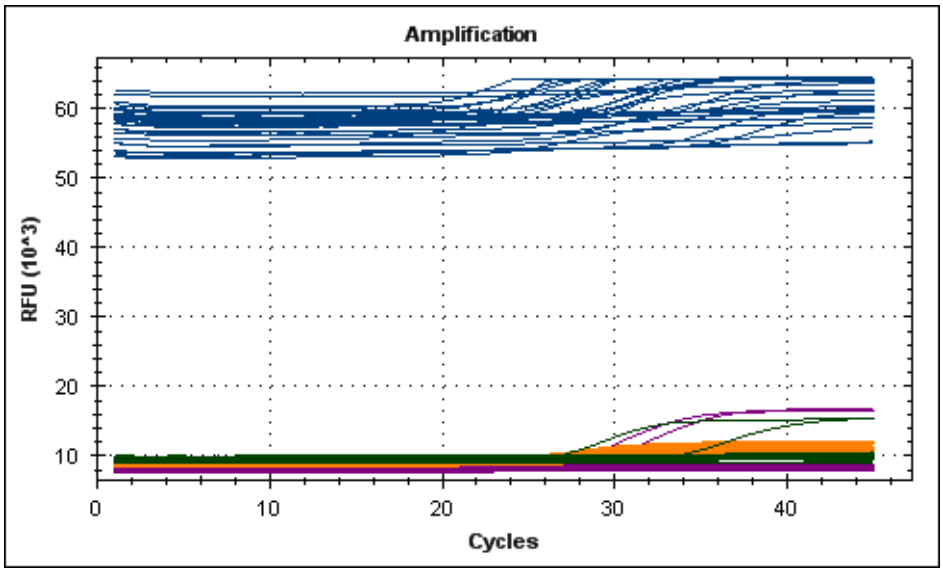

Fig. 27. The amplification curves obtained by amplifying 30 samples with assay 1 are shown in "no baseline subtraction mode". The signals measured in FAM channel are represented in blue, whereas those measured in JOE, Cy5 and ROX are in green, purple and orange respectively. FAM baseline is much higher than that of the other fluorophores (Biorad Dx, software v. 1.7).

Being the FAM baseline so high, amplification of FAM targets could easily bring the system to saturation. In such a case the amplification curve appears sharp-cornered, the corner corresponding to the point where the instrument is no more able to record fluorescence increase due to saturation. To limit this problem and keep the FAM fluorescence baseline as low as possible, the concentration of the corresponding probes had to be much lower than that of the HPV probes labeled with the other fluorophores (Fig. 28).

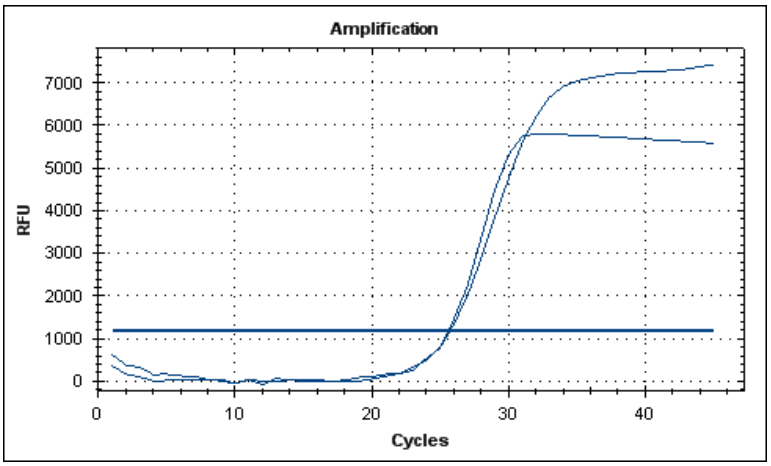

Fig. 28. The amplification curves for a sample positive for HPV 52 (FAM signal) is presented. The sharp-cornered curve was obtain using FAM probes at the same concentration of the other HPV probes. The sigmoid curve was obtained with FAM probes at a lower concentration.

3.3.2. PERFORMANCE OF ASSAY 1

3.3.2.1. Validation on clinical samples: clinical sensitivity and specificity, and genotype concordance

The assay was meant to work on ABI 7500 Fast Dx and Biorad Dx, thus it was validated on both Real-time instruments. A total number of 320 clinical samples was used. The sample composition was as represented in the pie chart below (Fig. 29).

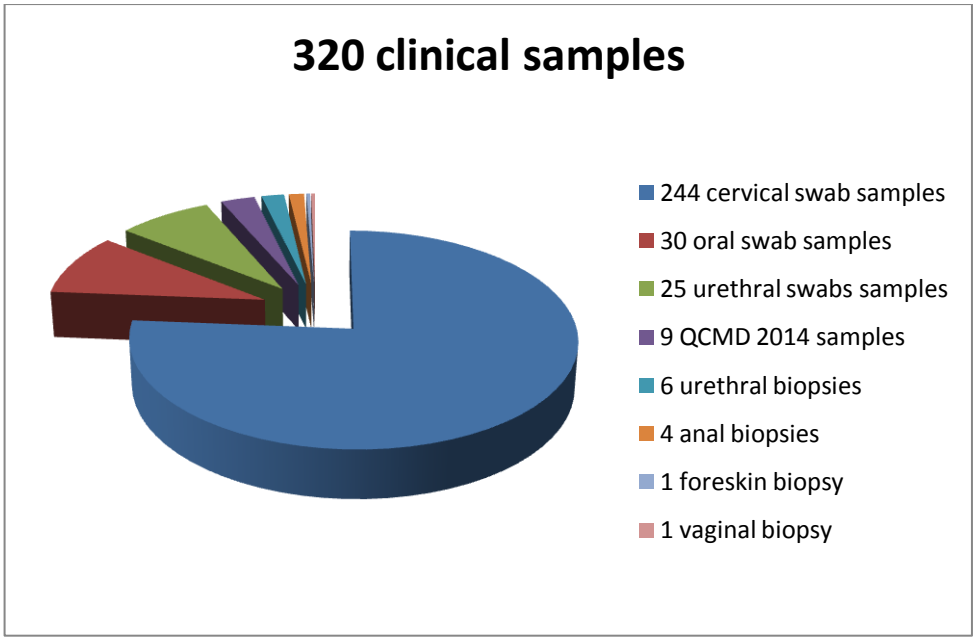

Fig. 29. Pie chart representing the composition of the 320 clinical samples used to validate assay 1.

The samples were tested both with assay 1 and a reference method (AMPLIQUALITY HPV-TYPE EXPRESS, v. 3.0; AB ANALITICA). In case of discordant results, sequencing was used to confirm the genotype composition of samples. In particular, if one method detected a genotype not detected by the other, the sample was amplified in simplex, using primers specific for the genotype of interest, and then the PCR product was sent for sequencing. The chosen reference method is a full-genotyping method based on end-point PCR and RLB, thus, in case it detected a genotype not detected by assay 1, it was immediately clear which one it was. The opposite situation was more complicated

as assay 1 only provides partial genotyping information. This means that, when an unexpected JOE or Cy5 signal was observed, the presence of HPV 16 or 18 was obviously suspected; whereas, in case of FAM unexpected signals, the responsible genotype could have been any of the 12 genotypes detectable in that channel. A possible way to confirm the presence of one of the "FAM genotypes", in such a circumstance, would have been to amplify the sample with 12 simplex reactions, one for each of the candidate genotypes, and then proceed with sequencing. To preserve samples, though, I decided to opt for a different approach, that only required to perform five simultaneous Real-time PCR reactions. Each of the five reactions was performed using all the primers from assay 1, but only a subset of probes. A scheme of how probes were allocated among the five reactions, called A, B, C, D and E, is represented in Fig. 30. In brief, mix A contained the probes

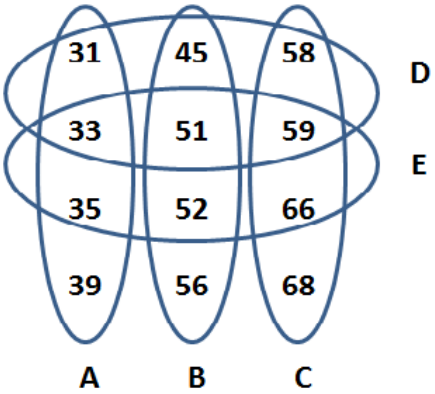

Fig. 30. Scheme representing how FAM probes of assay 1were allocated among mixes A, B, C, D and E.

for HPV 31, 33, 35 and 39; mix B the probes for HPV 45, 51, 52 and 56; mix C the probes for HPV 58, 59, 66 and 68; mix D the probes for HPV 31, 45, 58, 33, 51 and 59; and mix E the probes for HPV 33, 51, 59, 35, 52 and 66. According to this scheme, if present in mono-infection, each genotype would have given an univocal pattern of positivity among the five mixes. For example, with an HPV 31 positive sample, a FAM signal would have been observed only from mixes A and D; with HPV 33 the FAM signals would have been from A, D and E; with HPV 35 from A and E; with HPV 39 only from A and so on for all the other genotype. Once understood which genotype was responsible for the signal, it was amplified in simplex and send for sequencing. In Fig. 31, is the amplification plot obtained by amplifying, with mixes A, B, C, D and E, a sample that resulted negative for

the reference method, but produced a signal in the FAM channel when tested with assay 1. Mixes C and D gave a positive signal indicating the presence of HPV 58, later confirmed by sequencing.

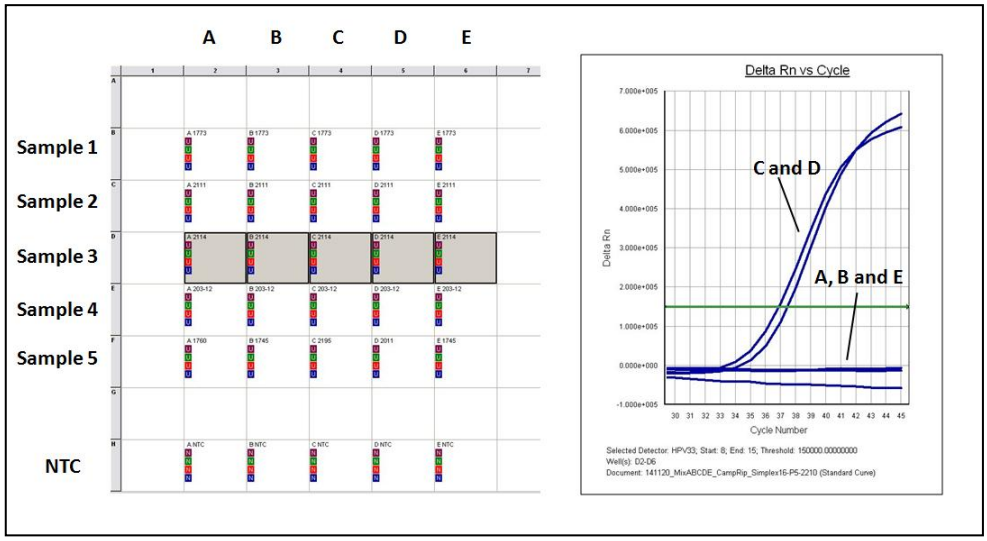

Fig. 31. On the left side of the picture, is a sample plate showing 5 samples that were all amplified with mixes A, B, C, D and E. On the right side, is the amplification plot obtained for sample 3 (gray wells on the sample plate). Only mixes C and D produced an amplification curve indicating the presence of HPV 58.

Once analyzed all the 320 samples, clinical sensitivity and clinical specificity of assay 1 could be calculate. Clinical sensitivity corresponds to the number of samples correctly identified by assay 1 as positive divided by the number of all the positive samples; whereas specificity corresponds to the number of samples correctly identified as negative by assay 1 divided by the number of all the negative samples. In this context, positives samples were considered to be those samples that resulted positive, for one of the 14 genotypes comprised in assay 1 panel, either with both assay 1 and the reference method or with one of the two methods and sequencing. All the other sample, comprising both HPV negative samples and samples positive for HPV genotypes different from the 14 comprised in assay 1 panel, were considered as negative. According to the above definitions, positive samples were 141 and negative samples were 179. Clinical sensitivity and specificity on the ABI 7500 Fast Dx instrument were 99.3% and 98.9% respectively; Clinical sensitivity and specificity on Biorad Dx were 97.8% and

99.4% respectively. According to the validation plan[9], any result above 95% would have been considered adequate.

With data obtained from the 320 samples, also genotype concordance between assay 1 and the reference method could be calculated. This parameter corresponds to the number of HPV genotypes identified by both methods divided by the total number of identified genotypes (genotypes identified by both assays + extra genotypes identified with assay 1 + genotypes not identified with assay 1). Genotype concordance on ABI 7500 Fast Dx was 92 %, whereas on Biorad Dx was 90.6 %.

3.3.2.2 Analytical sensitivity

As detailed in previous paragraphs, the WHO considers proficient an HPV genotyping assay able to detect at least 50 genome equivalents (GE) / 5 µl of HPV16 and HPV18 DNA, and 500 GE / 5 µl of the DNA of HPV 31, 33, 35, 45, 51, 52, 56, 58, 59, 66 and 68. Also, as assay 1, requires 5 µl of DNA extract per reaction, the above concentrations can be expressed as GE/rx.

The analytical sensitivity of assay 1 was assessed with several approaches. For HPV 16 and 18, it was calculated with probit analysis. To produce a sufficient number of data for the model, artificial samples containing the genotype of interest at five different concentratios (7.5 – 5 – 2.5 – 1.25 – 0.5 GE/rx) were tested in 8 replicates for 3 consecutive runs. The estimated limits of detection (LODs) for HPV 16 and 18 were 3.4 GE/rx ($p = 0.05$) and 4 GE/rx ($p = 0.05$) respectively (Fig. 32). The exact LODs for the other HPV targets were not calculated. However, assay 1 was tested with the "Global HPV LabNet 2013 HPV DNA Genotyping Proficiency Panel" (WHO proficiency panel in this writing). This panel comprises 46 artificial samples containing purified whole genomic plasmids of HPV 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68a and 68b in a background of human cellular DNA. The samples are prepared to include single types and mixtures at limit concentrations and 10-fold lower concentrations. The composition of the panel is summarized in table 7. All samples in the panel were correctly identified. Therefore, the limit of detection of

[9] A document compiled before starting the validation process (for details see section 5).

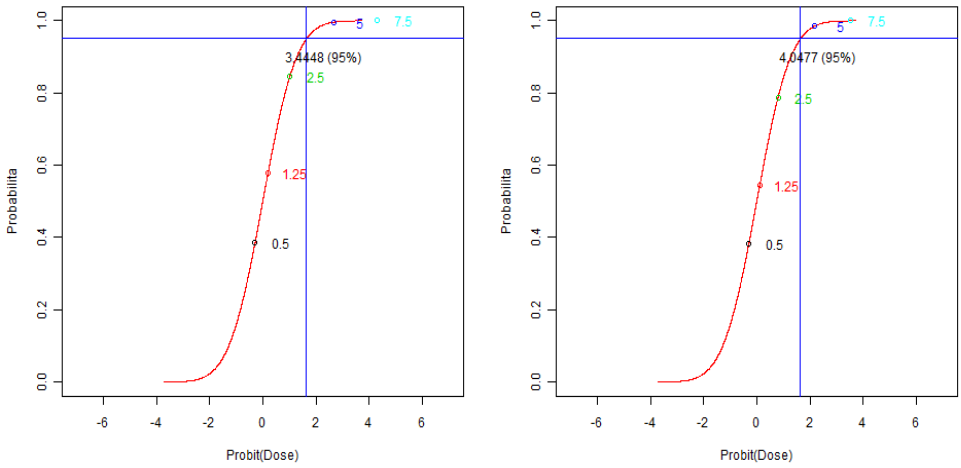

Fig. 32. Probit analysis output showing the limit of detection of assay 1 for HPV 16 (graph on the left; 3.4 GE/rx (p = 0.05)) and HPV 18 (graph on the right; 4 GE/rx (p = 0.05)) (R software).

assay 1 for the 14 detectable genotypes is below the limit concentrations required by WHO. It is worth noting that sample 30 contains 5 GE/5µl (i.e. 5 GE/rx) of HPV 16 plasmid DNA and was correctly genotyped, thus, confirming the result obtained with probit analysis.

WHO SAMPLE	GENOTYPE	CONCENTRATION (GE/5µl)
1	*58*	*50*
2	*18*	*50*
3	*39, 45, 52, 56, 68b*	*500*
4	*33*	*50*
5	*52*	*500*
6	*68b*	*500*
7	*6*	*500*
8	*39*	*50*
9	*66*	*500*
10	*35, 59, 66, 68a*	*500*
11	*16*	*50*
12	*31*	*50*
13	*52*	*50*
14	*68a*	*500*
15	*negative*	*0*
16	*51*	*500*
17	*31*	*500*
18	*11, 31, 33, 58*	*500*
19	*56*	*50*
20	*68b*	*50*
21	*35, 59, 66, 68a*	*50*
22	*6*	*50*
23	*45*	*50*

WHO SAMPLE	GENOTYPE	CONCENTRATION (GE/5µl)
24	*59*	*50*
25	*39, 45, 52, 56, 68b*	*50*
26	*18*	*5*
27	*35*	*50*
28	*59*	*500*
29	*6, 16, 18, 51*	*500*
30	*16*	*5*
31	*35*	*500*
32	*58*	*500*
33	*11, 31, 33, 58*	*50*
34	*11*	*50*
35	*45*	*500*
36	*66*	*50*
37	*6, 16, 18, 51*	*50*
38	*11*	*500*
39	*33*	*500*
40	*39*	*500*
41	*56*	*500*
42	*68a*	*50*
43	*51*	*50*
A	*none*	*0*
B	*16*	*2500*
C	*16*	*25*

Table 7. Composition of the WHO proficiency panel from 2013.

The WHO proficiency panel was from 2013; when I used it, it was too late submit the output data an receive an official response from WHO. Assay 1, though, could participate to a different external quality assessment (EQA) program i.e. the QCMD (Quality Control for Molecular Diagnostics) 2014 Human Papillomavirus DNA EQA Programme. The QCMD panel comprised 10 samples, composed as summarized in table 8. Assay 1 was able to correctly genotype all 6 core samples and 9 out of 10 samples, when considering also the educational samples (Fig. 33). Moreover, it has to be outlined that the only sample that was not correctly identified is described as "low viral load HPV 16 (SiHa)" and, as the QCMD referred, did not result positive also when analyzed by two external testing laboratories using the Cobas HPV assay (ROCHE) and the HC2 assay (QIAGEN) respectively. The QCMD also referred that only the 87.2% of participant reported 100% of core samples correctly. Data relative to this EQA program, thus,

represent a further confirmation that the analytical sensitivity of assay 1 is adequate for the scope of genotyping.

SAMPLE	MATRIX	CONTENT	STATUS	TYPE
HPV14-02	Pre servCyt + BSM	HPV16 (Caski)	Positive	Core
HPV14-06	Pre servCyt + BSM	HPV18 (Hela)	Positive	Core
HPV14-01	Pre servCyt + BSM	HPV45 (CC10b)	Positive	Core
HPV14-09	Pre servCyt + BSM	HPV16 /18 (Caski/Hela)	Positive	Core
HPV14-10	Pre servCyt + BSM	Lo w Vi r al Lo ad HPV16 (SiHa)	Positive	Educational
HPV14-08	Pre servCyt + BSM	HPV5 1&5 2 (clinical sample)	Positive	Educational
HPV14-04	Pre servCyt + BSM	HPV5 2 &5 6 (clinical sample)	Positive	Educational
HPV14-07	Pre servCyt + BSM	HPV5 4&5 6 (clinical sample)	Positive	Educational
HPV14-03	Pre servCyt + BSM	HPV Negative (BSM)	Negative	Core
HPV14-05	Pre servCyt + BSM	HPV Negative (BSM)	Negative	Core

Table 8. Composition of the QCMD panel from 2014.

Analysis of your laboratory's performance on all proficiency samples:

Your laboratory's qualitative results and performance scores

Sample	Sample Content	Qualitative			
		Sample Status	Sample Type	Your qualitative result	Your qualitative score
HPV14-02	HPV16 (Caski)	Positive	Core	positive	0
HPV14-06	HPV18 (Hela)	Positive	Core	positive	0
HPV14-01	HPV45 (CC10b)	Positive	Core	positive	0
HPV14-09	HPV16/18 (Caski/Hela)	Positive	Core	positive	0
HPV14-10	Low Viral Load HPV16 (SiHa)	Positive	Educational	negative	1
HPV14-08	HPV51&52 (clinical sample)	Positive	Educational	positive	0
HPV14-04	HPV52&56 (clinical sample)	Positive	Educational	positive	0
HPV14-07	HPV54&56 (clinical sample)	Positive	Educational	positive	0
HPV14-03	HPV Negative (BSM)	Negative	Core	negative	0
HPV14-05	HPV Negative (BSM)	Negative	Core	negative	0
Sum Qualitative Panel Score					1

Fig. 33. Snapshot from the QCMD individual report relative to assay 1 performance in the context of the QCMD 2014 Human Papillomavirus DNA EQA Programme.

3.3.2.3 Analytical specificity

In order to assess the analytical specificity of assay 1, it was necessary to test it with samples positive for possibly cross-reactive targets such as HPV types different from those detected by the assay and other pathogens, especially those infecting the ano-genital district.

As for the first subset of samples, the validation data could be used. Among the samples regarded as negative, there were also 54 samples positive for possible high risk and low risk HPV types (HPV 6, 11, 26, 40, 42, 43, 44, 53, 54, 61, 62, 67, 70, 73, 81, 82, 84, 87, 89and 90). Of these, two samples (3.7 %) resulted positive (late FAM signal) once analyzed on the ABI 7500 Fast Dx instrument. The genotypes detected with the reference method were HPV 6 and 87 in one case and HPV 40 and 84 in the other. The presence of an extra high risk genotype could not be confirmed by sequencing as no positive signals were obtained with mixes A, B, C, D and E (see paragraph 3.3.2.1). Cross-reactivity with those genotypes, however, was excluded as other samples positive for the same genotypes did not produced unexpected FAM signals.

As for the second subset of samples, those positives for other pathogens, 18 samples were analyzed (table 9). Only sample 14, positive for *Trichomonas vaginalis,* gave an unexpected positive signal. Being a clinical sample, it could also have been positive for an high risk HPV. Therefore, it was analyzed with another method (AMPLIQUALITY HPV-TYPE EXPRESS, v. 3.0; AB ANALITICA) and the presence of an high risk HPV type (HPV 66) was confirmed (Fig. 34). As other samples positive for *Trichomonas vaginalis* did not produce aspecific signals, cross-reactivity with that pathogen, as with the others tested, was exclude. The assay performance relative to analytical sensitivity was regarded as adequate.

SAMPLE	TYPE	PATHOGEN
1	Artificial sample	*Adenovirus*
2	Artificial sample	*Candida albicans*
3	Urine	*Cytomegalovirus*
4	Urine	*Chlamydia trachomatis*
5	Artificial sample	*Chlamydia trachomatis / Neisseria gonorrhoeae*
6	Artificial sample	*Gardnerella vaginalis*

SAMPLE	TYPE	PATHOGEN
7	Artificial sample	*Herpes simplex 1*
8	Urethral swab	*Herpes simplex 1-2*
9	Urethral swab	*Herpes simplex 2*
10	Artificial sample	*Mycoplasma genitalium*
11	Artificial sample	*Mycoplasma hominis*
12	Artificial sample	*Neisseria gonorrhoeae*
13	Vaginal discharge	*Trichomonas vaginalis*
14	Vaginal swab	*Trichomonas vaginalis*
15	Vaginal discharge	*Trichomonas vaginalis*
16	Urethral swab	*Ureaplasma parvum*
17	Artificial sample	*Ureaplasma parvum*
18	Artificial sample	*Ureaplasma urealyticum*

Table. 9. Samples positive for pathogen different from HPV.

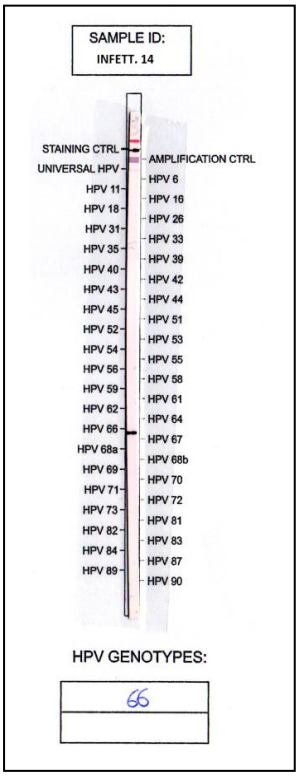

Fig. 34. Reverse line blot output for sample 14 (AMPLIQUALITY HPV-TYPE EXPRESS, v. 3.0).

3.3.2.4. Intra and inter-assay variability

Clinical samples carrying HPV mono-infections were used to calculate the intra and inter-assay variability for the 14 genotypes detected by assay 1. As for HPV

68, samples for both HPV 68a and HPV 68b variants were used, the total number of analyzed samples is 15 instead of 14.

INTRA-ASSAY VARIABILITY

To measure inter-assay variability, the 15 samples were amplified in eight replicates in a single run. For each genotype, the coefficient of variation (CV) was calculated as the ratio of standard deviation multiplied for 100 to the mean of the Cycle threshold (Ct) values of the eight replicates. The Ct values for the eight replicates of the sample positive for HPV 16 are reported in table 10.

REPLICATE	Ct
1	29.5701
2	29.579
3	29.7908
4	29.7193
5	29.7651
6	29.6254
7	29.7672
8	29.7279

Table 10. Ct values for the eight replicates of the sample positive for HPV 16 used to calculate the intra-assay CV value.

The standard deviation (σ) for values in table 10 is 0.09 and the mean (μ) is 29.69. Thus, the intra-assay CV ($\frac{\sigma * 100}{\mu}$) for HPV 16 is 0.3%. The intra-assay CV values for all the other genotypes are reported in table 12.

INTER-ASSAY VARIABILTY

To measure inter-assay variability, the 15 samples were amplified in three consecutive runs. For each genotype the CV was calculated as the ratio of standard deviation multiplied for 100 to the mean of the Cycle threshold (Ct) values of the three runs. The Ct values for the three runs of the sample positive for HPV 16 are reported in table 11.

RUN	Ct
1	29.7858
2	29.8157
3	29.7882

Table 11. Ct values corresponding to the sample positive for HPV 16, measured in three consecutive runs.

The standard deviation (σ) for values in table 11 is 0.02 and the mean (μ) is 29.80. Thus, the inter-assay CV ($\frac{\sigma * 100}{\mu}$) for HPV 16 is 0.06%. The inter-assay CV values for all the other genotypes are reported in table 12.

TARGET	Intra-assay CV (%)	Inter-assay CV (%)
HPV 16	0.30	0.06
HPV 18	0.17	0.15
HPV 31	0.63	1.48
HPV 33	0.48	0.59
HPV 35	1.03	0.43
HPV 39	0.40	0.93
HPV 45	1.28	0.92
HPV 51	0.89	0.86
HPV 52	0.76	0.90
HPV 56	0.42	0.17
HPV 58	0.71	0.39
HPV 59	0.67	0.62
HPV 66	0.84	0.77
HPV 68a	0.71	0.16
HPV 68b	0.63	0.45

Table 12. Intra-assay CV and inter-assay CV values for 15 genotypes.

The CV values for assay 1 are all below 1.5%, indicating that the assay has high repeatability.

3.3.3. PERFORMANCE OF ASSAY 2

3.3.3.1. Validation on clinical samples: clinical sensitivity and specificity

As assay 1, also assay 2 was meant to work on ABI 7500 Fast Dx and Biorad Dx, thus it was validated on both Real-time instruments. A total number of 192 clinical samples was used. 165 were cervical swab samples, whereas 27 were urethral swab samples.

The same approach used for assay 1 validation was used also here. All samples were tested with a reference method (AMPLIQUALITY HPV-TYPE EXPRESS v. 3.0). HPV composition of discordant samples was verified by end point PCR followed by sequencing.

In case of unexpected FAM signals from assay 2, the genotype generating the signal was identified using four simultaneous reactions (called F, G, H, I). Each of the four reactions contained all the primers from assay 2 but only a subset of probes. A scheme of how probes were allocated among the four reactions is represented in Fig. 35.

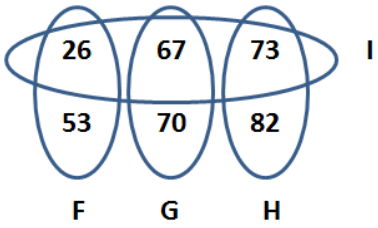

Fig. 35. Scheme representing how FAM probes of assay 2 were allocated among mixes F, G, H, and I.

According to this scheme, if present in mono-infection, each genotype would have given an univocal pattern of positivity among the four mixes. For example, with an HPV 26 positive sample, a FAM signal would have been observed only from mixes F and I.

In this context, positives samples were considered to be those samples that resulted positive, for one of the 8 genotypes comprised in assay 2 panel, either with both assay 2 and the reference method or with one of the two methods and sequencing. All the other sample, comprising both HPV negative samples and samples positive for HPV genotypes different from the 8 comprised in assay 2 panel, were considered as negative. According to the above definitions, of the 192

tested samples, 83 were positive and 109 were negative. All samples were tested on ABI 7500 Fast Dx. 81 out of 83 samples were correctly identified as positive, thus, clinical sensitivity was 97.6%; whereas 108 out of 109 samples were correctly identified as negative, thus, clinical specificity was 99.1%. On Biorad Dx, a lower number of sample could be used due to sample depletion. Clinical sensitivity was 97.4% (76 correctly identified out of 78 positive samples); whereas clinical specificity was 99.0% (102 correctly identified out of 103 negative samples). According to the validation plan[10], any result above 95% would have been considered adequate.

3.3.3.2. Analytical sensitivity

HPV 6 and 11 are the only two genotypes comprised in assay 2 panel, that need to be detected with a certain level of sensitivity, according to WHO requirements for genotyping tests. Thus far, therefore, they were the sole genotypes for which analytical sensitivity has been assessed. In order to do that, the WHO proficiency panel from 2013 was used (for panel composition see paragraph 4.2.6). Assay 2 was able to correctly identify HPV 6 and 11 at a concentration of 50 GE/rx, which is a 10-fold lower concentration than that required by WHO.

[10] A document compiled before starting the validation process (for details see section 5).

4. MATERIALS AND METHODS

4.1 DEVELOPMENT OF THE NEW VERSION OF THE REALQUALITY RI-HPV STAR DEVICE

4.1.1. CERVICAL SWAB SAMPLES

Cervical swab samples were provided from the Department of Pathology of the Central Hospital of Bolzano. The samples had been collected in the context of the *follow up* of patients with intraepithelial lesion or during ambulatory care visits for gynecologic condition. Cervical cells had been dispersed in PreservCyt transport medium in order to perform liquid based cytology. Cytological reports associated to the samples were provided as well. Such samples, if conserved at 4-37 °C, are suitable for cytological analysis for up to 6 weeks after collection. Molecular analysis can be perform also several years after collection.

4.1.2. ARTIFICIAL SAMPLES

Artificial samples for HPV 6, 16, 18, 31, 33, 39, 51, 52, 53, 54, 59, 66, 68 and 73 were prepared by diluting plasmids carrying the L1 sequence of the different HPV types in genomic background to mime clinical samples. Genomic DNA was extracted from HPV negative cervical samples.

4.1.3. PCR / REAL-TIME PCR INSTRUMENTS

End-point PCR was performed on the Applied Biosystems 2720 Thermal Cycler instrument (Applied Biosystems). Real-time PCR was performed on StepOnePlus Real-Time PCR System (Applied Biosystems) and on 7500 Fast Dx Real-Time PCR System (Applied Biosystems). Run files produced with StepOnePlus Real-Time PCR System were analyzed with software version 2.2.2. Run files produced with 7500 Fast Dx Real-Time PCR System were analyzed with software version 1.4.

4.1.4. DNA EXTRACTION

Viral and genomic DNA from most cervical samples was extracted using the EZ1 Advanced XL instrument (QIAGEN) and the EZ1 DSP Virus kit (QIAGEN), following manufacturer instructions. Manual DNA extraction was performed for a few samples using the QIAamp DNA mini Kit (QIAGEN), following manufacturer instruction.

4.1.5. END-POINT PCR WITH PRIMER SET A

Primer set A was selected from literature and, initially, used following the protocol provided in the original paper (64). In an attempt to eliminate aspecific PCR products, the original thermal profile was, later, modified as reported in table 13:

TEMPERATURE	TIME	CYCLES
95 °C	10’	1
95 °C	30’’	5
43/44/45 °C	30’’	
72 °C	45’’	
95 °C	30’’	45
64 °C	30’’	
72 °C	45’’	
72 °C	10’	1

Table 13. Modified thermal profile for end-point PCR with primer set A. three different annealing temperature for the first 5 cycles were tested.

4.1.6. END POINT PCR WITH PRIMER SET B

Primer set B was selected from literature and used following the protocol provided in the original paper (65) except for the thermal profile that was modified as reported in table 14:

TEMPERATURE	TIME	CYCLES
20 °C	5’	1
95 °C	5’	1
95 °C	30’’	45
50 °C	30’’	
72 °C	30’’	
72 °C	5’	1

Table 14. Thermal used for end-point PCR with primer set B.

4.1.7. AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis was performed by loading 10 µl of PCR product on a 3% agarose gel. DNA-Marker pUC19/Msp I (Carl Roth) was employed (Fig. 36).

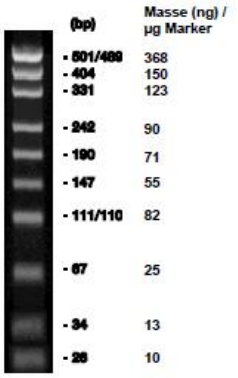

Fig. 36. DNA-Marker pUC19/Msp I (Carl Roth)

4.1.8. NEW VERSION OF REALQUALITY RI-HPV STAR

The new version of the REALQUALITY RI-HPV STAR device, as the old one, was based on Real-time PCR with intercalating dye and melting curve analysis. GP5+/GP6+ primers plus other primers selected from literature (primer set B (65)) were used for HPV amplification. *TST* was chosen as internal control (IC). The sequence of the primers used for *TST* amplification is covered by industrial secret and will not be disclosed. HPV primers were used at a higher concentration than *TST* primers in order to favor the amplification of the pathogen target. Precise primer concentrations are covered by industrial secret and will not be disclosed. Information on the employed mastermix and intercalating dye (named dye 1 in this writing) is covered by industrial secret and will not be disclosed. UNG (Uracil N-Glycosylase) enzyme was added to the mix in order to prevent carry-over contamination. The assay was meant to work on the StepOnePlus Real-Time PCR System (Applied Biosystems).The thermal profile for the PCR reaction is summarized in table 15.

TEMPERATURE	TIME	CYCLES
50 °C	2’	1
95 °C	10’	1
95 °C	30’’	45
50 °C	30’’	
72 °C	30’’	
72 °C	5’	1
Dissociation step (default profile)		

Table 15. Thermal profile the of new version of the REALQUALITY RI-HPV STAR device.

A typical melting plot for a positive sample was showing at least three peaks, the first corresponding to primer dimers, the second to HPV, and the third to *TST*. In order to avoid misinterpretation of results a range for HPV positivity was defined (71-80 °C).

4.1.9. NEW VERSION OF REALQUALITY RI-HPV STAR WITH *HBB* GENE AS INTERNAL CONTROL (discarded in the set up phase)

The same conditions as in the selected new version of the REALQUALITY RI-HPV STAR device (see paragraph 4.1.8) were used, except for that, instead of *TST* specific primers, *HBB* primers were used. The sequence of the primers used for *HBB* amplification is covered by industrial secret and will not be disclosed.

4.1.10. NEW VERSION OF REALQUALITY RI-HPV STAR WITH DYE 2 AS INTERCALATING DYE (discarded in the set up phase)

The same conditions as in the selected new version of the REALQUALITY RI-HPV STAR device (see paragraph 4.1.8) were used, except for that a different intercalating dye (named dye 2 in this writing) was employed. Information on intercalating dyes employed in AB ANALITICA devices is covered by industrial secret and will not be disclosed.

4.1.11. AMPLIQUALITY HPV-TYPE

The AMPLIQUALITY HPV-TYPE device (AB ANALITICA) allows genotyping of those samples that result positive after GP5+/G6+ PCR or a PCR performed using other primers targeting the same region in HPV genome. It is based on reverse line blot (RLB) technology. Basically, a membrane strip is spotted with probes reproducing a stretch of the HPV genome localized between GP5+ and GP6+ primers. Probes specific for 29 different genotypes (HPV 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82) are spotted at different heights on the strip to reproduce the band pattern showed in Fig. 37. An HPV universal band (HPV UNI)[11] is generated as well, spotting together several degenerated probes. A band specific for an internal control gene (*TST*), used in some of the AB ANALITICA's compatible kits is spotted above the UNI band and the HPV specific bands. Finally a "Control Staining" band is spotted at the top of the strip and represents a control for the colorimetric assay step. Once PCR has been performed, the amplification product is spread over the strip and let hybridize to the probes. Stringent washes allow the amplification product to be retained only where the complementary between the probes and the amplicons is perfect. The amplification products need to be

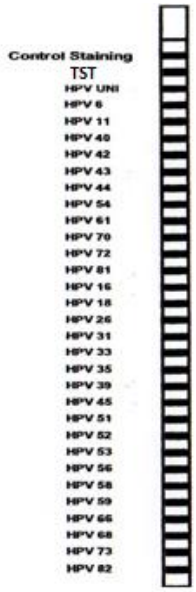

Fig. 37. Band pattern of the AMPLIQUALITY HPV-TYPE strip. Probes for 29 HPV genotypes are spotted at different heights. A HPV universal band, a TST specific band and a Control Staining band are spotted as well at the top of the strip.

[11] In case a sample is positive for an HPV type different from the 29 recognized by the device, the HPV UNI BAND will still signal that the sample is positive.

biotinylated for the following step to work. This can be achieved either by using biotinylated primers or by linking biotin to the amplicons after the amplification step. The biotinylation is necessary to perform a colorimetric assay, based on streptavidin conjugated alkaline phosphatase and NBT/BCIP substrate, which produces a dark band were the amplicons have hybridized (Fig. 38).

The device was used, according to manufacturer instruction, to genotype samples that resulted positive after amplification with the new version of the REALQUALITY RI-HPV STAR device.

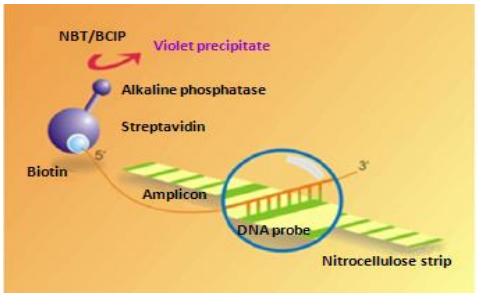

Fig. 38. Cartoon representing a RLB colorimetric assay based on biotin labeled amplicons, streptavidin coniugated alkaline phosphatese and NBT/BCIP substrate.

4.1.12. AMPLIQUALITY TYPE-EXPRESS V. 2.0 (REFERENCE METHOD)

AMPLIQUALITY TYPE-EXPRESS v. 2.0 (AB ANALITICA) was used, following manufacturer instructions, to genotype all sample that were later used to set up the reaction for the new version of the REALQUALITY RI-HPV STAR device and to verify its performance. This product is based on end-point PCR targeting the L1 region of HPV genome, followed by reverse line blot. It recognizes 29 different HPV types (HPV 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82).

4.2. DEVELOPMENT AND VALIDATION OF THE REALQUALITY RQ-HPV HR MULTIPLEX AND THE REALQUALITY RQ-HPV HR/LR MULTIPLEX DEVICES

4.2.1. CERVICAL SWAB SAMPLES

See paragraph 4.1.1.

4.2.2. URETHRAL, ANAL, FORESKIN AND VAGINAL BIOPSIES

Urethral, anal, foreskin and vaginal biopsies were purchased from Cerba Specimen Services (Saint Ouen l'Aumône, France).

4.2.3. URINE SAMPLES

Urine samples were purchased from Cerba Specimen Services (Saint Ouen l'Aumône, France)

4.2.4. DNA EXTRACTS FROM ORAL, URETHRAL AND VAGINAL SWAB SAMPLES AND FROM VAGINAL DISCHARGE SAMPLES

DNA extracts from oral, urethral and vaginal swab samples and from vaginal discharge samples were provided from the Department of Pathology "Gaetano Barresi" of the University of Messina (A.O.U. Policlinico G. Martino, Messina).

4.2.5. ARTIFICIAL SAMPLES

Artificial samples for HPV 16 and 18 were prepared by diluting plasmids carrying a fragment of the respective E6/E7 sequence in genomic background to mime clinical samples. Genomic DNA was extracted from HPV negative cervical samples. Plasmids were produced by GC cloning (Lucigen) of PCR fragments.
Artificial samples positive for other pathogens were either Acrometrix controls (Life Technologies) or Vircell controls (Vircell Microbiologists).

4.2.6. GLOBAL HPV LABNET 2013 HPV DNA GENOTYPING PROFICIENCY PANEL

AB ANALITICA received the panel from the WHO HPV LabNet in 2013 in the context of an international proficiency study for HPV genotyping test. The panel composition is summarized in table XX.

WHO SAMPLE	GENOTYPE	CONCENTRATION (GE/5µl)
1	*58*	*50*
2	*18*	*50*
3	*39, 45, 52, 56, 68b*	*500*
4	*33*	*50*
5	*52*	*500*
6	*68b*	*500*
7	*6*	*500*
8	*39*	*50*
9	*66*	*500*
10	*35, 59, 66, 68a*	*500*
11	*16*	*50*
12	*31*	*50*
13	*52*	*50*
14	*68a*	*500*
15	*negative*	*0*
16	*51*	*500*
17	*31*	*500*
18	*11, 31, 33, 58*	*500*
19	*56*	*50*
20	*68b*	*50*
21	*35, 59, 66, 68a*	*50*
22	*6*	*50*
23	*45*	*50*
24	*59*	*50*
25	*39, 45, 52, 56, 68b*	*50*
26	*18*	*5*
27	*35*	*50*
28	*59*	*500*
29	*6, 16, 18, 51*	*500*
30	*16*	*5*
31	*35*	*500*
32	*58*	*500*
33	*11, 31, 33, 58*	*50*
34	*11*	*50*
35	*45*	*500*
36	*66*	*50*

WHO SAMPLE	GENOTYPE	CONCENTRATION (GE/5µl)
37	*6, 16, 18, 51*	*50*
38	*11*	*500*
39	*33*	*500*
40	*39*	*500*
41	*56*	*500*
42	*68a*	*50*
43	*51*	*50*
A	*none*	*0*
B	*16*	*2500*
C	*16*	*25*

Table 16. Composition of the WHO proficiency panel from 2013.

4.2.7. QCMD 2014 HUMAN PAPILLOMAVIRUS DNA EQA PANEL

AB ANALITICA received the QCMD panel in the context of the QCMD 2014 Human Papillomavirus DNA EQA Programme. The panel had been developed to assess the proficiency of laboratories in the detection and genotyping of HPV. Its composition is summarized in table 17.

SAMPLE	MATRIX	CONTENT	STATUS	TYPE
HPV14-02	Pre servCyt + BSM	HPV16 (Caski)	Positive	Core
HPV14-06	Pre servCyt + BSM	HPV18 (Hela)	Positive	Core
HPV14-01	Pre servCyt + BSM	HPV45 (CC10b)	Positive	Core
HPV14-09	Pre servCyt + BSM	HPV16 /18 (Caski/Hela)	Positive	Core
HPV14-10	Pre servCyt + BSM	Lo w Vi r al Lo ad HPV16 (SiHa)	Positive	Educational
HPV14-08	Pre servCyt + BSM	HPV5 1&5 2 (clinical sample)	Positive	Educational
HPV14-04	Pre servCyt + BSM	HPV5 2 &5 6 (clinical sample)	Positive	Educational
HPV14-07	Pre servCyt + BSM	HPV5 4&5 6 (clinical sample)	Positive	Educational
HPV14-03	Pre servCyt + BSM	HPV Negative (BSM)	Negative	Core
HPV14-05	Pre servCyt + BSM	HPV Negative (BSM)	Negative	Core

Table 17. Composition of the QCMD panel from 2014.

4.2.8. PCR / REAL-TIME PCR INSTRUMENTS

End-point PCR was performed on the Applied Biosystems 2720 Thermal Cycler instrument (Applied Biosystems). Real-time PCR was performed on 7500 Fast Dx Real-Time PCR System (Applied Biosystems) and on Dx Real-Time System (BIO-RAD). Run files produced with 7500 Fast Dx Real-Time PCR System were analyzed with software version 1.4. Run files analysed with Dx Real-Time System were analyzed with software version 1.7.

4.2.9. DNA EXTRACTION

Viral and genomic DNA from cervical samples was extracted using the GENEQUALITY X120 instrument (AB NALITICA) and the GENEQUALITY X120 Pathogen kit (AB ANALITICA), following manufacturer instructions.
Biopsies were pre-treated with proteinase K before performing DNA extraction with the EZ1 Advanced XL instrument (QIAGEN) and the EZ1 DSP Virus kit (QIAGEN), following manufacturer instructions.
Urine samples were concentrated before extraction with the EZ1 Advanced XL instrument (QIAGEN) and the EZ1 DSP Virus kit (QIAGEN), following manufacturer instructions.

4.2.10. DESIGN OF GENOTYPE SPECIFIC PRIMERS AND PROBES

Primers and probes had to be genotype specific, thus, sequence relatedness issues had to be considered before selecting candidate oligos. The E6/E7 sequences of 32 HPV types (the 22 target genotypes plus other 10 low risk types) were retrieved from GenBank. Clustal Omega was used to align the sequences and generate a phylogenetic tree (Fig. 39). This tree was used as a guide to predict which types could have been, more likely, the cause of aspecific PCR products. For example, the design of HPV 18 primers and probe was done looking at the aligned sequences of HPV 18 and HPV 45, the closest genotype in the phylogenetic tree, in order to select DNA segments carrying mismatches for the non-specific sequence.

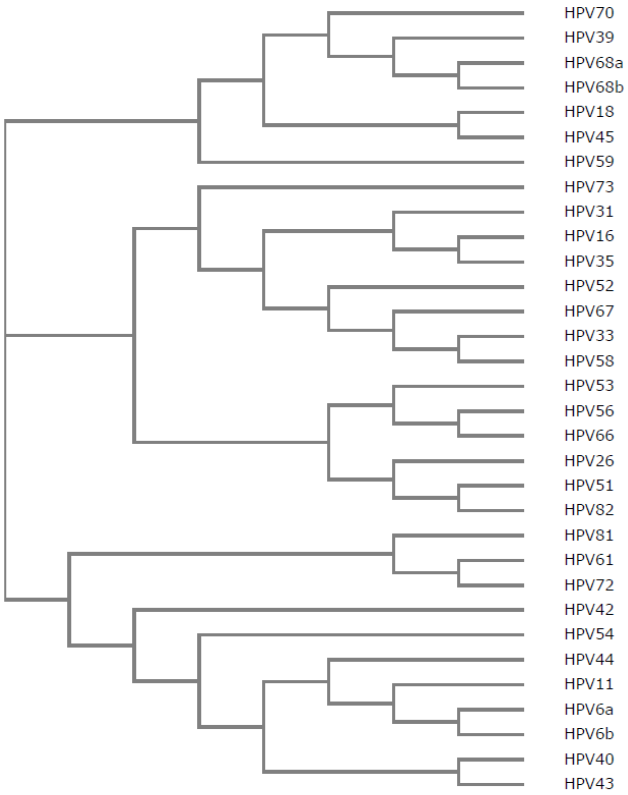

Fig. 39. Phylogenetic tree based on the E6/E7 sequences of 32 HPV genotypes (Clustal Omega).

Also, before starting the acutal design, several papers providing primer and probe sequences targeting the HPV E6/E7 region were consulted. Genotype specific primers form the literature tended to be concentrated in delimited portions of the E6/E7 sequence, suggesting they might have been favorable. When possible, primers and probe were designed in those regions. In Fig. 40, the E6/E7 sequence of HPV 18 is reported. Primers from four papers (66-69), all localizing in the lower portion of the sequence, are highlighted in different colors.

Another thing that had to be taken into account was that the thermal profile of the new PCR reactions had to be the same of all the other devices targeting infective pathogens in AB ANALITCA's catalogue (table 18). Primers and probe were all designed without the help of designing tools, however, to check whether the annealing temperature was correct, Primer3 was used.

HPV 18 E6/E7

ATTAATACTGGGTTTAACAATTGTAGTATATAAAAAAGGGAGTAACCGAAAACGGTCGGGACCGAAAACGGTGTATATAAAAGAT
GTGAGAAACACACCACAATACTATGGCGCGCTTTGAGGATCCAACACGGCGACCCTACAAGCTACCTGATCTGTGCACGGAACTG
AACACTTCACTGCAAGACATAGAAATAACCTGTGTATATTGCAAGACAGTATTGGAACTTACAGAGGTATTTGAATTTGCATTTAAA
GATTTATTTGTGGTGTATAGAGACAGTATACCCCATGCTGCATGCCATAAATGTATAGATTTTTATTCTAGAATTAGAGAATTAAGA
CATTATTCAGACTCTGTGTATGGAGACACATTGGAAAAACTAACTAACACTGGGTTATACAATTTATTAATAAGGTGCCTGCGGTG
CCAGAAACCGTTGAATCCAGCAGAAAAACTTAGACACCTTAATGAAAAACGACGATTTCACAACATAGCTGGGCACTATAGAGGC
CAGTGCCATTCGTGCTGCAACCGAGCACGACAGGAACGACTCCAACGACGCAGAGAAACACAAGTATAATATTAAGTATGCATGG
ACCTAAGGCAACATTGCAAGACATTGTATTGCATTTAGAGCCCCAAAATGAAATTCCGGTTGACCTTCTATGTCACGAGCAATTAA
GCGACTCAGAGGAAGAAAACGATGAAATAGATGGAGTTAATCATCAACATTTACCAGCCCGACGAGCCGAACCACAACGTCACAC
AATGTTGTGTATGTGTTGTAAGTGTGAAGCCAGAATTGAGCTAGTAGTAGAAAGCTCAGCAGACGACCTTCGAGCATTCCAGCAG
CTGTTTCTGAACACCCTGTCCTTTGTGTGTCCGTGGTGTGCATCCCAGCAGTAAGCAACAATGGCTGATCCAGAAGGTACAGACGG
GGAGGGCACGGGTTGTAACGGCTGGTTTTATGTACAAGCTATTGTAGACAAAAAAACAGGAGATGTAATATCAGATGACGA

Fig. 40. The E6/E7 sequence of HPV 18 is reported with primers form four papers highlighted in different colors (light green and light blue (66), yellow (67), grey (68), dark green(69)). In case of overlapping primers the color of the upstream primer is retain and the overlapping fragment is in bold.

TEMPERATURE	TIME	CYCLES
50 °C	2’	1
95 °C	10’	1
95 °C	15’’	45
60 °C	1’	

Table 18. Thermal profile of AB ANALITICA’s devices for the detection of infective pathogens.

Once selected a candidate primer or a probe, analytical specificity was evaluated *in silico* by BLAST analysis. Firstly, its sequence was aligned against the specific target organism (e.g. taxid: 333761 for HPV 18) to check whether there were genotype variants carrying mismatches in that stretch of DNA. If there was more than one mismatch for a group of variants or, only for primers, if a mismatch was located at the 3’end of the sequence, within the fourth nucleotide, then the oligonucleotide was discarded. If the first step went ok, the oligonucletide was blasted against all alphapapillomaviruses excluding the specific target (e.g. taxid:333750, excluded taxid: 333761). To be considered ok, the oligonucleotide had to align to other HPV types sequences with at least five dispersed mismatches or, only in case of primers, with at least three mismatches at the 3’end, within the fifth nucleotide. If ok, it was aligned against all organisms, excluding alphapapillomaviruse, and against human genomic DNA to check for other possible cross-reactivity phenomenon. The “five mismatch / three mismatch at the 3’end rule” was applied also here. If alignments with other organisms had occurred, they would have been taken into account only if those species could

have infected the same districts as HPV; however, such a circumstance never presented.

4.2.11. VERIFYING PRIMER ANALYTICAL SPECIFICITY: SIMPLEX REAL-TIME PCR WITH INTERCALATING DYE

For some genotypes, different primer pairs were ordered and tested in simplex by Real-time PCR with intercalating dye to select the best performing one, before ordering the respective probe. Information on primer/probe sequences, on primer/probe concentration and on the employed mastermix and intercalating dye is covered by industrial secret and will not be disclosed. The thermal profile was the same used in the final version of assay 1 and 2 (see paragraph 4.2.14).

4.2.12. VERIFYING PRIMER/PROBE ANALYTICAL SPECIFICITY: SIMPLEX REAL-TIME PCR

To check if primer pairs and probes were specific for the target genotypes, simplex Real-time PCR was performed. Information on primer/probe sequences, on primer/probe concentration and on the employed mastermix is covered by industrial secret and will not be disclosed. The thermal profile was the same used in the final version of assay 1 and 2 (see paragraph 4.2.15).

4.2.13. PARTIAL MULTIPLEX REAL-TIME PCR

Partial multiplex Real-time mixes were assembled by adding new target specific primers and probes two by two. The partial mixes were tested on known HPV positive and negative clinical samples in order to check whether the newly added oligos could interfere with the others. Information on primer/probe sequences, on primer/probe concentration and on the employed mastermix is covered by industrial secret and will not be disclosed. The thermal profile is the same used in the final version of assay 1 and 2 (see paragraph 4.2.15).

4.2.14. AGAROSE GEL ELECTROPHORESIS

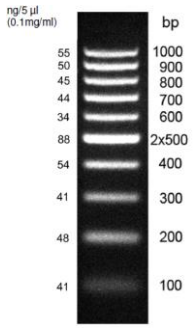

Fig. 41. 100 bp DNA Ladder ready-to-use (Bioron)

Agarose gel electrophoresis was performed by loading 10 µl of PCR product on a 3% agarose gel. 100 bp DNA Ladder ready-to-use (Bioron) was employed (Fig. 41).

4.2.15. ASSAY 1 REAL-TIME PCR

Assay 1 Real-time PCR includes primers and probes for HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68 as well as a primer pair and a probe for *HBB*. It is a four fluorophore Real-time reaction. The correspondence between targets and fluorophors is summarized in table 19.

Assay	JOE	CY5	FAM	ROX
1	HPV 16	HPV 18	HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	***HBB*** (Internal control)

Table 19. Correspondence between targets and fluorophors for assay 1.

Information on primer/probe sequences, on primer/probe concentration and on the employed mastermix is covered by industrial secret and will not be disclosed. UNG (Uracil N-Glycosylase) enzyme was added to the mix in order to prevent carry-over contamination. The thermal profile is summarized in table 20.
Assay 1 corresponds to the REALQUALITY RQ-HPV HR MULTIPLEX device.

TEMPERATURE	TIME	CYCLES
50 °C	2’	1
95 °C	10’	1
95 °C	15’’	45
60 °C	1’	

Table 20. Thermal profile of assay 1.

4.2.16. ASSAY 2 REAL-TIME PCR

Assay 1 Real-time PCR includes primers and probes for HPV 6, 11, 26, 53, 67, 70, 73 and 82 as well as a primer pair and a probe for *HBB*. It is a four fluorophore Real-time reaction. The correspondence between targets and fluorophors is summarized in table 21.

Assay	JOE	CY5	FAM	ROX
2	**HPV 6** (low risk)	**HPV 11** (low risk)	HPV **26, 53, 67, 70, 73, 82** (probabile high risk)	*HBB* (internal control)

Table 21. Correspondence between targets and fluorophors for assay 2.

Information on primer/probe sequences, on primer/probe concentration and on the employed mastermix is covered by industrial secret and will not be disclosed. UNG (Uracil N-Glycosylase) enzyme was added to the mix in order to prevent carry-over contamination. The thermal profile is the same used in the final version of assay 1 (see paragraph 4.2.15).
Assay 2, if associated to assay 1, provides the final user with a complete panel for HPV investigation. Assay 1 and 2, together, correspond to the REALQUALITY RQ-HPV HR/LR MULTIPLEX device.

4.2.17. A, B, C, D, E REAL-TIME REACTIONS

A, B, C, D, E Real-time reactions were used to verify the presence of an “assay 1 FAM genotype[12]” in samples that produced unexpected FAM signals during assay

[12] HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68.

1 validation[13]. All reactions contained the same primers as assay 1, but only a subset of probes. A scheme of how probes were allocated among the five reactions is represented in Fig. 42.

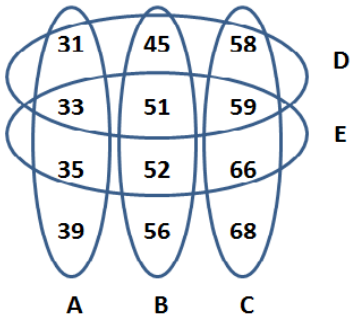

Fig. 42. Scheme representing how assay 1 FAM probes were allocated among mixes A, B, C, D and E.

If present in mono-infection, each FAM genotype gives an univocal pattern of positivity among the five mixes (table 22).

GENOTYPE	MIX
31	A, D
33	A, D, E
35	A, E
39	A
45	B, D
51	B, D, E
52	B, E
56	E
58	C, D
59	C, D, E
66	C, E
68	E

Table 22. Pattern of positivity in mixes A, B, C, D, E for the 12 FAM genotypes of assay 1.

Information on primer/probe sequences, on primer/probe concentration and on the employed mastermix is covered by industrial secret and will not be disclosed.

[13] Samples that were not positive for any of the "assay 1 FAM genotypes", according to the reference method (HPV-TYPE EXPRESS v. 3.0), but produced signals in the FAM channel, when tested with assay 1.

4.2.18. F, G, H, I REAL-TIME REACTIONS

F, G, H, I Real-time reactions were used to verify the presence of an "assay 2 FAM genotype[14]" in samples that produced unexpected FAM signals during assay 2 validation[15]. All reactions contained the same primers as assay 2, but only a subset of probes. A scheme of how probes were allocated among the four reactions is represented in Fig. 43.

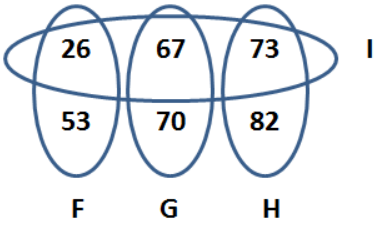

Fig. 43. Scheme representing how assay 2 FAM probes were allocated among mixes F, G, H, and I.

If present in mono-infection, each FAM genotype gives an univocal pattern of positivity among the five mixes (table 23).

GENOTYPE	MIX
26	F, I
53	F
67	G, I
70	G
73	H, I
82	H

Table 23. Pattern of positivity in mixes F, G, H, I for the 6 FAM genotypes of assay 2.

Information on primer/probe sequences, on primer/probe concentration and on the employed mastermix is covered by industrial secret and will not be disclosed.

[14] HPV 26, 53, 67, 70, 73, 82.

[15] Samples that were not positive for any of the "assay 2 FAM genotypes", according to the reference method (HPV-TYPE EXPRESS v. 3.0), but produced signals in the FAM channel, when tested with assay 2.

4.2.19. END-POINT PCR AND SEQUENCING OF DISCORDANT SAMPLES

Samples used for validating assay 1 and 2 were also tested with a reference method (AMPLIQUALITY HPV-TYPE EXPRESS, v. 3.0; AB ANALITICA). In case of discordant results, sequencing was used to confirm the genotype composition of the sample.

In case assay 1 or assay 2 detected a genotype not detected by the reference method, simplex end-point PCR was performed, with the primers for the genotype of interest[16] used in assay 1 or assay 2 reactions. The PCR product was then sent to BMR Genomics for sequencing. In case the reference method detected a genotype not detected by assay 1 or assay 2, the primers specific for the genotype of interest, employed in the reference method reaction, were used to perform simplex end-point PCR on the discordant sample. The PCR product was then sent to BMR Genomics for sequencing.

Information on primer sequences, on primer concentration and on the employed mastermix is covered by industrial secret and will not be disclosed.

4.2.20. PROBIT ANALYSIS

Probit analysis was used to determine the limit of detection of assay 1 for HPV 16 and HPV 18. Artificial samples containing the genotype of interest at five different concentrations (7.5 – 5 – 2.5 – 1.25 – 0.5 GE/rx) were used. To produce a sufficient amount of data for the probit model, each concentration was tested in 8 replicates for 3 consecutive runs. Each run output was converted to binary language, 1 representing a successful amplification and 0 representing a failed amplification. Table 24 is an example of a possible run output. Data were analyzed with R Software (p set to 0.05).

[16] In case of FAM unexpected signal the genotype had to be first identified with mixes A, B, C, D, E or mixes F, G, H, I.

	REPLICATE 1	REPLICATE 2	REPLICATE 3	REPLICATE 4	REPLICATE 5	REPLICATE 6	REPLICATE 7	REPLICATE 8
7.5 GE/ rx	1	1	1	1	1	1	1	1
5.0 GE/ rx	1	1	1	1	1	1	1	1
2.5 GE/ rx	1	1	0	0	1	1	1	1
1.25 GE/ rx	0	1	0	1	0	0	1	1
0.5 GE/ rx	1	0	0	0	1	0	1	0

Table 24. Run output converted to binary language for probit analysis (1 = successful amplification; 0 = failed amplification).

4.2.21. COEFFICIENT OF VARIATION

The Coefficient of Variation (CV) is a standardized measure of dispersion of a probability distribution or a frequency distribution. It is defined as the ratio of the standard deviation σ to the mean μ, but is often presented as the given ratio multiplied by 100. In this work it was used to give a measure of intra- and inter-assay variability of assay 1 and was calculated as follows:

$$CV = \left(\frac{\sigma * 100}{\mu} \right)$$

4.2.22. INTRA-ASSAY VARIABILITY FOR ASSAY 1

To measure intra-assay variability for assay 1, 15 samples were used, each positive for one of the following genotypes detected by assay 1: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68a and 68b. Each sample was amplified in eight replicates in a single run. The CVs for the Ct (Threshold Cycle) values produced by each genotype were then calculated.

4.2.23. INTER-ASSAY VARIABILITY FOR ASSAY 1

To measure inter-assay variability for assay 1, 15 samples were used, each positive for one of the following genotypes detected by assay 1: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68a and 68b. Each sample was amplified in three consecutive runs. The CVs for the Ct (Threshold Cycle) values produced by each genotype were then calculated.

4.2.24. HPV TYPE-EXPRESS V. 3.0 (REFERENCE METHOD)

AMPLIQUALITY TYPE-EXPRESS v. 3.0 (AB ANALITICA) was used, following manufacturer instructions, to genotype all sample that were later used to set up the reactions for assay 1 and assay 2 and verify their performance. This product is based on end-point PCR targeting the L1 region of HPV genome, followed by reverse line blot. It recognizes 40 different HPV types (HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68a, 68b, 69, 70, 71, 72, 73, 81, 82, 83, 84, 87, 89 and 90).

5. DISCUSSION

In the first part of my PhD, I worked on the prototype new version of a product recently developed at AB ANALITICA, the REALQUALITY RI-HPV STAR device. My task was to bring the original device to an higher level of analytical sensitivity for certain HPV genotypes as required by the World Health Organization.

The primers used in the original reaction mix were GP5+/GP6+ primers. These oligos are among those developed at the beginning of the HPV detection era. They target the most conserved region in HPV genome, i.e. L1, and are capable of detecting most HPV types in aggregate. However, the analytical sensitivity they provide is not equal for all the detected genotypes (62) and their performance was not in compliance with WHO requirements for genotyping tests.

First of all, the primer composition in the reaction mix was modified by adding other primers to the original pair. A multiple primer reaction required employing specific reagents and, at the end, the whole set up of the assay had to be modified. The occasion was used to also improve an ergonomic aspect and amplify the target pathogen and the internal control gene in multiplex instead of running two separate reaction as in the original assay.

Higher analytical sensitivity was achieved, but at the expense of robustness. Discrimination between positive and negative sample was based on melting curve analysis with intercalating dye technology. The presence of a melting peak in a defined range of melting temperature would have meant the sample was HPV positive. The genotype was than to be determine by RLB with the AMPLIQUALITY HPV-TYPE device.

Unfortunately, primer dimers related problems emerged. The corresponding melting peaks, in most cases, were located before the HPV positivity range. However, while evaluating clinical specificity on HPV negative samples, I randomly observed primer dimers peaks with shoulders invading the HPV positivity range. The Real-time PCR instrument never recognized those shoulders as actual peaks, nevertheless, they were clearly visible by eye. First of all, such an output could have generated confusion in the final user. Secondly it was not

possible to foresee whether the problem could have exacerbated under standard production conditions, that require frequent changes in the reagent batches used, and whether, at a certain point, the Real-time PCR instrument would have started to recognize those shoulders as real peaks. I tested different reaction conditions in order to solve the problem, with no improvements. On that premise, abandoning the project was considered the only feasible possibility, as the risk of releasing on the market a product generating an uncontrollable number of false positive results was not acceptable.

The use of intercalating dyes for Real-time PCR and melting curve analyses is convenient and cost effective, as it allows to detect multiple targets without the need to produce or order specific probes. On the other hand, with this technology, both specific and aspecific signals are visualized in the Real-time and melting plots. In a reaction with multiple primers, like that of the new version of the REALQUALITY RI-HPV STAR device, the generation of PCR by-products, such as primer dimers, was a highly probable event and, as expected, occurred with nearly every tested sample. The device could still have worked efficiently if the HPV positivity range had remained uninvolved, but this was not the case. Probably, melting curve analysis employing an intercalating dye did not represent the best choice for the intended application due to the above mentioned intrinsic limitations.

In the second part of my PhD, I worked at the design, development and validation of two new assays for HPV detection which also provide partial genotyping information. In this occasion, it was decided to abandon the RLB technology, which can result cumbersome and time consuming for the final user, and rely only on Real-time PCR, which, on the contrary, is a very popular technology in the field of diagnostics, as it is simple and does not require too much hand-on time for the final user. Melting curve analysis, however, was not considered as an option, this time, due to the problems experienced with the previous project. Instead, Real-time PCR with target specific primers and TaqMan fluorescent probes was employed.

Another significant difference with respect to the previous project is that the target region of the new devices is E6/E7 instead of L1. This choice was due to both

commercial and practical reasons. A key event in HPV-induced carcinogenesis is the integration of HPV genome in one of the host chromosomes, which can, in some rare cases, lead to the loss of L1, but not of E6/E7 (50). Since residual integrated HPV DNA can, ultimately, be the only form of viral DNA present in infected cells, devices based on L1 detection might miss a small, but yet significant, portion of infections, whereas E6/E7 targeting devices will not (63). Targeting E6/E7, thus, represents a strong marketing feature, especially considering that devices from most competitors target L1(11). On the practical side, being L1 sequence the most conserved in HPV genome, the design of type specific primers and probes in that region would probably have been much more difficult.

The two assay were design in order to target all the HPV genotypes necessary to ensure compliance with the WHO requirements for a genotyping assay (i.e. HPV 6, 11, 16, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) plus other possible high risk genotypes, according to IARC classification (31).

Once the reactions for the two assays were set up, they had to be validated. 320 samples were employed during the validation of the assays targeting HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68 (assay 1), whereas 192 samples were used to validate the assay targeting HPV 6, 11, 26, 53, 67, 70, 73 and 82 (assay 2). Both assays were validated on two instruments for Real-time PCR, ABI 7500 Fast Dx and Biorad Dx. Clinical sensitivity and specificity for both assays on both instruments resulted adequate, being above 95%. The 95% cut-off is not a universally established threshold for clinical sensitivity and specificity assessment for devices of this kind, however, is not arbitrary. Before starting the validation process in an industrial context, a validation plan has to be complied in which criteria for acceptable performance are stated. The 95% cut-off was established on the base of two main considerations: 1) all methods for HPV detection differ in the analytical sensitivity for different HPV types, thus, complete concordance on clinical samples is not a probable occurrence, especially when testing samples at limit concentrations; 2) competitors declare clinical sensitivity and specificity performance in the 95-99% range.

Data from the validation set were also used to calculate genotype concordance between assay 1 and the reference method. This resulted to be around 90%. Again

different analytical sensitivity for specific genotypes accounts for different results from the two methods. Moreover, for those cases in which assay 1 detected the presence of a high risk genotype (later confirmed by sequencing), while the reference method only detected low risk genotype or no HPVs at all, the result could be attributed to the fact that the reference method targets L1 and, therefore, can miss HPV infection in case of complete integration of viral genome with disruption of the L1 ORF. However, being those samples all associated to negative or ASCUS cytological reports and being complete integration associated to late stadiums of cancer progression, this hypothesis does not seem to fit circumstances.

As for analytical sensitivity, compliance with the WHO requirements was tested using the WHO proficiency panel from 2013. Not only all genotypes (HPV 6, 11, 16, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) were detected at the required limit concentration, but, also, they were correctly identified at a 10-fold lower concentration. For HPV 16 and 18, the two most dangerous HPV types, the exact LOD was established with probit analysis. The results confirmed that the analytical sensitivity for those genotypes is one order of magnitude higher than that required by WHO.

WHO proficiency panels are provided in the context of international proficiency studies. When the WHO release a panel, there is a window of time for result submission, then the panel composition is reveled and official responses on assay performance are sent to study participants. When I tested the two devices on the WHO panel from 2013, it was too late to submit the results. The panel for 2014 is not available yet due to production issues, however, as soon as it will, both assays will be tested on the new panel and results submitted in order to receive an official response.

In 2014, however, AB ANALITCA participated to another external quality assessment program, i.e. the QCMD 2014 Human Papillomavirus DNA EQA Program. Only assay 1 was ready by the time results could be submitted, thus, was the sole to be tested with the QCMD panel. This panel comprises both core samples, i.e. samples that have to be detected by a device to be declared proficient, and educational samples, i.e. samples that provide participants with additional information on their assay sensitivity. Assay 1 was capable to correctly

identify all core samples. Only one of the educational samples was erroneously identified as HPV negative, while being a low viral load HPV 16 positive sample, instead. It has to be outlined, however, that, as the QCMD referred, this sample did not result positive also when analyzed by an external testing laboratory using the Cobas HPV assay (ROCHE) and the HC2 assay (QIAGEN), both FDA approved and CE IVD marked methods.

Other performance parameters, such as analytical specificity and repeatability have already been assessed only for assay 1. Assessment for assay 2 is ongoing.

As for the analytical specificity of assay 1, both low risk HPV positive samples and samples positive for other pathogen were tested. Samples of the latter group all resulted negative except for one sample that was later demonstrated to actually contain high risk HPV DNA. Of the 54 samples positive for low risk HPV types, two produced unexpected FAM signals, with late Ct values, only on the ABI 7500 Fast Dx instrument. The presence of high risk genotypes, though, could not be demonstrated as subsequent amplifications finalized to sequencing did not produced any PCR product. According to the reference method, AMPLIQUALITY HPV-TYPE EXPRESS v. 3.0, one of the two samples was positive for HPV 6 and 87, the other for HPV 40 and 84. Cross-reactivity with these genotypes, however, can be excluded as other samples positive for the same types did not produce any amplification signal. HPV 6 samples, in particular, are also comprised in the WHO panel and, when amplified with assay 1, did not generate unexpected signals. A possible explanation to these result is that those two samples were actually positive for an high risk HPV type, but its concentration was below the limit of detection of the reference method and close the limit of detection of assay 1 that, therefore, produced non-reproducible results among instruments and in subsequent amplifications. On the base of these considerations, the analytical specificity of assay 1 was regarded as adequate.

As for repeatability, intra- and inter-assay coefficient of variation for all HPVs targeted by assay 1 were calculated and all resulted below 1.5%, proving that assay 1 is highly repeatable.

In conclusion assay 1 performance is largely in compliance with WHO requirements and with the acceptability criteria established in the validation plan, thus, this assay will be soon commercialized under the name REALQUALITY

HPV-HR MULTIPLEX. As for assay 2, some performance parameters have still to be assessed, but initial data are promising. This second assay, if associated to assay 1, provides the final user with a complete panel for HPV investigation. For this reason assay 1 and 2, together, will be proposed, as an alternative product, under the name REALQUALITY RQ-HPV HR/LR MULTIPLEX.

Both products will need CE IVD marking and FDA approval as AB ANALITICA intends to sell them in Europe and US. The combined product will also be submitted for CFDA (Chinese Food and Drug Administration) approval with a slight modification to the panel of detected genotypes. In brief, assay 1 will remain the same, whereas assay 2 will allow the pooled detection of HPV 6 and 11 (most relevant low risk types) in the Cy5 channel, the pooled detection of HPV 26, 53, 67, 70, 73 and 82 (possible high risk types) in the FAM channel and the pooled detection of HPV 40, 42, 43, 44, 55 and 83 (low risk types) in the JOE channel. The Chinese panel of detectable genotypes was design taking into consideration the suggestions from one of AB ANALITICA's Chinese distributors. The assessment of the performance of the Chinese product is ongoing.

As mentioned several times, compliance with WHO requirements for HPV genotyping assays has been a pivotal aspect of my whole work; however, assay 1 would also make a good screening device as it is high risk HPV specific and allows fast analysis of multiple samples. For these reasons, AB ANALITICA is considering to validate it for the scope of screening, following the instruction provided in the landmark paper written by Meijer *et al*. (61). In order to be considered adequate for screening, assay 1 should be tested on at least 60 histologically confirmed CIN 2+[17] positive samples and at least 800 histologically confirmed CIN 2+ negative samples. All samples should have been previously tested with an already approved screening method (e.g. HC2). Sensitivity and specificity of assay 1 with respect to the reference method should be of at least 90% and 98% respectively.

Beside cervical cancer, HPV seems to be responsible for a considerable proportion (40%) of head and neck cancers (27). While validating assay 1, also 30 oral swabs samples were tested. Three of these samples resulted positive for an

[17] CIN2+ category comprises CIN2, CIN3 and cancer.

high risk HPV type with both assay 1 and the reference method (already validated on this kind of specimens). All the other samples resulted negative with both devices. This result indicates that assay 1 could be adequate to assess HPV positivity also in oral swabs, however a higher number of positive sample will have to be tested in order to confirm the result.

APPENDIX

SUMMARY

The subject of this dissertation is the work I carried out at AB ANALITICA s.r.l. in the context of a doctoral program based on higher apprenticeship, an innovative form of PhD aiming at reducing the distance between academia and business.

In the first part of my PhD I worked on the new version of a product for HPV detection, recently developed at AB ANALITICA: the REALQUALITY RI-HPV STAR device. My task was to bring the original device to a higher level of analytical sensitivity for certain HPV genotypes as required by the World Health Organization (WHO).
This device was based on Real-time PCR and melting curve analysis with intercalating dye technology. The primers used in the original reaction mix were GP5+/GP6+ primers. These primers are capable of detecting most HPV types in aggregate. However, the analytical sensitivity they provide is not equal for all the detected genotypes and their performance was not in compliance with WHO requirements for genotyping tests. Thus, first of all, the primer composition in the reaction mix was modified by adding other primers to the original pair. A multiple primer reaction required employing specific reagents and, at the end, the whole set up of the assay had to be modified. The occasion was used to also improve an ergonomic aspect and amplify the target pathogen and the internal control gene in multiplex instead of running two separate reactions as in the original assay.
Higher analytical sensitivity was achieved, but primer dimers related issues emerged, which could have impaired the correct interpretation of the assay results, and the project was abandoned.

In the second part of my PhD, I worked at the design, development and validation of two new assays for HPV detection, based on Real-time PCR with target specific primers and TaqMan fluorescent probes.

The HPV types detected by the first of the two assays are the 14 most common high risk types, i.e. HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68. The second assay detects the two most common low risk types, i.e. HPV 6, 11, as well as other possible high risk types, i.e. HPV 26, 53, 67, 70, 73 and 82.
Assay 1 has already been validated and its performance resulted largely in compliance with WHO requirements and with the acceptability criteria established in the validation plan. This assay, therefore, is ready to be commercialized under the name REALQUALITY RQ-HPV HR MULTIPLEX.
As for assay 2, some performance parameters have still to be assessed; however, initial data are promising. This second assay, if associated to assay 1, provides the final user with a complete panel for HPV investigation. The two assays combined, therefore, will be soon proposed, as an alternative product, under the name REALQUALITY RQ-HPV HR/LR MULTIPLEX.

RIASSUNTO

Oggetto di questa tesi è il lavoro svolto presso l'azienda AB ANALITICA s.r.l. nell'ambito di un Dottorato di Ricerca in Alto Apprendistato, una forma innovativa di dottorato che mira ad avvicinare il mondo dell'università a quello del lavoro.

Nella prima parte del mio percorso di dottorato ho lavorato ad un progetto che aveva come obbiettivo lo sviluppo di una nuova versione del prodotto REALQUALITY RI-HPV STAR, un dispositivo per la rilevazione dell'HPV commercializzato da AB ANALITICA. La nuova versione doveva innanzitutto corrispondere ai criteri di sensibilità analitica definiti dalla World Health Organization (WHO) per i dispositivi di genotipizzazione dell'HPV.
La tecnologia su cui si basa il dispositivo originale, e su cui, pertanto, si sarebbe dovuta basare la nuova versione, è la PCR Real-time con intercalante e analisi delle Curve di Melting. I primer utilizzati nella mix del prodotto originale sono i primer GP5+/GP6+. Si tratta di primer consensus che permettono di amplificare la maggior parte dei genotipi (o "tipi") di HPV in aggregato. La sensibilità analitica

di un sistema basato su questi primer non è omogenea rispetto ai diversi tipi di HPV identificabili e, per alcuni di essi, è inferiore a quanto richiesto dal WHO. Per prima cosa, si è stabilito di aggiungere altri primer alla coppia originale. In conseguenza di ciò, si è reso necessario l'impiego di reagenti idonei ad una reazione con molteplici primer ed, alla fine, l'intero assetto del saggio è stato modificato. Con l'occasione si è deciso di modificare un altro aspetto del saggio ed amplificare in multiplex, invece che in due reazioni distinte, il target patogeno ed il controllo interno. Lo scopo di ottenere un più alto livello di sensibilità analitica è stato raggiunto. Tuttavia, nel corso della pre-validazione del nuovo prototipo, sono emerse delle problematiche relative allo sviluppo di dimeri di primer che rischiavano di compromettere la corretta interpretazione dei risultati e che hanno portato alla sospensione del progetto.

Nella seconda parte del mio dottorato ho lavorato alla progettazione, allo sviluppo e alla validazione di altri due dispositivi per la rilevazione dell'HPV, basati, questa volta, su PCR Real-time con primer genotipo-specifici e sonde di tipo TaqMan.

I genotipi di HPV rilevati dal primo dei due saggi sono i 14 genotipi ad alto rischio più diffusi ovvero HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 e 68. Il secondo saggio rileva invece la presenza dei due HPV a basso rischio più diffusi, HPV 6 e 11, e di altri HPV a possibile alto rischio, ovvero HPV 26, 53, 67, 70, 73 e 82.

Il primo saggio è già stato validato e le sue performance rientrano abbondantemente nei parametri stabiliti dal WHO e dal piano di validazione redatto in azienda. Tale saggio risulta, quindi, pronto per essere commercializzato come dispositivo per la detection degli HPV ad alto rischio con il nome REALQUALITY RQ-HPV HR MULTIPLEX.

La validazione del secondo saggio è in corso, ma i primi dati sono promettenti. Il secondo saggio, se associato al primo, permette di offrire un pannello di indagine dell'HPV estremamente completo. Accanto al prodotto corrispondente al primo saggio, ne verrà, pertanto, proposto un secondo, corrispondente ai due saggi associati. Tale prodotto prenderà il nome di REALQUALITY RQ-HPV HR/LR MULTIPLEX.

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