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CURRICULUM: MOLECULAR AND CELLULAR PHARMACOLOGY

XXXII CYCLE

DEVELOPMENT AND VALIDATION OF *IN VITRO* DIAGNOSTIC DEVICES FOR HPV AND HIV-1

Thesis realized with the financial support of BIOFIELD INNOVATION S.r.l.

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TABLE OF CONTENTS

PREAMBLE

The object of my PhD studies was the development and validation of two diagnostic *in vitro* medical devices based on the Real time Polimerase chain reaction (PCR) methodic targeting respectively HPV DNA and HIV-1 DNA. Those studies were carried out in collaboration with BIOFIELD INNVATION S.r.l., an innovative start-up involved in the fields of biotechnology, mechatronics and Information and Communication Technology.

Real time PCR technique allows a rapid detection of the target nucleic acids and since it has been used, it has greatly improved the performance in diagnostic laboratories. This technology represents a powerful tool in the diagnostic field because it is able to detect and quantify small amount of nucleic acids.

In the first part of the dissertation, I deal with the validation of an assay for the identification of 14 high risk HPV genotypes according to references guideline, in order to assess if it is suitable for primary cervical cancer screening.

Whereas, the focus of the second part of this study was the design and the validation of an assay able to identify and quantify HIV-1 DNA.

ABSTRACT

Sexually transmitted viral infections represent a global health problem. More than one million of these infections are estimated to be acquired everyday globally. HPV and HIV-1 infections are two of the most studied because of their large diffusion and their link with important diseases. The HPV prevalence and the mortality linked to cervical cancer have decreased thanks to vaccination and to screening programs. In this study, the performance of an assay detecting 14 high risk HPV genotypes, based on the Real time PCR methodic, has been tested, in order to verify if the device is suitable for the use in cervical cancer screening according to Meijer's guideline. The validation tests were performed in order to define the parameters of clinical specificity, sensitivity, intra- and inter-reproducibilities of this assay. These tests proved it performed well and it is suitable for the use in primary cervical cancer screening. Moreover, the evaluation of analytical and diagnostic performance suggests that this HPV detection device fits the requirements of the Directive 98/79/CE regarding the *in vitro* diagnostic medical devices, therefore it is suitable for commercialization.

Regarding HIV-1 infections, the quantification of viral DNA is a fundamental parameter for the therapeutic management of positive patients, however its application in the diagnostic routine is delayed by the lack of standardized systems available in commercial formulations. Particularly, the evaluation of HIV DNA levels before and during antiretroviral therapy can be useful to evaluate a therapy change or suspension and to assess its effectiveness. On this basis, the development and validation of a Real time PCR device for the identification and the quantification of the HIV-1 DNA able to satisfy the performance requirements for its use on the diagnostic field, were carried out. The assay's validation, which allowed evaluating its analytical and diagnostic performance, indicates that it is suitable for the use in the research field. Indeed, it has already been commercialized as a Research Use Only (RUO) device.

1. INTRODUCTION

Sexual transmitted infection (STI) pathogens are spread mainly by sexual activity including vaginal, anal and oral sex. Some STIs can also be transmitted through non-sexual means such as via blood or others body fluids. Many STI pathogens, including *Chlamydia, Neisseria gonorrhoeae*, primarily *hepatitis B*, *Human Immunodeficiency Virus* (HIV), and *Syphilis* can also be transmitted from mother to child during pregnancy and childbirth. About thirty different STIs are known, caused by over twenty types of microorganisms including bacteria, viruses, protozoa and parasites [1]. Eight of these pathogens are linked to the greatest incidence of sexually transmitted diseases. Among these eight infections, four are currently curable: *Syphilis, Neisseria gonorrhoeae, Chlamydia* and *Trichomoniasis*. The other four are viral infections and are incurable, but their symptoms or disease can be reduced through treatments. The viruses that cause that kind of infections are: *Hepatitis B*, *Herpes Simplex virus* (HSV), HIV, and *Human Papillomavirus* (HPV).

Sexually transmitted viral infections represent a global health problem. The World Health Organization (WHO) estimated that more than one million STIs are acquired everyday. The prevalence of HPV infections is estimated at 440 milion people worldwide, causing 510 thousand cases of cervical cancers [2]. According to WHO data, a total of 37.9 million people of all ages were living worldwide with HIV during 2018. And, among them, 25.7 million cases of HIV infections were noticed in Africa.

1.1. HUMAN PAPILLOMA VIRUS (HPV)

Human papillomaviruses (HPV) are non-enveloped double-stranded DNA viruses that belong to the *Papillomaviridae* taxonomic family [3]. They form a large and different group of viruses, with new HPV types being continuously found [4]. Several hundred species of papillomaviruses, traditionally referred to as "types" have been identified infecting not only mammals, but also other vertebrates such as birds.

The circular DNA of the Papillomaviruses (PVs) is approximately 8 kb in size and tipically contains eight different genes. In spite of their small size, their structure is very complex. The PVs genome is constituted by three domines: the Long Control Region (LCR) that regulates the expression and viral replication, the Early Region (E), composed by six different genes: E1, E2, E4, E5, E6, E7 that encode for proteins that interact with the host genome to replicate viral DNA and finally the Late Region (L), that encodes for structural proteins L1 and L2, expressed after DNA replication. The E1, E2, E4, E5, E6, E7, L1 and L2 constitute the open reading frame (ORF) of the PV genome. The E1, E2, L1, and L2 ORFs are particularly well conserved among all members of the family. There is also strong evidence that PV genomes are very static, indeed, sequence changes by mutation or recombination are rare events.

1.1.1. CLASSIFICATION OF PAPILLOMAVIRUSES (PVs)

The papillomaviruses had been originally grouped together with the polyomaviruses in one family, the *Papovaviridae*. This was based on similar, nonenveloped capsids and the common circular double-stranded DNA genomes. But later was recognized that those two virus groups have different genome sizes, completely different genome organizations and no similarities about major nucleotide or amino acid sequence, so they were then officially recognized by the International Committee on the Taxonomy of Viruses (ICTV) as two separate families, *Papillomaviridae* and *Polyomaviridae* [5]. At the beginning of the study about PVs, each research group started using its own nomenclature to name the different PV types they discovered, but it has brought confusion in this field. In the International Papillomavirus workshop held in Quebec in 1995, all the scientists working on PV taxonomy and diagnosis, defined that a new PV isolate is recognized as such if the complete genome is cloned and the DNA sequence of the L1 ORF differs by more than 10% from the closest known PV type. Differences between 2% and 10% homology define a subtype and less than 2% a variant [6]. With the introduction of PCR and new sequencing technologies, many

types of PVs were founded, so a complete taxonomic classification was needed. This kind of classification should have some objectives: it should establish the relationship between PV types; it should compare the term PV type against the taxonomic terms ‘‘species’’ and ‘‘genus’’, which are used for the systematics of all biological organisms; and it should investigate the relationship between the taxonomic classification and pathological and biological properties of the virus [5]. An understanding of relationship between PV types based on nucleotide sequence comparison began to emerge in 1995 when the first phylogenetic tree of PVs was designed by Chan *et al.,* based on the nucleotide sequences of the 291-bp L1 segments of 92 different PVs [7].The L1 ORF was selected to obtain phylogenetic information because it is the most conserved gene within the genome and it has been used for the identification of new PV types. In this classification, the different PVs founded were splitted into groups. (Figure 1). Unfortunately, the ICTV didn’t accepted this classification, but the PV researchers started using this kind of grouping PVs and it formed the base for the current classification.

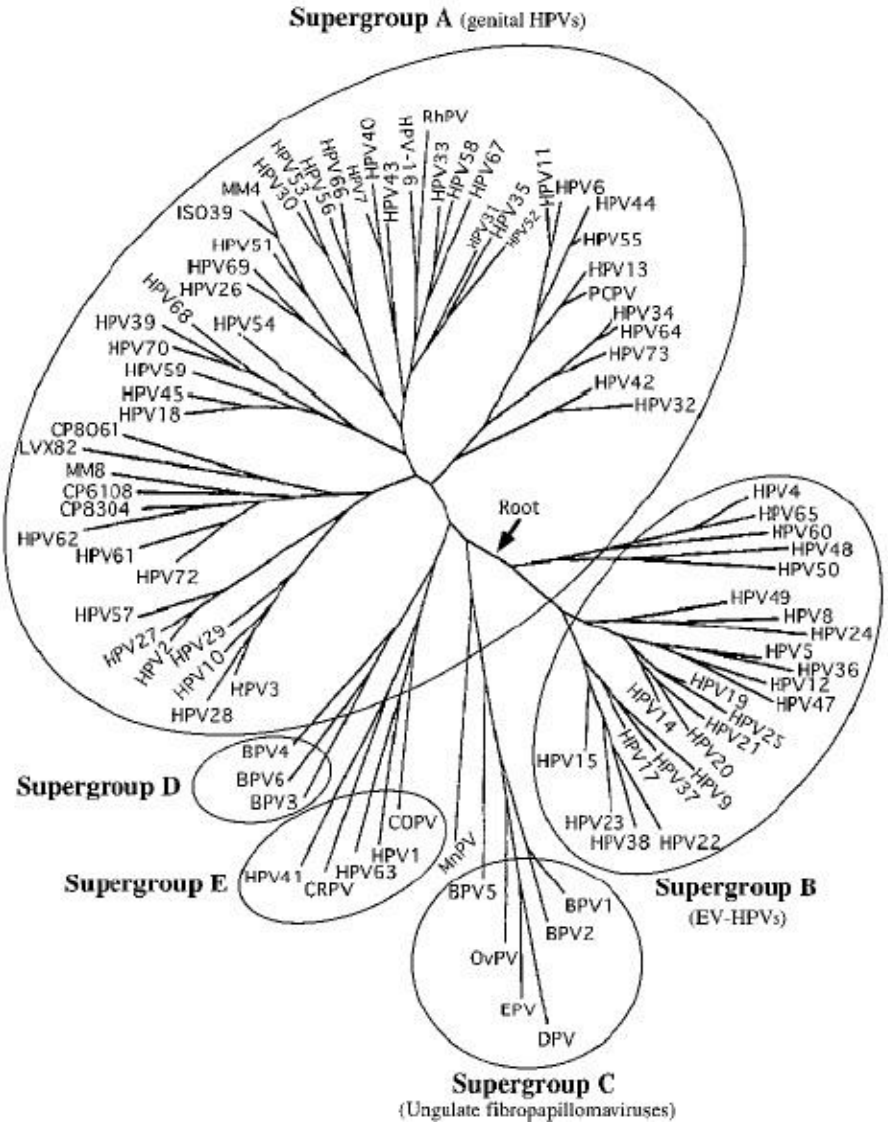

Figure 1. Chan et al., phylogenetic tree of 92 PV types based on the evaluation of the 291-bp L1 segment (from [7]).

In 2003, a group of researchers proposed a new PVs taxonomic classification very similar to the one proposed by Chan *et al.,:* a new phylogenetic tree was conceived analyzing the L1 sequences of 118 PVs and it grouped all the HPV types in genera [8]. The ICTV formalized the classification of the HPV genera (that stands today) that was designated on the basis of the De Villiers classification of PVs [8]. In this new classification, sixteen groups of PVs fulfilled the criterion of genera, and the Greek alphabet from the letters alpha to pi was used to create their nomenclature. According to this classification, human PVs were members of five genera (Alpha, Beta, Gamma, Mu and Nu) and two genera (Eta and Theta) were each comprised of a single bird PV (Figure 2). The remaining nine genera contained one or several PVs isolated from various mammals [9]. Among them, the largest group is the alpha group that contains

64 HPVs that mainly infect mucosal epithelia. Approximately 40 of these HPVs can infect the anogenital tract and include the approximately 14 high risk (hr) types (HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) [10] that have been classified as oncogenic and are found to cause anogenital cancers. The next largest group is the β-group HPVs that mainly infects cutaneous epithelia [11].

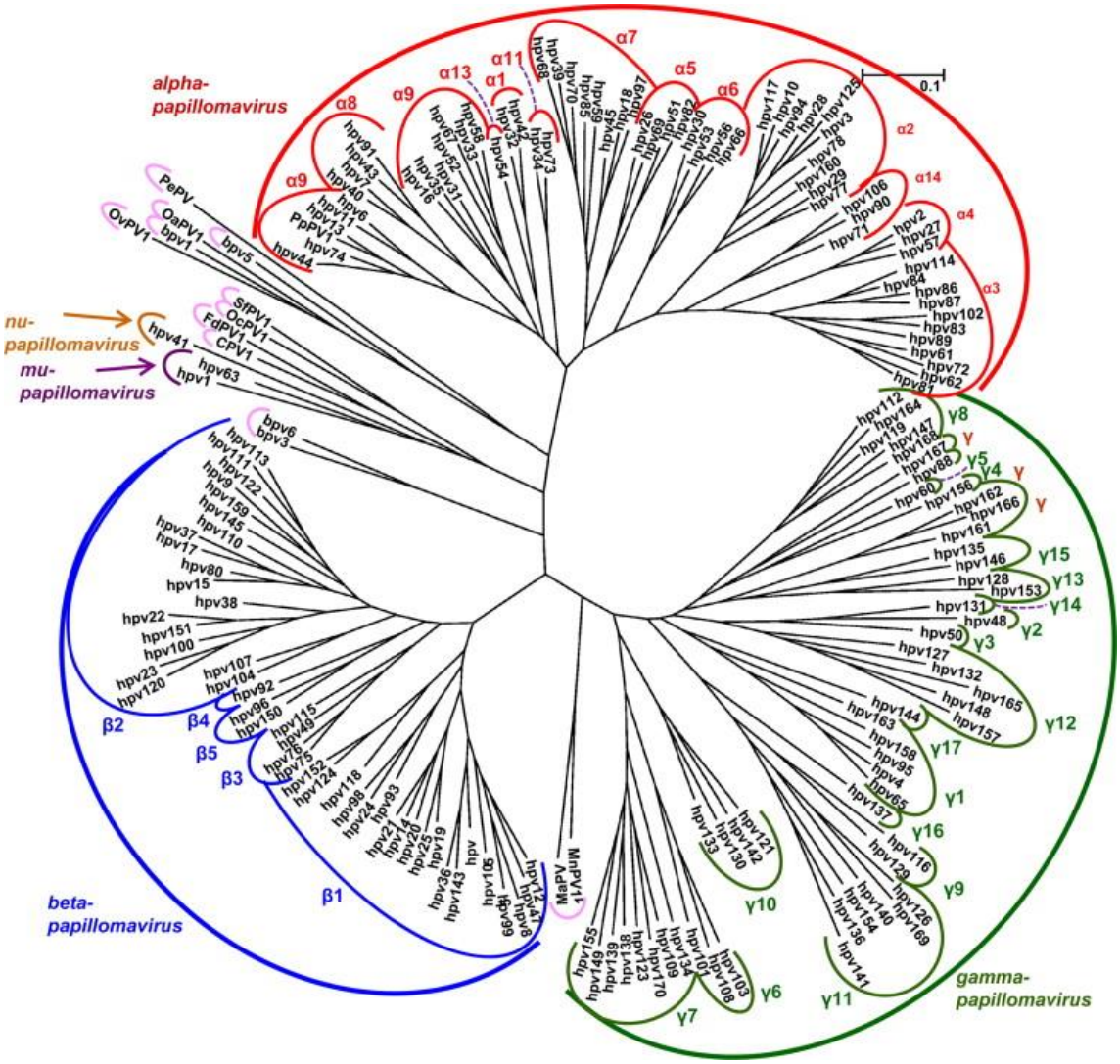

Figure 2. De Villiers., phylogenetic analysis based on the L1 ORF sequences of 170 HPV types, as well as single animal papillomaviruses, using the maximum likelihood method. [from ([12])].

1.1.2. STRUCTURE AND GENOME OF HUMAN PAPILLOMAVIRUSES

HPVs are non-enveloped DNA viruses with an affinity for the squamous epithelium. Each virus particle consists of an icosahedral capsid of about 60 nm in diameter, containing a single molecule of double stranded circular DNA of about 8,000 base pairs. Only one

strand of the double stranded DNA genome is used as a template for transcription and this coding strand contains three genomic regions, including approximately ORFs. The HPV genome (Figure 3), maintains the structure of the PVs previously described in paragraph 1.1. Among the early genes, E6 and E7 are very studied for their roles in inactivation of host tumor-suppressor genes and oncogenic progression. The other early genes play critical roles in viral replication, transcriptional regulation, and viral genome maintenance, all necessary processes for sustaining persistent HPV infection [13]. The early region (E) contains up to seven ORFs encoding viral regulatory proteins and the late (L) region encodes the two viral capsid proteins. Each ORF in the early region is designated “E” followed by a numeral, indicative of the length of the ORF. The third region of the genome has been referred to as the long control region (LCR), the upstream regulatory region (URR) or the noncoding region (NCR). This genomic region contains the origin of DNA replication, as well as transcription control sequences [14].

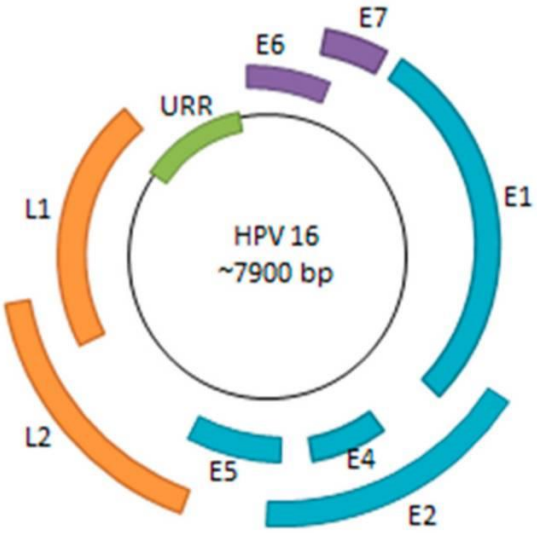

Figure 3. The structure of HPV genome. It consists of six early genes (E1, E2, E4, E5, E6, and E7) and two late genes (L1 and L2). Many of the early genes are implicated in viral replication, transcriptional regulation, genome maintenance along with the immune system evasion (from [13]).

1.1.3. THE REPLICATION MECHANISM

HPV penetrates into the organism via micro-injuries of mucosa membrane, which allows to reach the basal layer of the epithelium. HPV attaches itself to the cell membrane with the use of epidermal growth factor receptors and α-6 integrins. In the infected cells of the epithelium basal and para-basal layer, only E HPV genes encoding early viral proteins are expressed. However, HPV goes through a full development cycle only in differentiated keratinocytes that produce capsid proteins and make infectious progeny virions [15].

1.1.3.1. HPV CELL ENTRY

The non-enveloped human papillomaviruses specifically target epithelial cells of the skin and mucosa (Figure 4). Successful infection requires a lesion in the stratified tissue for access to the basal cells. HPV can get into the epithelium through microabrasions or by entering cells of the single layered squamous cellular junction between the endo- and ectocervix (this mechanism is used by high risk HPVs that infect the cervical epithelium). HPV virions attach to the cell through their major viral capsid protein, L1. The cellular receptor for HPV has not been unequivocally identified. Both cell surface heparan sulfate proteoglycans (HSPGs) and integrin proteins have been reported to play an important role in viral attachment and entry [16]. Many studies have proposed also the epidermal growth factor (EGF) and laminin as important receptors for hrHPVs entry into human cells. First of all, the HPV L1 capsid protein binds to cellular receptors present on the basement membrane or on the surface of basal layer cell. After that, a conformational modification occurs in the viral capsid and the N-terminus of the L2 component is exposed on the surface of the virion [17]. This kind of mechanism used by this virus, can depend on the HPV genotype or on the cell type that will be infected. The virus travels through membrane-bound cytoplasmatic components and its genome is transported to the nucleus by microtubules where it enters via nuclear pores or following breakdown of the nuclear membrane during mitosis [18].

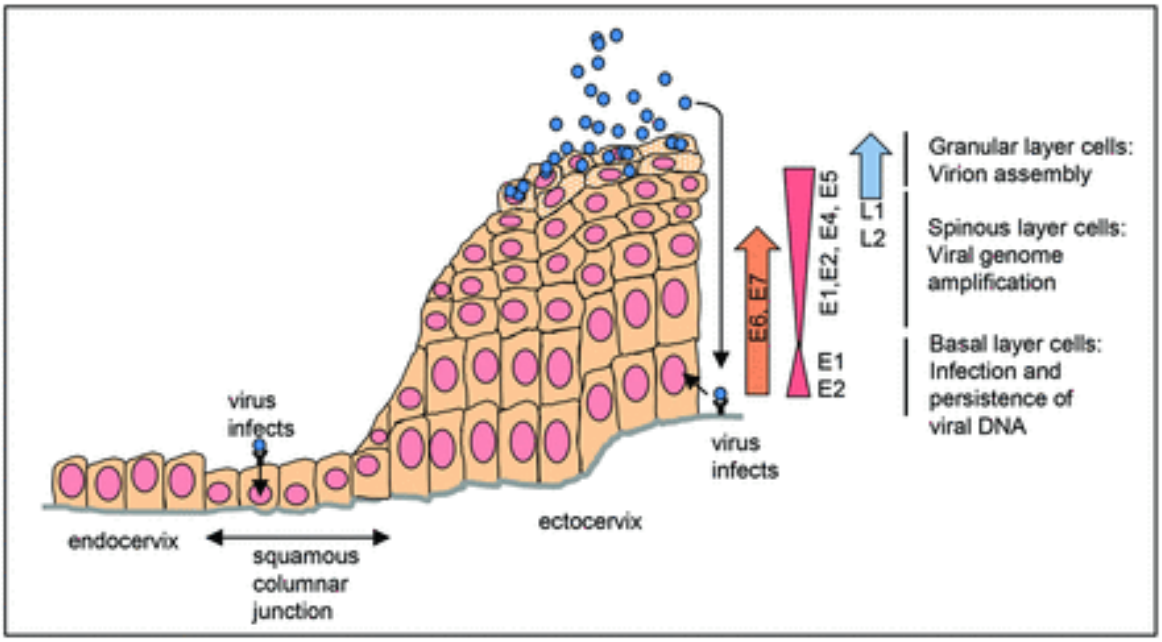

Figure 4. The virus infection of the cervix: HPV can bind receptors on the basement membrane and then it infects basal layer cells of the epithelium. Alternatively, HPV can enter cells in the squamous columnar junction (from [11]).

1.1.3.2. THE EARLY PHASE OF THE VIRAL REPLICATION CYCLE: E1 AND E2 PROTEINS

HPV has evolved such that the early genes are readily transcribed by the host cell transcription machinery without any need for viral protein synthesis. There are three steps of replication in the HPV life cycle. First of all, the genome amplification occurs when a viral particle first enters the host cell. In the second phase, the viral genome is keeped in a constant copy number in the proliferating basal cells and in the third phase, genomes are amplified in differentiated cells to produce progeny virions [19]. E1 and E2 are the only two HPV proteins required for replication of the circular DNA genome, with the remaining functions being provided by the host cell proteins [20]. The E1 protein is a helicase, member of the helicase superfamily 3 and it is a classical initiator protein that plays many roles in the initiation of viral DNA synthesis, in particular it initiates the DNA unwinding to obtain the template for the synthesis of DNA. First of all, E1 recognizes the specific segment of the viral genome called origin of DNBA replication (ori) and after this recognition, the E1 protein is turned into its ezimatically active form, that is able to unroll the ori and the DNA ahead of the replication fork [21]. The E2 protein is an important trascriptional regulator of the HPVs replication and its main function is recruiting cellular factors to the viral genome, which activate or repress the transcriptional processes depending on the site of binding in the viral genome and on the nature of the associated cellular factors [19]. E2 participates in the first step of the replication of viral genome by loading the E1 helicase onto the ori, that contains an E1 binding site and an E2 binding site. E1 is the primary replication protein but E2 enhances and supports the functions of E1. After E1 is loaded, it converts to a double-hexameric helicase and E2 is displaced. E2 also displaces nucleosomes from the origin to alleviate repression [19]. In this phase, viral proteins are probably expressed at low levels in infected basal cells to avoid activating the immune response, in this way HPV is able to maintain the infection of the epithelial cells for a long period [22]. In infected basal cells, viral replication occurs coordinately with cellular replication, and stable copy numbers are maintained. The E2 protein is also important for the segregation of newly replicated viral DNA with mitotic chromosomes, that allow a similar distribution of viral genomes in the daughter cells. E2 is associated with viral DNA during mitosis, and that leads to the equal partitioning to the daughter cells [23].

1.1.3.3. ROLE OF E6 AND E7 PROTEINS

In HPV infected epithelial cells the E6 and E7 oncoproteins, through their interaction with the tumor suppressing proteins p53 and pRB respectively [24], regulate the cell cycle by the reactivation of cell division and the inhibition of apoptosis and epithelial differentiation. Although the designation as oncoproteins, their expression is essential for the normal replicative HPV life cycle. Indeed, the E6 protein is required for episomal genome maintenance and for E7 expression. That is important because it activates the G_1 to S-phase checkpoint in keratinocytes that would normally undergo terminal differentiation, thus expanding the compartment of epithelial cells active in DNA replication [11]. The main 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). E6 and E7 proteins have potent transforming activities, indeed they reprogramme the cellular enviroment to be conductive to viral replication and they are necessary but not sufficient to make their host squamous epithelial cell tumorigenic [25].

1.1.3.4. THE LATE PHASE OF THE VIRAL REPLICATION CYCLE

The late phase of the viral life cycle leads to the virion formation. Increased expression of the viral E1 and E2 proteins is required to accomplish this phase. The late stage of the life cycle is marked by activation of the viral major late promoter that is situated in the *E7* gene region. This results in increased expression, not only of E1 and E2, but also of E4 and E5. Late stage DNA replication, probably using a rolling circle mechanism, yields many thousands of progeny viral genomes. The E4 proteins are the most abundant viral regulatory factors and play essential roles in the differentiated keratinocytes which support viral genome amplification and late events in the life cycle [11]. The E4 proteins make easier the viral genome amplification, the regulation of late gene expression, the control of virus maturation and the mediation of virus release.

1.1.4. THE VIRAL INFECTIONS: BENIGNANT AND MALIGNANT LESIONS

HPVs can infect both skin and mucous membranes with different manifestation: from benignant warts to malignant lesions. HPV has emerged over the past decade as the

major candidate to be the sexually transmitted aetiological factor in cervical cancer. Among all the HPVs classified until now, the genotypes that cause cervical cancer are called high risk HPV (hrHPV) to distinguish them from the low risk HPV (lrHPV) that commonly cause benign epithelial lesions [26] (figure 5). The criteria to put a new HPV type into the low risk group or into the high risk group is based on molecular epidemiologic studies that provide risk estimates and on functional evidences of the oncogenic potential of different HPV types [27].

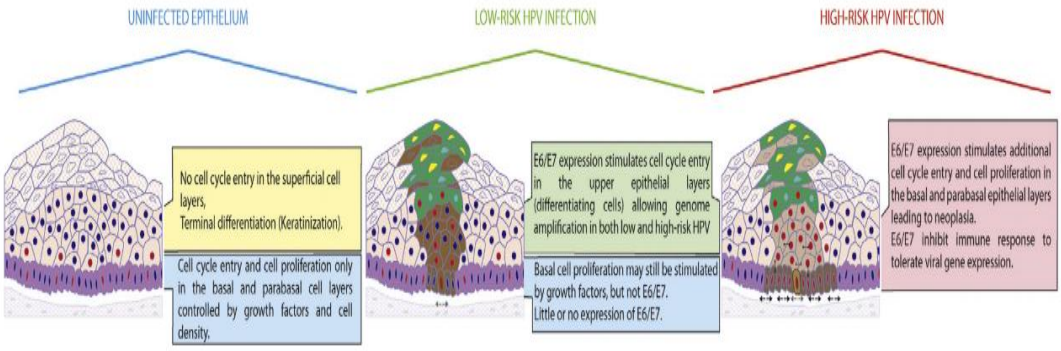

Figure 5. The different infections caused by lrHPVs and hrHPVs (from [26]*).*

One of the main features of the HPV group is the preference of particoular genotype for distinct anatomical sites where they cause lesions with different clinical pathologies. Most of the sexually active individuals will acquire one of the HPV genotypes that cause anogenital infections during their lifetime. HPV infections are ubiquitous and many genotypes are present on the skin of immunocompetent individuals without creating any sign of lesions [28].

1.1.4.1. LOW RISK HPVs

LrHPVs have been less studied than hrHPVs because of their lower impact as carcinogenic agents, but nowadays the majority of the known HPVs are proper low risk. LrHPVs cause many different benign mucosal and skin lesions, including genital warts (caused by HPV 6 and 11), common warts (caused by HPV 2, 27 and 57), flats warts (caused by HPV 3 and 10), verrucas or myrmecia (HPV 1) and other many skin lesions [26]. These infections are quite frequent, in particular the benign cutaneus lesions are relatively common in the general population, especially in children (33%)

[29] who may be encountering HPV types for the first time and in immunosuppressed individuals. In the most cases this kind of benign lesions will be cleared by a cell mediated immune response. Although their classification as low risk, HPV 6 and HPV 11 were detected in invasive cervical and anal cancers, but it is quite rare [30], anyway lrHPV infections are not considered a significant agent for the developing malignant transformation in the general population, and the link between lrHPV and carcinoma remains unclear except in immunosuppressed individuals and in certain genetic backgrounds [26]. From the molecular biology point of view, the lrHPV are different from the hrHPV because the lrHPV E6 and E7 proteins, and particularly those encoded by low risk Alpha HPV types, have often been regarded as less potent versions of the hrHPV proteins, which neglects their important role in driving successful lrHPV infections [26].

1.1.4.2. HIGH RISK HPVs

HrHPVs are the causative agents of approximately 5.2% of all human cancers worldwide and hrHPV associated cervical carcinoma is the fourth most common cancer among women globally [14]. Epidemiological evidence for the carcinogenicity of hrHPVs has been extensively discussed and established in particular for the cervical cancer. During the 1970s Harald zur Hausen, a German virologist, studied the human papillomavirus and its possible relation with the development of cervical cancer [31] and his discoveries lead him to receive the Nobel Prize in 2008.

HPV 16 and HPV 18 have been classified as cervical carcinogens since 1995 by the The International Agency for Research on Cancer (IARC) and ten years later, in 2005, the group of cervical carcinogens was expanded to thirteen types. In 2007 the IARC described in depth the HPVs and its determinant role in the developing of cancer in the Volume 90 of the IARC Monograph on the Evaluation of Carcinogenics Risks to Human. In 2009 the WHO released a manual for laboratories that perform HPV genotyping in which the number of high risk HPV genotypes was extended to fourteen (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68).

1.1.5. CANCERS RELATED TO HUMAN PAPILLOMAVIRUSES

Many biological studies and epidemiological evidences assesed that some types of HPVs are the main cause of invasive cervical cancer [32] and cervical intraepithelial neoplasia [33]. However HPVs are not the direct cause of only the cervical cancer but also of other malignancies like the head and neck cancers [34], the non melanoma skin cancer (NMSC) [35] and tumors located on the anogenital tracts (anus, penis, vagina and vulva). While almost all cervical cancers are associated to HPV infections, 64–91% of vaginal, 40–50% of vulvar, 88–94% of anal and 40–50% of penile cancers are HPV-positive [36].

In 2009 the IARC declared that there was a strong evidence in the aetiological cause of HPV 16 in the development of oropharynx and tonsil cancers [37]. The incidence of HPV positive oropharyngeal cancers (OPCs), that are a subset of head and neck squamous cell cancers (HNSCCs), is steadily increasing. About 70% to 80% of OPCs are now attributed to HPV in the United States and western Europe [34].

The connection between HPV and NMSC was founded in patients with Epidermodysplasia verruciformis (EV), indeed in the 90% of cutaneous squamous cell carcinomas were founded HPVs [35]. NMSC is caused by UV radiation but many epidemiological studies have shown that HPV play an important role in the pathogenesis of these cancers [36]: the DNA-damage in keratinocytes made by UV radiation is emphasized by the anti-apoptotic activities made by HPV and that leads to the persistence of DNA-damaged keratinocytes and subsequently to skin cancer.

1.1.5.1. CERVICAL CANCER

Cervical cancer is an important global public health problem because it is the fourth [14] most common tumor in women worldwide with more than 85% of the cases registered in developed countries. The screening programs introduced in many high-resource countries, were useful to decrease the cervical cancer incidence and mortality. HPV carcinogenicity has been convincingly established for cervical cancer and it is generally accepted that HPV infection is necessary for the development of invasive cervical cancer (ICC) [38]. Among the 200 different types of HPV already identified, only 40 (all belonging to the alpha genus) are able to infect the mucosal epithelium of the cervix and of these, less than twenty have been actually correlated to cervical cancer development (the hrHPV types).

The carcinogenic type related in the aetiology of invasive cervical carcinoma are HPV

16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68.

Cervical carcinomas, caused by infection made by hrHPV types can be classified in two main histotypes, squamous cell carcinomas (SCCs; 80%) and adenocarcinomas (AdCAs; 15–20%) [39].

HPV16 is the most type found in both SCCs (59.3%) and AdCAs (36.3%) around the world [30]. HPV18, the second most common genotype, has been found in a higher proportion of AdCAs (36.8%) compared to SCCs (13.2%) [30].

1.1.5.2. PHATOGENESIS OF CERVICAL CANCER

Invasive cancer is not an immediate consequence of HPV infection. HPV-induced carcinogenesis takes years or decades to occur, and there is an increasing evidence that additional tumor-promoting steps are necessary [36].

It has been already established that the immune system plays a key role in mucosal as well as cutaneous HPV-associated carcinogenesis. In immunocompetent individuals, up to 90% of anogenital HPV infections are cleared within two years by the immune system and this is thought to be due to innate immunity as well as adaptive $CD8^+$ T cell-mediated responses directed against viral early proteins [40]. Instead, immunocompromised patients, such as transplant individuals or HIV patients, show higher prevalences of HPV infection and HPV-related diseases, underlining the importance of immune system in the carcinogenesis linked to HPV [41].

HPV infections that escape immune control can persist and a certain proportion progresses to cancer. At early stages, the role of the immune system is manteined and the virus has adopted strategies to evade the immunosurveillance in order to persist in the epithelium. Nevertheless, at later stages of the disease, HPV transformed cells reprogram the local immune microenvironment and rather initiate chronic stromal inflammation, which leads to the progression of precursor lesions to invasive cancer [36].

The transformation of HPV infected cells to cancer cells is a multi-step process where there is a deregulated expression of E6 and E7 proteins of hrHPV types that lead to a non productive infection [42]. It has also been proposed that the consequence of viral genome integration that can result in oncogenic progression is the loss of the expression of the E2 protein that plays a critical role in regulating the activation and repression of viral promoters. Indeed, the E2 protein, by binding to sites proximal to the E6/E7 promoter, is able to displace other transcriptional factors and thus prevent the formation of a transcription initiation complex. The loss of E2 expression is

associated with the oncogenic progression of HPV because it leads to the increase of the expression of viral oncogenic proteins, E6 and E7, which are known to disrupt tumor-suppressor proteins p53 and pRB, respectively [13]. These tumor suppressor proteins normally control signaling pathways that regulate the cell cycle and protect the integrity of the genome [43].

1.1.5.3. LEVELS OF CERVICAL LESIONS

HPV penetrates the epithelium of the transformation zone through microabrasions and infects epithelial stem cells that are located in the basal epithelial cell layer of the cervix. Infection with all mucosal HPV types, when persistent, can lead to the development of epithelial lesions. Only hrHPV types lead to the development of precancerous and cancerous lesions that can be classified by two different methods, using cervical cytology or the histological data. According to the 2001 Bethesda System, that analyze the specimen's cytology, there are different levels of squamous intra-epithelial lesion:

- the atypical cells of undetermined significance (ASCUS) used for abnormalities suggesting a low grade intraepithelial squamous lesion;
- the atypical cellular findings not permitting exclusion of a high grade intraepithelial squamous lesion (ASCH) that is proposed for those unconfirmed, although suspected cases of high grade intraepithelial squamous lesion;
- the LSILs (low-grade squamous intraepithelial lesions) that generally represent non-neoplastic productive HPV infections that have a low risk of progression to malignancy;
- the HSILs (high grade squamous intraepithelial lesions) comprise abortive virus infections in which there is deregulated expression of HPV early genes in basal epithelial cells and a greater risk of progression to invasive disease (Figure 6) [44].

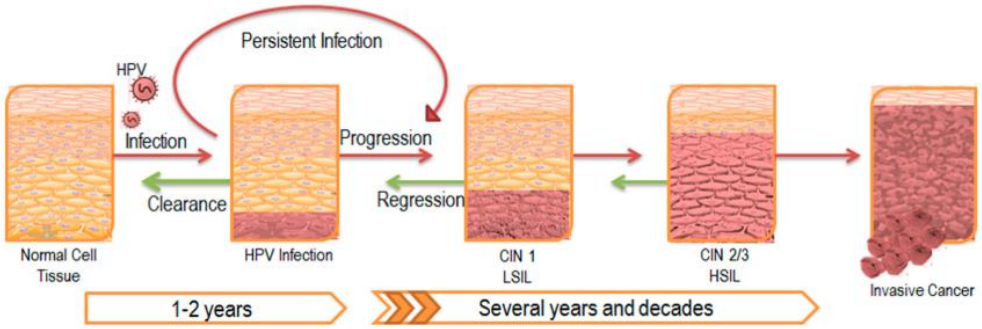

Figure 6. Progression of HPV infection. HPV typically establishes infection in the basal epithelial layer. A majority of these infections are transient and cleared by the immune system within two years. However, 10–20% of infections persist latently, leading to disease progression (from [13]*).*

The second method to classified cervical lesions is the Cervical intraepithelial Neoplasia (CIN) System that allow the analysis of histological samples. The traditional grading of CIN (described in Table 1) is based on the proportion of epithelial thickness occupied by undifferentiated basaloid cells, reflecting the progressive loss of epithelial maturation and glycogenation decrease occurring at the same time as the increase in lesion severity [45].

CIN GRADE	FEATURES
CIN 1	Mild dysplasia. Good maturation with minimal nuclear abnormalities. Undifferentiated cells are confined to the deeper layers (lower third) of the epithelium. Mitotic figures are present, but not very numerous.
CIN 2	Moderate dysplasia. Abnormal cells extend into the basal and intermediate layers, with more marked nuclear abnormalities than in CIN 1.
CIN 3	Severe dysplasia and carcinoma in-situ. Dysplastic cells occupy the whole of the epithelium. Many mitotic figures have abnormal forms. Stratification may be totally absent or present only in the superficial quarter of the epithelium.

Table 1. Histological classification of the cervical intraepithelial neoplasia.

The discrimination between low and high grade CIN specimens is very important because the low grade cases are treated differently than high grade cases. Indeed, low grade CIN cases are usually reversible if treated properly, but high grade CIN cases are evolving lesions that might need surgical intervention. Failure in distinguishing the CIN grade can compromise treatment's overall efficacy [46].

High grade CIN (CIN 2–3) is a precancerous lesion and can progress to cervical cancer if untreated. Conization has been widely accepted as both a diagnostic and therapeutic modality for the management of CIN 2–3. Cold knife conization (CKC) and the loop electrosurgical excision procedure (LEEP) are the most common procedures for the treatment of CIN 2-3. However, the prevalence of persistent or recurrent high grade lesions after conization varies between 5% and 25%, leading to frequent follow-up and retreatment [47].

Comparing the two different classifications for the analysis of cervical lesions, the LSIL category corresponds to CIN 1, whereas the HSIL category corresponds to CIN 2-3. Only HSIL/CIN 2-3 lesions are considered precancerous lesions.

1.1.6. HPV VACCINES

Vaccination with HPV vaccines available for the prevention of cervical cancer induces a systemic immune response against HPVs. Vaccination programs against HPV are being implemented for young adolescent girls and boys in many countries.

There are currently three prophylactic HPV vaccines with FDA (Food and Drug Administration) approval:

- the bivalent vaccine (that target hrHPV types 16 and 18) that was approved in 2009 for young women;
- the quadri-valent vaccine (that target HPV types 6, 11, 16, and 18) that was approved in 2006 for young women and in 2009 for young men;
- the nine-valent (that target HPV types 6, 11, 16, 18, 31, 33, 45, 52, 58) that was approved in 2014 for young women and men.

These vaccines target viral genotypes that are associated with a high risk of cervical cancer (HPV 16, 18, 31, 33, 45, 52, and 58) and those associated with genital warts (HPV 6 and 11) [48]. Literature evidences asses that vaccination programs has greatly reduced the prevalence of HPV in young women and men [49], providing encouragement for a future reduction in cervical cancer mortality and giving the assurance that the extention of the vaccination could deliver substantial health economic benefits [50].

1.1.7. SCREENING PROGRAM

Even though there are many evidences that the vaccination programs have reduced the HPV prevalence and also the mortality linked to cervical cancers, early detection of precancerous cervical lesions through screening program remains a critical health care service intervention for reducing cervical cancer incidence and mortality particularly in low-resource countries where HPV vaccination coverage is poor [51]. In comparison to developing countries with poor vaccination coverage and lack of organized cervical cancer screening programs, developed countries with good organized cervical cancer screening programs have gained significant reduction in cervical cancer incidence and mortality [52].

Cervical cancer screening is traditionally made by using the Papanicolaou test (Pap test) that consists in the collection of cells from the cervix with a spatula and the examination of the collected cells under the microscope by a cytologist. Using this test, the detection of cytological abnormalities of Pap smears and subsequent treatment of women with high grade cytological abnormalities avoids development of cancer. European guidelines reccomended the colposcopy for ASCH and HSIL positive patients. Both colposcopy and repeating the Pap test are acceptable strategies for LSIL positive patients and hrHPV test triage or Pap test repetition or direct colposcopy are strategies reccomended for ASCUS positive patients.

European guidelines [53] have not established a defined procedure for HPV DNA testing implementation in primary screening program yet, however many applications for HPV DNA detection have been discussed in these guidelines: first of all the use in the primary screening for oncogenic HPV types alone or in combination with cytology; then the use for women with equivocal cytological results and also for the follow-up of women treated for CIN to predict success or failure of treatment [53]. HPV infections are very common and usually clear spontaneously, therefore the detection of HPV DNA could lead to unnecessary colposcopies, psychological distress and the possibility of overdiagnosis. Therefore, the HPV DNA detection could be very useful because evidence from randomised studies shows that HPV DNA testing is more sensitive and equally specific in finding high grade CIN compared with repeat cytology. Moreover the high sensitivity of current HPV DNA detection methods yields high negative predictive values even for adenocarcinoma precursors that often escape cytological detection [53].

1.1.8. HPV MOLECULAR DETECTION METHODS

The most widely used and extensively investigated biomarker in the management of cervical disease is HPV DNA testing that is actually made by a wide range of HPV detection techniques [54].

At the beginning of the use of HPV molecular detection methods, the assays that were firstly introduced was based on the techniques of southern blotting, *in situ* hybridization, and dot-blot hybridization using radio labeled nucleic acid hybridization [55]. Unfortunately, these assays recquired a large amount of purified DNA and also had low sensitivity.

Nowadays, different technologies, with higher sensitivity and easier to perform are available. The HPV DNA detection methods most used are based either on signal amplification technologies or on target amplification technologies.

At the first category belong the Hybrid Capture 2 HighRisk HPV DNA test (QIAGEN, HC2), that is a non-radioactive signal amplification method based on the hybridization of the target HPV DNA to labeled RNA probes in solution [54]. It is an assay FDA approved, and it is often used in primary cervical cancer screening.

The second category of the HPV DNA detection techniques that is actually very used, is made up by the nucleic acid amplification methods that are based on the Polymerase chain reaction (PCR) techniques. Nowadays, many Real time PCR based assay are currently available and many of them are used in primary cervical cancer screening programs. Among them, we can mention the RealTime High Risk HPV test (Abbott), the Cobas 4800 HPV test (Roche) and the Anyplex II HPV HR Detection (Seegene). Also the assay object of this dissertation is based on the Real time PCR technique and the aim of this study is properly to evaluate if it is suitable for primary cervical cancer screening.

1.1.9. MEIJER'S GUIDELINES

Based on the fact that there is an absolute aetiologic link between hrHPV and cervical cancer, the detection of hrHPV DNA is now being considered as an alternative for cytology in cervical cancer screening. However, before cost-effective implementation of population based hrHPV testing in cervical cancer screening can be conceived, any candidate HPV testing technologies must offer an optimal balance between clinical sensitivity and specificity for detection of CIN grade 2 or 3 and treatable cancer (≥CIN

2) to minimize excessive follow-up procedures. Meijer's guidelines [56] are useful instructions to follow for the development of hrHPV DNA test intended for use in primary cervical cancer screening. These guidelines define a clinical validation strategy based on the comparison of the performance of the assay under evaluation with that of an already clinically validated reference HPV test, on samples originating from a population-based screening cohort. They also required the assessment of the device inter and intra-laboratory reproducibility.

The key issue for hrHPV DNA testing in cervical screening is to detect hrHPV infections that are associated with or develop into ≥CIN 2 and to differentiate them from transient hrHPV infections. This implies that there should be a balance between clinical sensitivity and specificity for detection of ≥CIN 2 [56].

Meijer *et al*., based their studies on two assays, the FDA approved Hybrid Capture 2 (HC2; QIAGEN) and the GP5+/6+-PCR enzyme immunoassay (GP5+/6+-PCR, EIA) because they have repeatedly demonstrated clinical sensitivity of about 90–95% for the detection of ≥CIN 2 in large prospective cohorts or randomized controlled trials.

According to Meijer's guidelines, a HPV detection assay should fulfill the following requirements:

- The candidate test should have a clinical sensitivity for ≥CIN2 not less than 90% of the clinical sensitivity of the already validated assay in women of at least 30 years.
- The candidate test should have a clinical specificity for ≥CIN2 not less than 98% of the clinical specificity of the already validated assay in women of at least 30 years of age.
- The candidate test should display intra- and inter-laboratory reproducibilities with a lower confidence bound not less than 87%.

1.2. HUMAN IMMUNODEFICIENCY VIRUS (HIV)

The Human Immunodeficiency virus (HIV) belongs to the genus *Lentivirus* within the family of *Retroviridae*, subfamily *Orthoretrovirinae* [57]. Lentiviruses cause chronic persistent infections in various mammalian species, including bovines, horses, sheep, felines, and primates [58]. The HIV research community worked on names informally until a formal meeting was held in September 1999 at the Santa Fe Institute in New Mexico, where a set of rules for naming primate lentiviruses HIVs and SIVs (simian immunodeficiency viruses) was decided [59]. Genetically, HIV is characterized by a wide diversification and a very rapid evolution [60]. The defining feature of HIV is its exceptional genetic diversity. This high diversity derives from four sources: high substitution rates, a rather small genome, short generation times, and high recombination frequency [61]. HIV was classified into the types 1 and 2 (HIV-1, HIV-2) and they are distinguished on the basis of their genome organization and phylogenetic relationships, clinical characteristics, virulence, infectivity and geographic distribution [61]. Among the many theories about the origin of HIV, the most likely explanation is that HIV was introduced to humans from monkeys. It has been established that the original source of HIV-1 was a subspecies of chimpanzee native to west equatorial Africa. The researchers believe that the virus crossed over from monkeys to humans when hunters became exposed to infected blood [58]. African primates are naturally infected with over 40 different SIVs, two of which have crossed the species barrier and generated HIV-1, HIV-2. Unlike the human viruses, SIVs do not cause acquired immunodeficiency syndrome (AIDS) in their natural host [62]. Phylogenetic evidence has showed that HIV-1 and HIV-2 are the product of several cross-species transmission events between chimpanzee (*Pan troglodytes troglodytes*) SIV (SIVcpz) with humans [61].

1.2.1. HIV-1

Human immunodeficiency virus type 1 (HIV-1) was first identified as the causative agent of the acquired immunodeficiency syndrome (AIDS) in 1984 when a retrovirus was isolated from the peripheral blood of patients suffering from a progressive and often fatal disease involving the quantitative depletion of the $CD4^+$ T-cell population [63].

1.2.1.1. HIV-1 CLASSIFICATION: THE GROUPS

It is now well established that SIVs were infecting west central African chimpanzees and western gorillas comprises the progenitors of HIV-1 [64]. This virus is classified into four groups, each of which arose from different transmissions of SIVs from nonhuman primates into humans. These groups are: the goups M (for main), N (for non-M/non-O), O (for outlier) and P [65] (Figure 7). It has been established that HIV-1 bases its origin in the African continent. On the basis of phylogenetic analysis, groups N and M are very closely related to SIVcpz strains from southern Cameroon, indicating that they are of chimpanzee origin. HIV-1 group N appears to be emerged in the area of south-central Cameroon, whereas the pandemic form, group M, likely originated in an area flanked by the Boumba, Ngoko, and Sangha rivers in the southeastern corner of Cameroon [58]. The HIV-1 M group spread far more extensively in humans than groups N, O, or P did and it is the primarily cause of the AIDS pandemic [59]. Existing phylogenetic data support a gorilla origin of HIV-1 group P from Cameroon and despite its rarity, group P shows evidence of adaptation to humans [66]. As well, it has been defined that HIV-1 group O came from western lowland gorillas in Cameroon [59]. From the beginning in the of HIV pandemic there was a strong evidence that there was great diversity between viruses isolated from patients sampled in the developed world, and patients sampled in Africa [59].

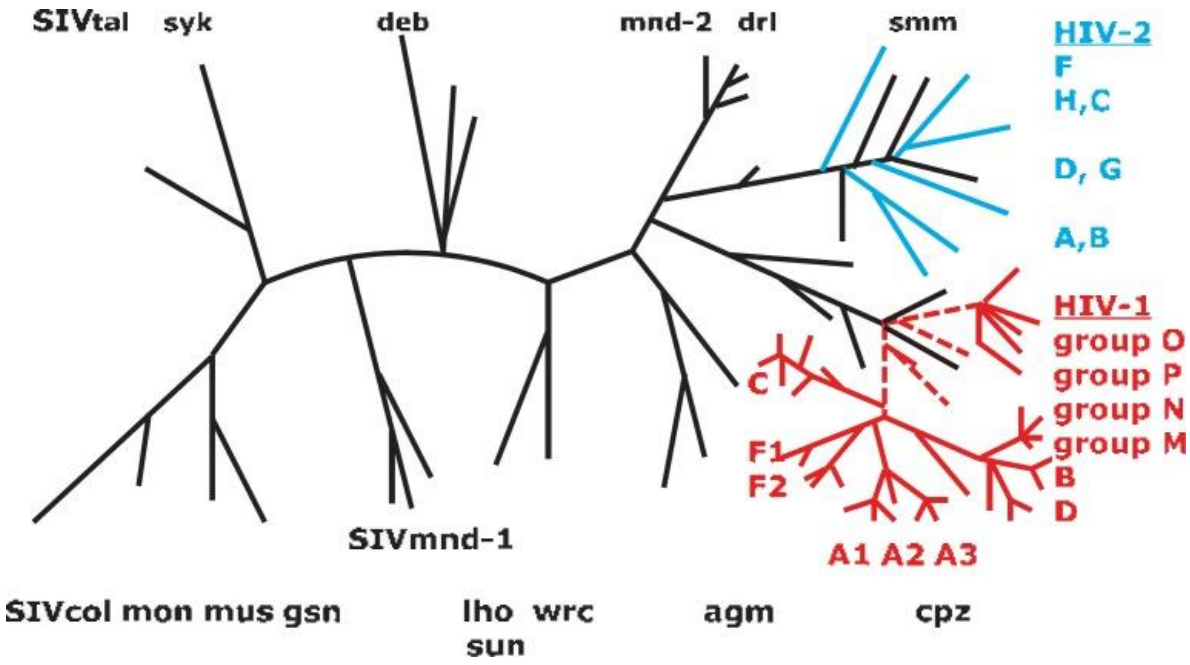

Figure 7. Scheme of the phylogenetic tree of human and simian Lentiviruses using the analysis of a pol region of Lentiviruses. The HIV-1 groups M, N, O and P are underlined in red (from [67]*).*

1.2.1.2. HIV-1 CLASSIFICATION: THE SUBTYPES AND SUB-SUBTYPES

HIV-1 group M is the most common globally, being responsible for over 95% of infections worldwide [68]. The group M is divided into nine phylogenetically distinct subtypes (A, B, C, D, F, G, H, J and K) and into five sub-subtypes (A1, A2, A3, F1 and F2) [69]. In evolutionary terms, groups A and D are the oldest viruses. The peculiarity of HIV-1 is that recombination can occur between viruses of the same subtype and between viruses of different subtypes. The recombination is the most important mechanisms contributing to HIV-1 variability, leading to the rapid generation of viral variants with high replicative capacity and to the development of drug resistance. Multiple infections of individuals with viruses of different subtypes, a prerequisite for intersubtype recombination, may result from a single transmission of genetically different viruses or a subsequent HIV infection acquired by an already infected individual [68]. Recombinant HIV, derived from various subtypes, are named CRF (circulating recombinant form). Approximately 20% [68] of group M viruses belong to these viruses. Up to now, more than 70 different epidemiologically stable CRF have been described [69], and the development of additional CRF is to be expected. The recombination is due to template switching during the reverse transcription process. Two copies of the viral RNA genome are present in a virus particle, whenever a cell is coinfected by two or more viruses, the daughter virions can contain two RNA strands from different categories. Then recombinant genomes are generated by template switching of the reverse transcriptase while producing cDNA [70]. The first CRF of HIV-1 identified was CRF01_AE and it represents a putative recombinant between subtypes A and E. Although CRF01_AE contains a subtype E *vif*, *vpr*, *env*, *nef*, and long terminal repeat (LTR), most or all of the remaining genome derives from subtype A [71].

1.2.1.3. HIV-1 GENOMIC STRUCTURE

The HIV-1 genome consists of two identical single-stranded RNA molecules [67] that are included in the core of the virus particle.The HIV-1 genome is composed of nine viral genes [72] (*gag*, *pol*, *vif*, *vpr*, *tat*, *rev*, *vpu*, *env*, and *nef*) that are required for all processes in the viral replicative cycle (Figure 8). Even though HIV encodes for few proteins, many physical interactions occur between pairs of them, providing essential mechanisms for HIV to achieve efficient viral replication at different stages of the HIV life cycle [73].

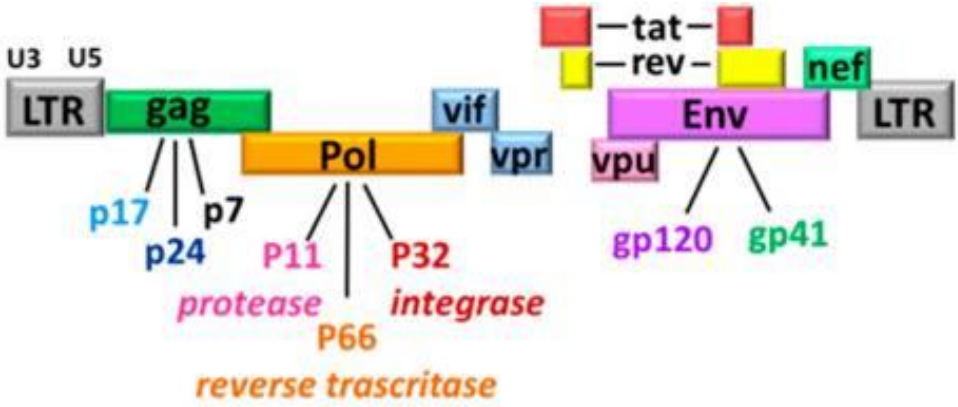

Figure 8. HIV-1 genomic structure: the nine genes (gag, pol, vif, vpr, tat, rev, vpu, env, and nef) that compose HIV-1 genome (modified from [74]*).*

The HIV DNA genome is flanked at both ends by LTR (long terminal repeat) sequences. The 5′ LTR region codes for the transcription promotor of the viral genes. In the direction 5′ to 3′ the reading frame of the *gag* gene follows, encoding the proteins of the outer core membrane (MA, p17), the capsid protein (CA, p24), the nucleocapsid (NC, p7) and a smaller, nucleic acid-stabilising protein. Then the *pol* reading frame encodes for the enzymes protease (PR, p12), reverse transcriptase (RT, p51) and RNase H (p15) or RT plus RNase H (together p66) and integrase (IN, p32). Next to the *pol* gene, the *env* encodes for the two envelope glycoproteins gp120 and gp41 that are essential for the viral entry [67]. Besides the structural proteins, the HIV genome encodes for many regulatory proteins: Tat (transactivator protein) and Rev (RNA splicing-regulator) are necessary for the initiation of HIV replication and the other regulatory proteins Nef (negative regulating factor), Vif (viral infectivity factor), Vpr (virus protein r) and Vpu (virus protein unique) are important for viral replication, virus budding and pathogenesis (Figure 9). The genomic structure of the immunodeficiency viruses of chimpanzees (SIVcpz) and gorillas (SIVgor) is identical to that of HIV-1.

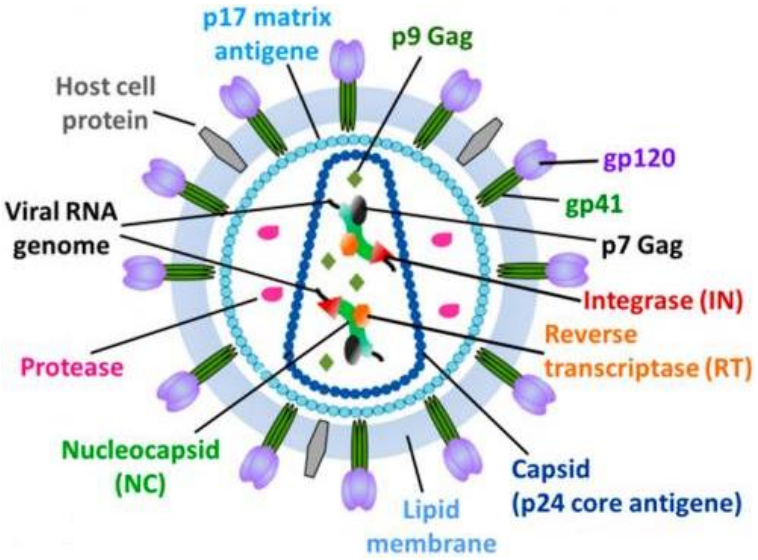

Figure 9. Structure of the HIV-1 viral particle (modified from [74]*).*

1.2.1.4. REPLICATION CYCLE

Retroviruses are distinguished from other viruses by two main steps in the viral replication cycle. The first is reverse transcription, which results in the production of a double-stranded DNA copy of the viral RNA genome, and the second is integration, which leads the formation of covalent attachment of the DNA copy to host cell DNA [75]. The HIV-1 replication cycle is divided in many steps that are all necessary for the release of the mature and infectious virions.

1.2.1.4.1. HIV-1 VIRAL ENTRY

Like all other viruses, HIV-1 must enter a susceptible cell to begin the replication cycle. As an enveloped virus, HIV-1 must fuse the phospholipid bilayer surrounding it with a host cell membrane in order to deliver the genome to the cytoplasmic compartment of the cell [76]. Host cells, likeT-helper cells, monocytes, macrophages, and dendritic cells, which express the CD4 (cluster of differentiation 4) glycoprotein on the cell surface, are the main HIV targets [73]. In this phase, the gp120 and the gp41 play an essential role for the viral entry. For the HIV-1 entry into the cell, an essential requirement is needed: into the host cell surface must be present a receptor for the gp120 that is the T-lymphocyte receptor CD4 which has a high affinity for the gp120. On HIV surface, gp120 physically interacts with gp41 to create a trimeric Env structure via noncovalent interactions [77]. After the attachment of viral gp120 to the host CD4 cell receptor, a shift of gp120 subunits occurs

to disrupt noncovalent interactions between gp120 and gp41. That leads to expose coreceptor binding sites [77] (Figure 10) of the CXCR4 and CCR5 that have been identified as the major coreceptors for the HIV-1 cell entry. After the binding, the gp120 undergoes structural rearrangements involving conformational changes that lead to a change in the gp41 from a nonfusogenic to a fusogenic state [63]. This change is important to make the cellular membrane and viral envelope closer, thereby facilitating membrane fusion between the virus and target cell, leading to the introduction of the content of the virion into the host cell. Then the viral core enters the host cell cytoplasm, and during this viral entry process the reverse transcriptase starts the conversion of viral genomic RNA into a double-stranded DNA proviral genome.

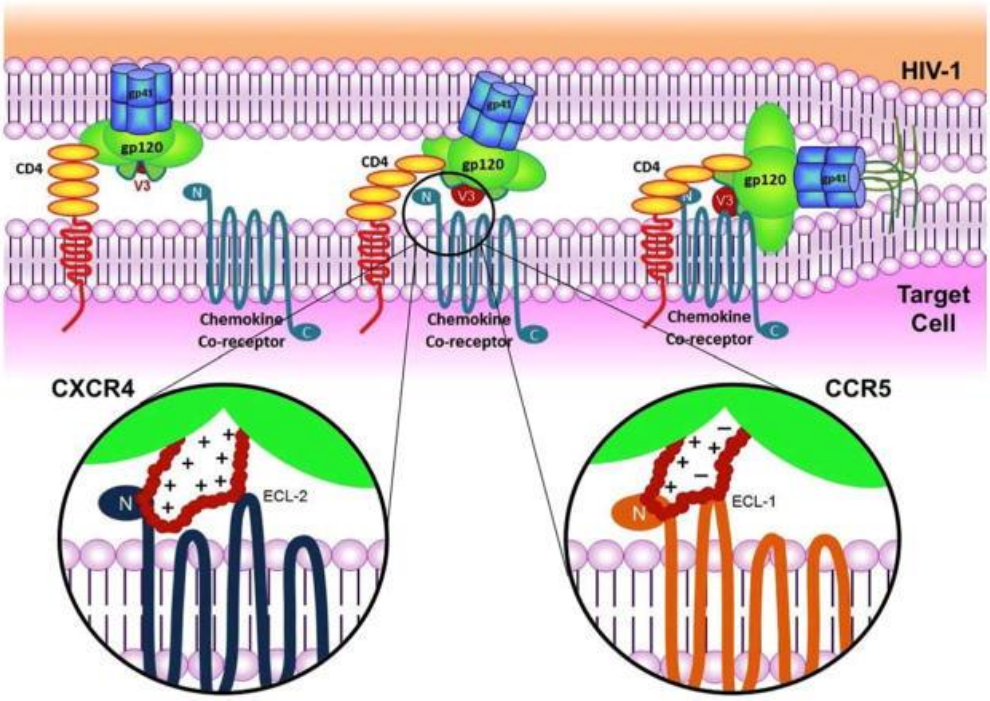

Figure 10. Representation of the interaction between the gp120 and the coreceptors CCR5 and CXCR4, that is an essential step in viral entry (From[63]*).*

1.2.1.4.2. REVERSE TRANSCRIPTION

The reverse transcription phase occurs in the newly infected cells after the viral entry thanks to a specific DNA polymerase, the Reverse Transcriptase (RT). During HIV reverse transcription, RT produces a double-stranded DNA (dsDNA) genome from a single-stranded RNA genome that will be integrated into the host cell chromosome [78]. After viral entry, a series of events takes place in the viral core that lead the construction of the reverse transcriptase complex (RTC) (Figure 11). The HIV-1 RTC may consist of RT,

protease, integrase, matrix, capsid, nucleocapsid, Vif, Tat, Nef, Vpr, and host proteins, although the exact composition of the RTC remains debated. During reverse transcription, the RTC produces viral dsDNA with a high content of uracil that protects viral dsDNA from viral autointegration [73].

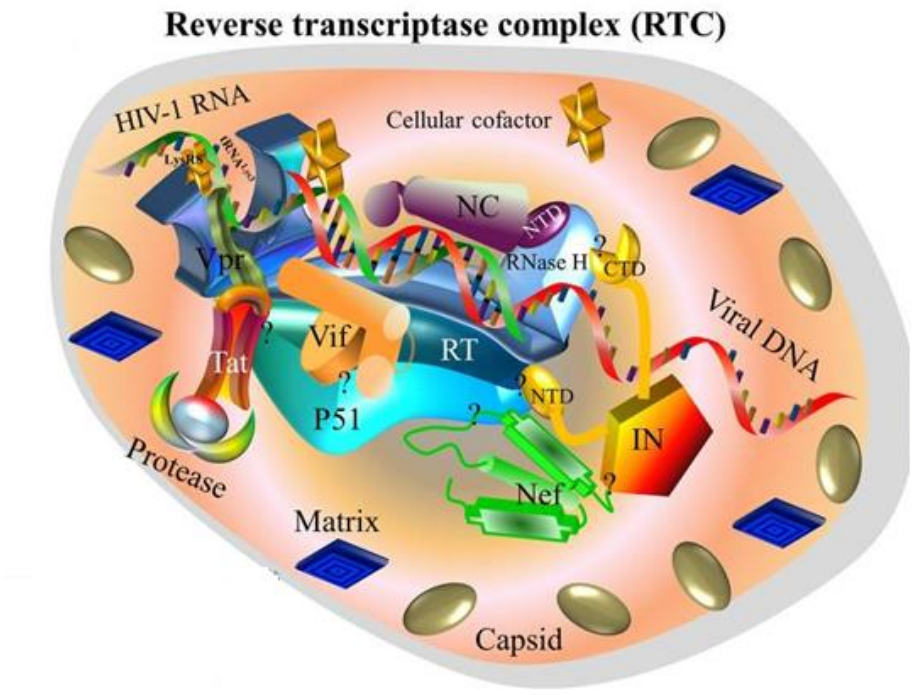

Figure 11. Composition of the Reverse Transcriptase Complex. It consists of reverse transcriptase, protease, integrase, matrix, capsid, nucleocapsid, Vif, Tat, Nef, Vpr and many cellular proteins (modified from [73]*).*

1.2.1.4.3. VIRAL INTEGRATION

The integration phase is a central step of the HIV cycle [79]. During this phase a copy of the viral genome is integrated into the host cellular DNA. The viral integration is mediated by the virus-encoded integrase protein, which is introduced into cells during infection [75]. The integrase protein binds the just synthesized viral DNA and other viral and host proteins to form a high molecularweight nucleoprotein complex, the preintegration complex (PIC). The integration process starts when the integrase hydrolyzes a dinucleotide from both end of HIV-1 DNA [80] to generate a reactive intermediate that contains a 3′-hydroxyl group. Then the PIC is imported from the cytoplasm to the nucleus through nucleus pore complexes and it is localized in the host chromosomal domains with high transcriptional activity. This step is mediated by cellular cofactors such as lens epithelium-derived growth factor (LEDGF)/p75b [80] that helps the binding between the PIC and host chromosomes. After that, the viral dsDNA is inserted into host chromosomes

through the integrase strand transfer reaction. And finally the gap repair occurs [73]: unpaired regions between HIV and host dsDNA are repaired under the assistance of cellular cofactors.

1.2.1.4.4. VIRAL TRANSCRIPTION AND TRANSLATION

Transcription of the HIV-1 proviral DNA into mRNA is a critical step in the viral life cycle, as the mRNA serves not only as the template for the synthesis of all viral structural and accessory proteins but also as the genome for the next generation of viral particles [81]. The transcription step is mediated by host RNA polymerase II (Pol II) and by the virus-encoded transcriptional transactivator Tat protein that lead the formation of a large number of viral mRNAs synthesized from viral dsDNA integrated into host chromosomes [73]. Then the translation occurs: viral mRNAs are then processed (polyadenylation, methylation, capping, and splicing) to become protein.

1.2.1.4.5. VIRAL ASSEMBLY AND BUDDING

The assembly step of the viral replication consists in the preparing of the viral particle with all its components required for infectivity: two copies of the positive sense genomic viral RNA, cellular tRNA molecules to prime cDNA synthesis, the viral envelope Env protein, the Gag polyprotein, and the three viral enzymes: protease, reverse transcriptase, and integrase (Figure 12). Assembly is driven by the main viral structural polyprotein, Gag, which is formed by four domains, namely, matrix (MA), capsid (CA), nucleocapsid (NC), and p6, and of two short spacer peptides, SP1 and SP2 [82]. The viral enzymes are packaged as domains within the Gag-Pro-Pol polyprotein. HIV-1 virion assembly occurs at the plasma membrane, within specialized membrane microdomains. HIV-1 assembles as an immature particle, wherein CA is released from the Gag polyprotein upon proteolytic cleavage during maturation and self-assembles to form the closed, conical structure [83]. The HIV-1 Gag (and Gag-Pro-Pol) polyprotein itself plays essential roles in virion assembly, including binding the plasma membrane making the protein–protein interactions necessary to create spherical particles, concentrating the viral Env protein, and packaging the genomic RNA. The budding phase occurs after the assembling and these two steps are considered inextricably linked processes. Virus budding can be divided into two stages [84]: the first is the membrane deformation, when the membrane is packed around the assembling virion and the second is the membrane fission, when

the bud neck is severed. Although Gag itself can bind membranes and assemble into spherical particles, the budding event that releases the virion from the plasma membrane is mediated by the host ESCRT (endosomal sorting complexes required for transport) machinery [85].

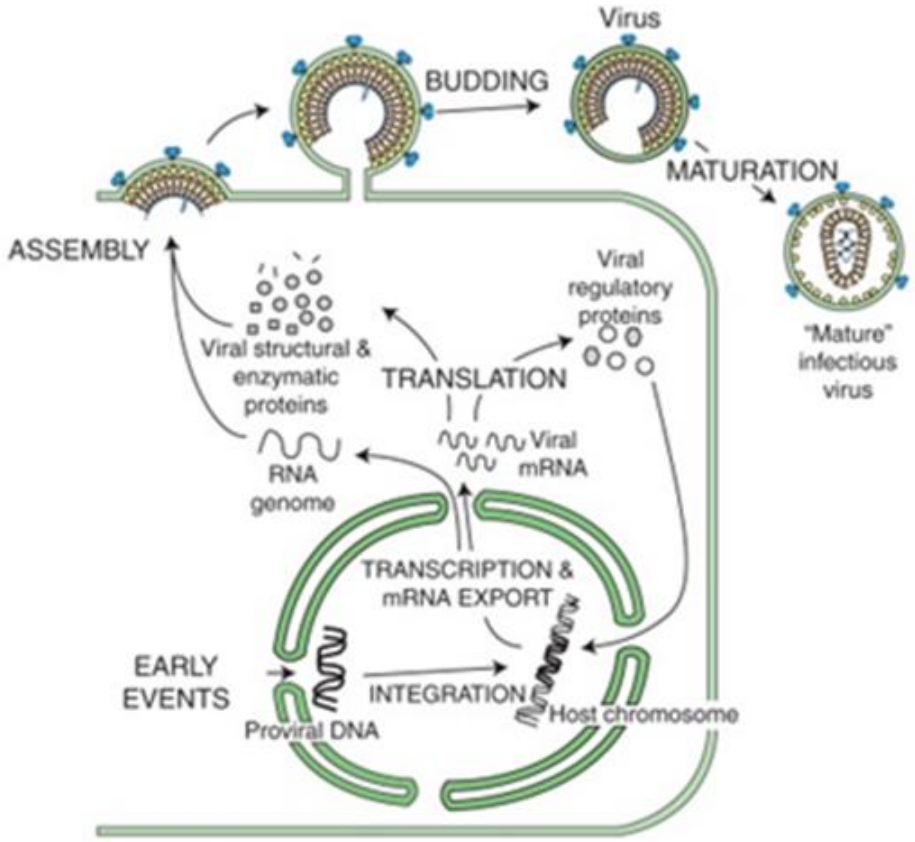

Figure 12. Graphical representation of viral assembly, budding and maturation (modified from [85]*).*

1.2.1.4.6. VIRAL MATURATION

Viral maturation begins simultaneously with (or immediately after) budding, and it occurs when viral protease cleaves the Gag and Gag-Pro-Pol polyproteins at ten different sites, producing the fully processed MA, CA, NC, p6, PR, RT, and IN proteins [85]. During maturation, these processed proteins rearrange dramatically to create the mature infectious virion, with its characteristic conical core. At the beginning the spherical, noninfectious and immature viral particle is organized by a layer of Gag proteins that are associated with the inner viral membrane. Upon budding, the immature particles rearrange to create infectious and mature virions. The interaction between the positively charged NC domain and negatively charged RNA is responsible for the encapsidation of the RNA genome within particles [86].

1.2.2. HIV-2

Even though HIV-1 infection is the cause for most of the global AIDS pandemic, HIV-2 is also an important cause of diseases in some regions of the world. It was first isolated in1986 [87] and in terms of epidemiology, infection with HIV-2 remains largely confined in West Africa, but recently an increasing number of cases have been discovered also in Europe, India, and the United States [88]. Eight different HIV-2 groups were identified, from A to H. Group A is widespread throughout the sub-Saharan region, whereas group B is mainly reported in the Ivory Coast. Groups C to H are categorized as “dead-end” [89] because they produce no subsequent infections. HIV-2 shares many similarities with HIV-1 in genetic and biological properties, such as genome arrangement, CD4+ cell depletion [90], modes of transmission and intracellular replication pathways [91]. However, HIV-2 exhibits much longer clinical latency periods, significantly lower rates of disease progression and transmission and lower viral load in the asymptomatic phase as compared to HIV-1 infection. Moreover, HIV-2 infection is characterized by higher CD4 cell counts, lower viral RNA levels [88] and lower AIDS-related mortality rate [87] than that seen in HIV-1 infection. When clinical progression occurs, infections from both HIV-1 and HIV-2 demonstrate very similar pathological processes, although progression in HIV-2 occurs at higher CD4 counts [91]. Another difference between the two viruses is the treatment, indeed most of the antiretrovirals developed, inhibit HIV-1 replication and many of them are not active against HIV-2.

1.2.3. HIV EPIDEMIOLOGY AND TRANSMISSION

The time between HIV infection and the diagnosis of HIV varies between individuals. Therefore, it is difficult to calculate the number of people newly infected with HIV per year to determine the incidence of HIV infections by exclusively using newly diagnosed HIV cases. From the WHO data, since the beginning of the epidemic, 75 million people have been infected with HIV and about 32 million people have died for HIV. In the 2018, 37.9 million [32.7–44.0 million] people were living globally with HIV, distributed mainly in the African continent, indeed, Southern Africa remains the epicentre of the pandemic and still have high rates of new HIV-1 infections (Table 2).

REGION	NUMBER OF PEOPLE (million)
Africa	25.7 [22.2-29.5]
Americas	3.5 [3.0-4.2]
South-East Asia	3.8 [3.1-4.9]
Europe	2.5 [2.3-2.8]
Western Pacific	1.9 [1.7-2.1]
Eastern Mediterranean	0.5 [0.29-0.57]

Table 2. Estimated number of people living with HIV during 2018 from WHO data.

HIV is present in some human fluids (blood, semen, rectal and vaginal fluids and breast milk). For transmission, fluids infected with HIV must get into the bloodstream through a mucous membrane, open cuts or by direct injection [92]. Therefore, HIV is spread by sexual intercourses, by sharing needles or syringes, by receiving blood transfusion, from mother to child during pregnancy, birth or breastfeeding. Heterosexual transmission remains the dominant mode of transmission and accounts for about 85% [93] of all HIV-1 infections. In spite of the different ways by which HIV-1 can be transmitted between individuals, there is generally an orderly and reproducible appearance in the blood of viral and host markers of infection following the transmission event.

1.2.4. HIV ASSOCIATED DISEASE

There are different pathogenetic ways for HIV to cause human disease [94]:

- The progressive destruction of CD4+ T cells that lead to the reduction of cell-mediated immunity, with development of opportunistic infections. CD4+ T-cells are the central facilitators for both cellular and humoral immune responses against exogenous antigens and they are kept constant in the human body by homeostatic mechanisms. HIV binds to the CD4 molecule on the surface of helper T-cells and replicates within them. This leads to the destruction of CD4+ T-cells and to a steady decline in this population of T-cells [90]. Many tumours are just the consequence of the reduced immune surveillance occurring in HIV disease;
- The direct tissue damage by HIV via mononuclear cell activation;
- The systemic indirect tissue damage via endothelial cell dysfunction and immune activation. HIV untreated patients have enhanced immunological activation, both

innate and adaptive systems, with higher inflammatory markers in body fluids. The other systemic process is damage to endothelia in chronic HIV infection that causes intimal proliferation and vascular stenoses [94].

1.2.5. STAGES OF HIV INFECTIONS

HIV-1 infections can be divided in three different phases with different symptoms and manifestations.

1.2.5.1. PHASE 1: ACUTE HIV INFECTION

Acute HIV infection is the earliest stage of HIV infection, immediately following acquisition of the virus and before the seroconversion. For HIV-1, This period generally lasts 3–4 weeks. In this phase there is a high viral load in the blood and people are very countagious [95]. Many individuals in this phase show symptoms of the acute retroviral infection, that are often mild and not specific like flu symptoms and this is the reason why often HIV infection is not immediately recognized. During this primary infection, the number of HIV-1 particles in plasma increases rapidly, reaches a peak, and then declines until it reaches a set point level [96]. The period between the moment when the first cell is infected and when virus is first detectable in the blood is termed the eclipse phase. Based on clinical evidences, it has been estimated that the ecplipse phase lasts for about 7-21 days [92]. During this phase that is clinically silent, virus increases exponentially in blood plasma and it is propagated in $CD4^+$ T cells in mucosa, submucosa, draining lymphatics, and less in gut-associated lymphoid tissue and systemic lymphatic tissues [92]. Fiebig *et al.,* [97] developed a classification of primary HIV-1 infection (Figure 13) using sequential assay reactivity to identify six different laboratory stages of acute/early HIV-1 infection over approximately a 3 months period following HIV-1 acquisition [98]. The stages [99] are based on the sequential appearance in plasma of HIV-1 viral RNA (stage I); the gag p24 protein antigen (stage II); virus-specific antibodies detectable first by recombinant protein-based enzyme-linked immunosorbant assay (ELISA) (stage III); virus-specific antibodies detectable by Western immunoblotting (stage IV); diagnostic banding pattern but missing p31 reactivity (stage V) and diagnostic banding pattern with p31 reactivity (stage VI).

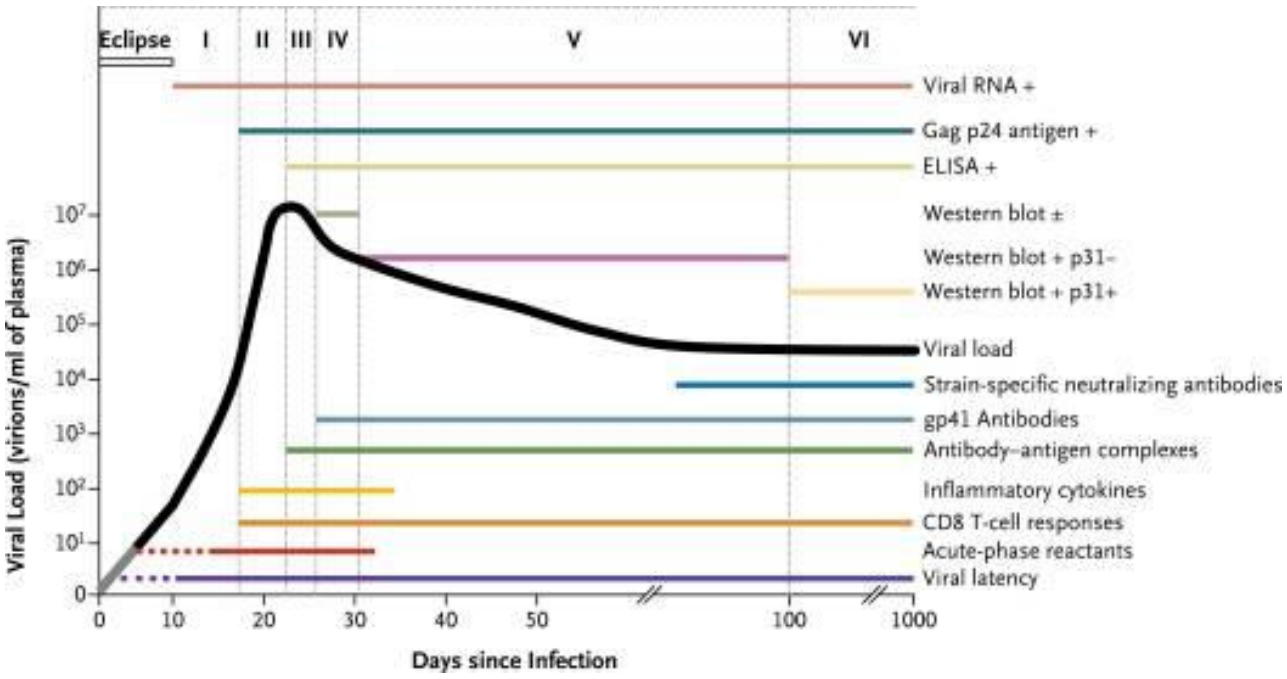

Figure 13. Fiebig classification of HIV-1 primary infection (From[99]*).*

1.2.5.2. PHASE 2: CLINICAL LATENCY

The second stage of HIV infections is an asymptomatic period in which HIV is still active but reproduces at low levels. Without treatment this phase can last from 3 to over 10 years. People who are taking antiretoviral medicines may stay in this phase for many decades and keep an undetectable viral load. In the case of untreated individuals, HIV infection will cause severe damage to the immune system. At the end of this phase, a person's viral load increase and the CD4 cell count begins to go down and that lead to the progression of AIDS.

1.2.5.3. PHASE 3: AIDS

The acquired immune deficiency syndrome (AIDS) is one of the most devastating infectious diseases affecting humankind and it is the most known manifestation of the HIV infections. It was first recognized in the summer of 1981 when an increasing number of young men began falling ill, especially they showed the symptons of the Kaposi's sarcoma [100] and non-Hodgkin lymphoma [101]. This patients experienced a rapid downhill course and death as their doctors vainly treated one opportunistic infection after another [102]. The AIDS is the last stage of HIV infection and it occurs when the number of the CD4+ T cells falls below 200 cells per cubic millimeter of blood [90] (200 cells/mm^3). The

decline of CD4 T cells can lead to opportunistic infections, and it increases mortality. AIDS occurs when the HIV infection is not treated and lead to the development of many other infections caused by bacteria, viruses and parasites that are normally controlled by the immune system. Moreover, peolpe with AIDS have an increased risk to develop viruses related tumor: including lymphoma, that is usually associated with human herpesvirus 8 [103] and cervical cancer caused by hrHPV infections [104].

1.2.6. ANTIRETROVIRAL THERAPY (ART) and HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART)

Actually, a definitive cure for HIV-1 has not yet been found, but many treatment options are still available. HIV-1 infection can be kept under control by the ART therapy, a combination of antiretroviral drugs. Standard ART is comprised of the highly active antiretroviral therapy [89] (HAART) that is constituted by at least three different medicines. This therapy can dramatically decrease the progression of HIV disease [105], improving health outcomes, reducing the risk of transmission and extending life expectancy. The HAART therapy is essential to interrupt the natural infection progression because it reduces the viral replication and so it slows the immune system failure. Moreover, a key point of the HAART treatment is that it can reduce the HIV transmission by as much as 96% [106]. However, the access to HAART is inequitable around the world: only about 30% of people living with HIV in developing countries receive treatment. Also people who are exposed to HIV-positive bodily fluids should begin the prophylactics using antiretroviral therapy as soon as possible because they are at risk of infection [107]. Unfortunately, a treatment failure may occur due to drug resistence. Low compliance, drug side-effects or drug-drug interactions can lead to suboptimum drug concentrations, resulting in viral rebound [93].

1.2.6.1. CLASSES OF FDA-APPROVED HIV DRUGS

FDA approved HIV drugs are divided into different classes based on the method by each drug interfers with the viral lyfe cycle.

- Reverse Transcriptase Inhibitors (RTIs): are a group of drugs, which can bind and inhibit the reverse transcriptase enzyme, by inducing conformational changes, to reduce the multiplication of HIV. There are two types of RTIs: non-nucleoside

reverse transcriptase inhibitors (NNRTIs) that directly bind the RT enzyme [108] and nucleoside reverse transcriptase inhibitors (NRTI) that compete with natural deoxynucleotides for incorporation into the growing DNA chain [109].

- Protease Inhibitor (PIs): are drugs that block the functioning of HIV protease enzymes [110] in acutely and chronically HIV-infected CD4 cells leading the release of noninfectious viral particles.
- Fusion Inhibitors (FIs): are a class of drugs that prevent HIV entry by blocking the fusion of the virion to the host cell [89].
- Chemokine Receptor 5 Antagonists: are a group of drugs that antagonize the CCR5 coreceptors preventing the HIV viral entry into host cells [111].
- Integrase Inhibitors (INs): are a group of drugs that prevent the integration of viral DNA into the host genome of CD4 cells by the inhibition of the integrase enzyme [112].
- Post-Attachment Inhibitors: are a class of drug that binds CD4 receptors on the surface of certain immune cells and consequently they blocks the attachment of HIV to the CCR5 and CXCR4 coreceptors preventing the HIV viral entry into cells [113].
- Pharmacokinetic Enhancers: are drugs used in a HIV regimen therapy to increase the effectivness of other HIV medicines. They inhibit the human CYP3A cytochrome, that is an enzyme responsible for the metabolism of most HIV drugs, increasing plasma concentration of other anti-HIV drugs [114] they are paired with.

1.2.7. HIV TESTING

HIV testing is a key point for HIV prevention and treatment. It is important in order to identify HIV infections as early as possible, to find the best treatment for each individual and to reduce the risk of HIV transmission. HIV-1 RNA, anti-HIV antibodies in serum or in plasma, and HIV-1 capsid protein (p24 antigen) are the main viral markers used to detect HIV-1 infections [115]. Current techniques of HIV antibody detection are very sensitive and they can detect HIV antibody within or after 1 or 2 weeks of HIV infection. Additional confirmatory tests are also available for the accuracy of results [116]. In primary HIV screening, enzyme immunoassay (EIA) tests are the most used assay for the detection of HIV antibody [117]. In a small number of early seroconverters who are still in the diagnostic window, that is the period between the exposure to HIV and the point where biochemical measureable markers like antibodies can be detected, the p24 antigen may become positive because it can be identified 5 days before seroconversion. For this reason, HIV-

1 p24 tests are used in combination with anti-HIV antibody testing for early detection of HIV-1 infection.

Since 1980s, after the start of HIV testing, EIA tests became more sensitive and automated, but less specific and that leads to an increasing of false positive outcomes in most cases and this is the reason why the first test should be confirmed by a confirmatory test that consists in another type of assay. The most common confirmatory tests are based on the technique of Western Blot, a highly specific immunoblot that allows the visualization of antibodies for the structural polypeptides of HIV. These tests are also performed in patients who are at the beginning of drug therapy for monitoring its effectiveness.

To evaluate the effectiveness of the treatment also the HIV RNA tests are used, indeed, if the confirmatory test is positive, the RNA test can be used to quantify the HIV RNA by PCR to determine the viral load that can be used to predict the infection clinical progression. Moreover, they are also used to diagnose HIV infection in children aged less than 18 months, since the HIV serological tests are not reliable because they cannot distinguish between maternal HIV antibodies transeferred passively during pregnancy and HIV antibodies produced by the infant.

Regarding the therapeutic drug monitoring, recent studies, have been demonstrate that also the quantification of HIV DNA is important, indeed in patients under tretment, it has been established a direct link between the HIV DNA viral load and the viral reservoir [118].

1.2.8. HIV DNA

Due to monitor the evolution of the infection, an important marker to measure during HAART therapy is HIV DNA that constitutes the latent reservoirs of the provirus from which infectious virions can potentially be released. Viral reservoirs consist of anatomical stocks and a small pool of infected long-lived memory T lymphocytes [93]. HIV-1 latency in long-lived cell populations is an obstacle for the HIV eradication because antiretroviral combination treatments can fail to eliminate integrated proviruses from resting cells. This reservoir is generated at the beginning of the infection, during the acute phase by reversion of the infected CD4+ T cells into quiescent memory lymphocytes [119]. These infected cells are transcriptionally silent but they can produce new infectious virions when activated again. Reactivation of these latent provirus is responsible for the rebound viraemia [120] that occur in patients who interrupt antiretroviral therapy and this is the reason why it is very important to measure the HIV DNA in order to monitor the progression of the infection. Furthermore the quantification of the HIV DNA at different

stage of the antiretroviral therapy, could be used to evaluate the effectiveness of the treatment and to predict the failure of the therapy at early stage [119]. HIV DNA can be detected in lymphoid tissues and in peripheral blood mononuclear cells (PBMC) [119], indeed after HIV enters the body and local replication occurs, it quickly disseminates to the lymph node and later on the bloodstream. The lymphoid tissues are the most important sites of viral replication during active infection, and HIV DNA can still be detected in the lymph nodes after years of antiretroviral therapy [120].

Many recent evidences suggest that the quantification of HIV DNA represents an important element in the pianification and monitoring of the therapy in patients who are treated with antiretroviral drugs. Indeed, total HIV DNA load influences the course of the infection and is therefore clinically relevant [121], especially, it is predictive of progression to AIDS and death, independently of HIV RNA load. Actually, the identification and quantification of HIV DNA is made by Real time PCR *in-house* methodic and the standardized and validated assays are poorly available or difficult to use. So, it has been emerged the need of the standardization of this type of assay to provide relevant information for HIV treatment.

2. AIM OF THE STUDY

The aim of this project is the development and the validation of two different *in vitro* diagnostic devices for the identification of two sexually transmitted virus.

In the first part of the study the focus was on the Human Papillomavirus (HPV), in particular I deal with the validation of a new hrHPV detection assay usable in primary cervical cancer screening, on the instrument AriaDx (Agilent Technologies).

This device was previously designed for the qualitative detection of the DNA of 14 hrHPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 e 68) by Real Time PCR with the simultaneous genotyping of HPV 16 and HPV 18. This assay has already been validated for the use in primary cervical cancer screening on the instrument Applied Biosystems 7500 Fast Dx Real Time PCR Systems (ABI 7500 Fast Dx - Applied Biosystems). In this study, the clinical validation, according to Meijer guidelines [56], was carried out on AriaDx instrument and in particular, the following parameters were evaluated:

- Clinical specificity
- Clinical sensitivity
- Intra-laboratory reproducibility
- Inter-laboratory reproducibility

Moreover, were tested the analytical and the diagnostic performace of this assay, evaluating:

- Analytical sensitivity
- Analytical specificity
- Diagnostic sensitivity
- Diagnostic specificity

The last purpose of the first part of this study was the evaluation of eventually interfering substances that may derive directly from the sample (swab in this case) or be introduced during its processing or during the extraction of nucleic acid. Indeed, the Real time PCR is sensitive to the action of inhibitory substances that may affect the perofmance of the assay, or even lead to false negative results. For this reason, it is important to consider the substances that can eventually affect the sample analysis.

Since it has been established the importance of measuring the HIV DNA in order to monitor the progression of the infection and to evaluate the effectiveness of the treatment, in the second part of the project, I worked on the development and

validation of a device for the identification and the quantification of the HIV-1 DNA able to satisfy the requirements of sensitivity and specificity for its use in the diagnostic field. The purpose of this second part of the study was first of all, to design a Real time PCR methodic for the detection of HIV-1 DNA group M, by selecting specific primer and probes. Then the aim was to evaluate the performance of this prototype, in particular, were analyzed:

- Cut off value of internal control (IC) Ct
- Analitycal Specificity
- Analytical sensitivity
- Precision
- Diagnostic specificity
- Diagnostic sensitivity

This performance evaluation aims to define if the assay is suitable for the use in the research field.

3. PART I: HPV

3.1. MATERIALS AND METHODS

3.1.1. COLLECTION AND STORAGE OF CERVICAL SAMPLES

The cytologic cervical samples used to evaluate the performance of the hrHPV detection assay object of this study, were collected from the Cervical Cancer Screening Program that was made in the district of Bari in the period from September 2015 to October 2016. The samples were selected retrospectively from the files in the Cytopathology Department of the Hospital Di Venere, that represents a reference laboratory for cervical cytology.

After collection, all cytological cervical samples used to assess the clinical performance of the assay were stored in ThinPrep PreservCyt Solution (Hologic Inc., Madison, WI, USA) at Room temperature.

According to Meijer *et al.,*[56] a total of 910 samples divided in the following histological and cytological categories (Table 3) were selected.

Number of Samples	Cytological/Histological status
73	≥CIN2 histology
600	Negative cytology result in two consecutive screening rounds
192	Negative cytology result in three consecutive screening rounds
45	Abnormal cytology result with <CIN2 histology

Table 3. Cytological/histological status of the 910 samples selected for the study.

3.1.2. 2014 WHO LabNet Proficiency Panel

The HPV Laboratory Network (LabNet) has designed international proficiency studies that can be issued regularly and in a reproducible manner. The 2014 LabNet Prociciency Panel was used in this study to determine the analytical sensitivity of the assay under validation. This panel contained 41 coded samples, composed of purified plasmids of sixteen HPV types (HPV 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68a and 68b) and 3 extraction controls. The WHO consider proficient an HPV genotyping assay able to detect at least 50 International Units of HPV 16 and HPV 18 and 500 genome equivalents for the other 14 HPV types, with at least 97% specificity [122].

3.1.3. EXTRACTION METHOD

All the cytologic cervical samples analyzed for the evaluation study, were extracted with GENEQUALITY X120 Pathogen kit (AB ANALITICA, Padova) on the instrument GENEQUALITY X120 (AB ANALITICA, Padova). Specifically, the extractions have been performed by processing 400 µL of sample and eluting in 90 µL.
The CE-IVD marked product GENEQUALITY X120 Pathogen kit is used for the extraction and purification of bacterial and viral nucleic acids, based on magnetic particle technology, starting from different types of biological samples. The GENEQUALITY X120 extraction system automatically performs all the steps of the nucleic acids purification and can process up to 64 samples in one session.
The extraction technology is based on silica-coated paramagnetic beads that are able to bind nucleic acids with high affinity and can be easily integrated into automated extraction systems. The use of magnetic particles enables a high quality purification of nucleic acids which can be used directly in molecular biology assays.
The purification procedure requires the following 4 steps:

- **Lysis of cells:**
  The lysis is performed under highly denaturating conditions at an elevated temperature and in the presence of proteinase K and lysis buffer under continuos shaking. This ensures lysis of cells, digestion of proteins and inactivation of nucleases.

- **Binding to magnetic particles:**
  The binding buffer is added to the lysed samples to obtain the specific condition for binding. The pH and saline environment permit to avoid the binding of proteins and other contaminants, which can inhibit PCR and other enzymatic reactions downstream, to the magnetic particles.

- **Washing of magnetic particles:**
  Substances not bound to the magnetic particles, like proteins, cell debris and PCR inhibitors are removed thanks to repeated washing with washing buffers.

- **Elution of nucleic acids**
  The highly purified nucleic acids are eluted with the elution buffer in a single step at a controlled temperature.

The extraction from cytologic cervical samples was performed without any pretreatment. The HPV DNA extracted after the extraction sessions were collected and stored at temperature -20°C/ -30°C.

3.1.4. hrHPV DETECTION ASSAY

The hrHPV detection assay object of this dissertation is an assay for the qualitative detection of the DNA of 14 high risk oncogenic genotypes of the Human Papillomavirus (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 e 68) by Real time PCR, targeting the E6 and E7 region of HPV genome. In addition, the assay is able to specifically genotyping the HPV 16 and HPV 18 types. The PCR method was the first method to amplify *in vitro* a specific part of DNA (target sequence) by a thermostable DNA polymerase. This technique proved to be a valuable and versatile instrument of molecular biology. It allowed a more efficient research of new genes and their expression, and has revolutionized, among others, the fields of laboratory diagnostics and forensic medicine. Real time PCR technology represents an advancement of the basic PCR technique since, it allows visualizing the amplified target DNA molecules while the reaction proceeds. The detection of amplicons is based on the use of labeled probes or fluorescent intercalating agents able to bind to the double helix DNA molecules produced at each amplification cycle. Fluorescence is recorded in real time through the use of a thermocycler equipped with an optical detector. The main advantage of the Real time PCR compared to conventional techniques of DNA amplification is the possibility to perform a semi-automated amplification. This means, extra steps necessary to visualize the amplification product can be avoided and the risk of contamination by post-PCR manipulation is reduced.

The hrHPV detection assay object of this study includes a ready-to-use PCR master mix that contains all reagents needed for the reaction as well as the dUTP/UNG system that allows to prevent contaminations from previous amplifications, being able to remove uracil residues embedded in single or double helix DNA molecules. The kit includes also a positive control that contains nucleic acid fragments that correspond to the amplified gene regions. The assay also allows to evaluate the presence of reaction inhibitors in the extracted DNA and to monitor the extraction process by amplification of the β-globin gene (BG - internal control) in multiplex with the target pathogens. This valid tool helps the operator to recognize false negative results. The sequence specific probes of the assay are marked with FAM, JOE, Cy5 and ROX fluorophores for the detection of the pathogen target nucleic acid and the internal control DNA (BG) (Table 4).

DNA	Target region	Fluorophores
HPV 16	E7 gene	JOE
HPV 18	E7 gene	CY5
HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	E6-E7 genes	FAM
BG	-	ROX

Table 4. Target regions and fluorophores corresponding to the different HPV genotypes detected by the assay.

For the amplification reaction, 20 µL of the specific Real time mix included in the assay the and 5 µL of target nucleic acid were used.

To guarantee the correct interpretation of the results, it is necessary to use, in each Real time PCR sessions, the positive control that is included in the kit and a negative control (water). If both positive and negative controls show the expected results (Table 5), samples can be analyzed.

	RESULT	INTERPRETATION
Positive control	Amplification signal in all channels	Control and PCR worked correctly
	No amplification signal in one or more channels	No amplification of HPV target gene and/or *BG* gene **repeat the analysis**
Negative control	Amplification signal* in one or more channels	Contamination **repeat the analysis**
	No amplification signal in any channel	Control and PCR worked correctly

Table 5. Scheme of the correct interpretation of Positive and Negative control signals.

To determine samples' s results, it is necessary to evaluate first of all, the threshold cycle (Ct) value of the BG (Table 6): a sample is negative for HPV only when the amplification curve of the internal control has a Ct lower than or equal to 34. If the Ct of BG is higher, it is not possible to exclude that the sample negativity for the pathogen target is due to issues regarding the sample itself or the extraction procedure, thus the sample has to be considered unsuitable for the analysis. With positive samples a delayed Ct in the β-globin gene amplification signal can occur because the assay has been designed to favor the amplification reaction of the pathogenic target.

RESULT	INTERPRETATION	
BG	**HPV negative sample**	**HPV positive sample**
Amplification signal with Ct ≤ 34	Suitable sample	Suitable sample
Amplification signal with Ct > 34	Unsuitable sample	Suitable sample

Table 6. Interpertation of the Ct values of the BG signals.

Once the BG signals have been evaluated, it is possible to proceed with the analysis of the target pathogens.

This assay has already been validated for the use in primary cervical cancer screening on the instrument Applied Biosystems 7500 Fast Dx Real Time PCR Systems (ABI 7500 Fast Dx - Applied Biosystems). In this study, the clinical validation, according to Meijer guidelines [56], was carried out on AriaDx.

The same cut-off previously identified on ABI 7500 Fast Dx produced satisfactory results during the clinical validation on AriaDx. Thus, in order to guarantee homogeneity of analysis among instruments, it was chosen as cut-off value also on this instrument. The Ct cut-off value for all targets was set to 40, therefore, samples with Ct higher than 40 must be considered as negative. Results were interpreted according to the following table (Table 7):

BG channel (ROX)	**HPV channels (FAM and/or JOE and/or Cy5)**	**Interpretation**
Ct signal ≤ 34	**Ct signal < 40**	HPV positive
Ct signal ≤ 34	**Ct signal ≥ 40 or undetectable**	HPV negative
Ct signal > 34 or undetectable	**Ct signal < 40**	HPV positive
Ct signal > 34 or undetectable	**Ct signal ≥ 40 or undetectable**	Sample unsuitable for analysis

Table 7. Cut-off values for each fluorophore.

3.1.5. REAL TIME PCR INSTRUMENT: AriaDx Real-Time PCR System (AriaDx - Agilent Technologies)

The AriaDx Real Time PCR System is a fully integrated quantitative PCR amplification, detection, and data analysis system for nucleic acids samples. The system design combines a thermal cycler, an optical system with a LED excitation source, and data analysis software. The instrument has six dyes (ROX, FAM, HEX, CY5, CY3 and ATTO 425) and it provides a closed- tube PCR detection format that can be used with a variety of fluorescence detection chemistries including fluorogenic probe systems as TaqMan probes. A specific thermal profile was used for the validation experiments on AriaDx (Table 8).

Step	Repeats	Time	(°C)
1	1	02:00	50.0
2	1	10:00	95.0
3	45	00:15	95.0
		01:00	60.0*

Table 8. Specifications of thermal profile.

* Fluorescence detection step

All the experiments were analyzed in linear scale using the settings of baseline and threshold described in the following table (Table 9):

INSTRUMENT	CHANNEL	BASELINE	THRESHOLD
AriaDx	FAM	automatic	150
	JOE	automatic	100
	Cy5	automatic	350
	ROX	automatic	25

Table 9. Settings used for the analysis of each run.

The baseline of the Real time PCR reaction refers to the signal level during the initial cycles of PCR in which there is low variation in fluorescent signal. The low-level signal of the baseline can be equated to the background or the “noise” of the reaction and it is used to normalize all amplification signals. The cycle range in which baseline is calculated should be set carefully to allow accurate determination of the threshold

cycle. This range should comprise enough cycles to eliminate the background noise found in the early cycles of amplification but should not include the cycles in which the amplification signal begins to rise above background. The choice of the baseline range is strictly depending on the type of fluorophores and their concentration in the PCR reaction. AriaDx employs an automatic baseline setting.

The threshold of the Real time PCR reaction is the level of signal that reflects a statistically significant increase over the calculated baseline signal. It is set to distinguish relevant amplification signal from the background noise. Usually, Real-Time PCR instrument software automatically sets the threshold at 10 times the standard deviation of the fluorescence value of the baseline. However, the positioning of the threshold can be set at any point in the exponential phase of the reaction.

The choice of threshold is depending on the kinetics of the reaction that determines the amplification plots (caused by type and concentration of fluorophores, specific design of the assay). The threshold values reported in the table were chosen empirically. They were evaluated and definitively set after the analysis of the performance evaluation study, in order to assure optimal results in terms of diagnostic sensitivity and specificity and analytical sensitivity.

3.1.6. THE REFERENCE METHOD: HYBRID CAPTURE 2 (HC2)

Hybrid capture 2 (HC2), an FDA approved assay for diagnostic testing, was used as reference method and was performed previously on all the 910 samples used in this study. It is an *in vitro* nucleic acid hybridization assay with signal amplification and chemiluminescence for the qualitative detection of 13 hrHPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) in cervical specimens.The data obtained on these samples were then compared with the results obtained on the same samples analyzed with the assay object of this study.

3.1.7. AMPLIQUALITY HPV TYPE-EXPRESS v.3.0

AMPLIQUALITY HPV TYPE-EXPRESS v.3.0 (AB ANALITICA) was used to analyze the samples that resulted discordant with the two methodics. This product is based on end-point PCR targeting the L1 region of HPV genome, followed by reverse line blot (RLB) and 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, 90).

The RLB technique is based on the fact that the PCR products are hybridized on a solid surface (strip) that was previously coated with highly specific probes which are complementary to the amplified nucleic acid sequences.

The hybridization on a membrane includes the following steps:

- Denaturation: the labeled PCR product is denatured chemically or thermically (incubation at 95 °C).
- Hybridization: the membrane (strip) is incubated under specific conditions (temperature, shaking, pH) with a solution containing the labeled and denatured PCR product.
- Washing: unbound PCR product is washed away. This step is an important factor determining the stringency of the hybridization.
- Visualization: the hybrid is visualized because of the labelling of the bound product with the enzyme alkaline phosphatase. This colorimetric reaction leads the appearance of a band pattern on the strip.

3.1.8. STATISTICAL ANALYSIS

To compare the clinical sensitivity and specificity for ≥CIN2 of the assay object of this study to that of HC2, a non-inferiority score test with a power of at least 80% was performed. The relative sensitivity and specificity thresholds used were 90% and 98% respectively, according to Meijer guidelines; a P value <0.05 was considered statistically significant. Percentages of agreement were used to test intra- and inter-laboratory reproducibilities.

3.2. RESULTS

3.2.1. CLINICAL PERFORMANCE

In order to assure that a new HPV DNA test is suitable for primary cervical cancer screening, Meijer *et al.*, [56] defined a clinical validation strategy based on the comparison of the performance of the assay under evaluation with that of an already clinically validated reference HPV test, on samples originating from a population-based screening cohort. They also required the assessment of the device inter and intra-laboratory reproducibility.

In this study, the clinical sensitivity and specificity of the assay object of this study were compared to those of the reference method (HC2) using a non-inferiority test, while percentage of agreement were used to evaluate inter and intra-laboratory reproducibility.

3.2.1.1. CLINICAL SPECIFICITY for <CIN2

Clinical specificity is the definition of the probability that the device produces results non-inferior to those of an already clinically validated device on <CIN2 samples. For clinical specificity, 837 <CIN2 samples were tested with both the hrHPV detection assay object of this study and the reference method, HC2. The table below summarises the obtained results (Table 10).

	HC2 (reference method)		
hrHPV detection assay object of this study	**Positive**	**Negative**	**Total**
Positive	67	15	82
Negative	13	742	755
Total	80	757	837

Table 10. Results obtained with both the assay object of the study and HC2 on 837 <CIN2 samples.

The non-inferiority test applied to the above data produced a T value (interpreted as a z statistics) of 2.28 with a p value of 0.011. With a significance level of 0.05, the null hypothesis of inferiority is rejected.

3.2.1.2. CLINICAL SENSITIVITY for ≥CIN2

Clinical sensitivity is the definition of the probability that the device produces results non-inferior to those of an already clinically validated device on ≥CIN2 samples. For clinical sensitivity, 73 ≥CIN2 samples were tested with both the hrHPV detection assay object of this study and the reference method, HC2. The table below (Table 11) summarises the obtained results.

	HC2 (reference method)		
hrHPV detection assay object of this study	**Positive**	**Negative**	**Total**
Positive	71	0	71
Negative	0	2	2
Total	71	2	73

Table 11. Results obtained with both the assay object of the study and HC2 on 73 ≥CIN2 samples.

The non-inferiority test applied to the above data produced a T value (interpreted as a z statistics) of 2.81 with a p value of 0.002. With a significance level of 0.05, the null hypothesis of inferiority is rejected.

The clinical specificity and sensitivity for ≥CIN2 of the hrHPV detection assay object of this study on the instrument AriaDx are at least as good as those of the reference test, HC2.

3.2.1.3. INTRA- LABORATORY REPRODUCIBILITY

For intra-laboratory reproducibility, that is the percentage of agreement between two consecutive test rounds performed in the same laboratory, 536 samples were tested in two separate occasions with the hrHPV detection assay object of this study. The table below (Table 12) summarises the obtained results.

	First test round		
Second test round	**Positive**	**Negative**	**Total**
Positive	168	4	172
Negative	0	364	364
Total	168	368	536

Table 12. Results obtained on 536 samples tested in two consecutive rounds in the same laboratory.

From these results, intra-laboratory reproducibility was calculated as shown below:

$$\textbf{Intra} - \textbf{laboratory reproducibility}: \frac{\mathbf{168 + 364}}{\mathbf{168 + 0 + 4 + 364}} \times \mathbf{100} = \mathbf{99.3}\%$$

The corresponding Clopper-Pearson 95% confidence interval was 98.1%-99.8%. To define this interval the online tool MedCalc was used [123] .

With a lower confidence bound higher than 87%, the intra-laboratory reproducibility on AriaDx of the hrHPV detection assay object of this study was adequate, according to international guidelines.

3.2.1.4. INTER-LABORATORY REPRODUCIBILITY

For inter-laboratory reproducibility, that is the percentage of agreement between two test rounds performed in two different laboratories, 534 samples were tested with the hrHPV detection assay object of this study in two different laboratories: the first ltest was carried out at BIOFIELD INNOVATION laboratories and the second was done in a laboratory partner. The table below (Table 13) summarises the obtained results.

	First laboratory		
Second laboratory	**Positive**	**Negative**	**Total**
Positive	167	2	169
Negative	0	365	365
Total	167	367	534

Table 13. Results obtained on 534 samples tested in two consecutive rounds in two different laboratories.

From these results, inter-laboratory reproducibility was calculated as shown below:

$$\textbf{Inter} - \textbf{laboratory reproducibility}: \frac{\mathbf{167 + 365}}{\mathbf{167 + 0 + 2 + 367}} \times \mathbf{100} = \mathbf{99.6}\%$$

The corresponding Clopper-Pearson 95% confidence interval was 98.7%-100.0%. To define this interval the online tool MedCalc was used [123] .

With a lower confidence bound higher than 87%, the inter-laboratory reproducibility on AriaDx of the hrHPV detection assay object of this study was adequate, according to international guidelines.

3.2.2. EVALUATION OF ANALYTICAL PERFORMANCE

3.2.2.1. THE ANALIYTICAL SENSITIVITY: WHO PROFICIENCY

Analytical sensitivity represents the lower concentration of a given substance that can be detected by the assay.

The 2014 WHO LabNet Proficiency Panel has been tested on AriaDx with the device object of this study. A total of 41 samples were tested. All the genotypes were present in two different concentrations (500 and 50 Genome Equivalents per reaction), except for HPV 16 and HPV 18 that were present in the concentrations of 5 and 50 International Units per reaction.

All the tested samples were at low concentrations in order to verify if the assay is able to correctly identify also the sample's concentration in its sensitivity limit.

The available sample still allowed the evaluation of proficiency according to WHO criteria.

The following table (Table 14) shows the obtained results:

Sample ID	HPV content	Concentration per genotype (IU or GE / rx)*	BG status	Ct HPV Rx-1	Ct HPV 16	Ct HPV 18	Results
1	16, 33, 45, 51	500	Suitable	28.48	29.57	NA	Correct
2	negative	0	Suitable	NA	NA	NA	Correct
3	51	50	Suitable	33.76	NA	NA	Correct
5	39	500	Suitable	32.05	NA	NA	Correct
6	58	500	Suitable	30.19	NA	NA	Correct
7	35, 39, 59, 66, 68b	500	Suitable	28.25	NA	NA	Correct
8	68a	500	Suitable	30.61	NA	NA	Correct
9	51	500	Suitable	30.51	NA	NA	Correct
10	31	500	Suitable	32.84	NA	NA	Correct
11	16	5	Suitable	NA	36.17	NA	Correct
12	35	50	Suitable	33.46	NA	NA	Correct
13	66	50	Suitable	33.31	NA	NA	Correct
14	6, 56, 58, 68a	50	Suitable	31.89	NA	NA	Correct
15	11	50	Suitable	NA	NA	NA	Correct
16	45	50	Suitable	34.76	NA	NA	Correct
17	58	50	Suitable	33.33	NA	NA	Correct
18	68b	50	Suitable	34.18	NA	NA	Correct
19	6	500	Suitable	NA	NA	NA	Correct
20	39	50	Suitable	34.34	NA	NA	Correct
21	66	500	Suitable	30.31	NA	NA	Correct
22	11, 18, 31, 52	50	Suitable	32.53	NA	32.55	Correct
23	31	50	Suitable	35.89	NA	NA	Correct
24	56	500	Suitable	29.78	NA	NA	Correct
26	33	50	Suitable	33.9	NA	NA	Correct
27	6, 56, 58, 68a	500	Suitable	28.39	NA	NA	Correct
28	11	500	Suitable	NA	NA	NA	Correct
29	52	500	Suitable	30.49	NA	NA	Correct
30	11, 18, 31, 52	500	Suitable	29.05	NA	28.97	Correct
31	18	5	Suitable	NA	NA	37.26	Correct
33	59	50	Suitable	33.02	NA	NA	Correct
34	35, 39, 59, 66, 68b	50	Suitable	31.25	NA	NA	Correct
36	56	50	Suitable	33.46	NA	NA	Correct
37	68a	50	Suitable	33.93	NA	NA	Correct
38	6	50	Suitable	NA	NA	NA	Correct

Sample ID	HPV content	Concentration per genotype (IU or GE / rx)*	BG status	Ct HPV Rx-1	Ct HPV 16	Ct HPV 18	Results
39	**59**	**500**	Suitable	30.66	NA	NA	Correct
41	**16, 33, 45, 51**	**50**	Suitable	32.03	33.09	NA	Correct
42	**33**	**500**	Suitable	30.65	NA	NA	Correct
43	**35**	**500**	Suitable	30.41	NA	NA	Correct
A	**16**	**2500**	Suitable	NA	25.1	NA	Correct
B	**16**	**25**	Suitable	NA	32.57	NA	Correct
C	**negative**	**0**	Suitable	NA	NA	NA	Correct

Table 14. Results (HPV content, concentration, BG status and Ct values) of each sample of the 2014 WHO LabNet Proficiency Panel tested with the hrHPV detection assay object of this study.

* Concentration is expressed as International Units (IU) per reaction for HPV 16 and 18, and as Genome Equivalents per reaction for all of the other genotypes.

All samples were correctly identified by the hrHPV detection assay object of this study.
As for all the other high risk genotypes, the assay was able to correctly identify the reference concentration for proficiency (500 Genome Equivalents per reaction). It was also able to correctly identify the respective samples with a 10 times lower concentration.
The assay produced the anticipated results also for multi-infection samples.
Being able to detect at least 50 International Units per reaction for HPV 16 and 18, and at least 500 Genome Equivalents per reaction for the other hrHPVs, the device object of this study is proficient on AriaDx according to WHO criteria.

3.2.2.2. EVALUATION OF ANALYTICAL SPECIFICITY

The analytical specificity is the evaluation of phenomena of cross-reactivity with DNA belonging to other potentially cross-reactive microorganism to sequence homology, phylogenetic proximity or infecting the same biological district;
The analytical specificity of the device object of this study is guaranteed by an accurate choice of specific primers and probe, as well as by specific amplification conditions. Primer and probe alignments in the most common databases revealed the absence of non-specific pairing.
Specificity was also checked using 18 samples positive for one or more of the following pathogens, potentially cross-reactive because they infect the same districts:

- *Chlamydia trachomatis,*
- *Neisseria gonorrhoeae,*
- *Ureaplasma parvum,*
- *Herpes simplex 1,*
- *Herpes simplex 2,*
- *Mycoplasma hominis,*
- *Mycoplasma genitalium,*
- *Ureaplasma urealyticum,*
- *Ureaplasma parvum,*
- *Candida albicans,*
- *Gardnerella vaginalis,*
- *Adenovirus,*
- *Cytomegalovirus,*
- *Trichomonas vaginalis,*

Also other 54 samples were tested. They were positive for one or more of the following HPV genotypes: HPV 6, 11, 40, 42, 43, 44, 53, 54, 55, 61, 62, 67, 70, 73, 81, 82, 84, 87, 89, 90, potentially cross-reactive due to genetic similarity with the types detected by the assay under evaluation.

None of the tested pathogens resulted cross-reactivewith the hrHPV assay object of this study.

3.2.3. EVALUATION OF DIAGNOSTIC PERFORMANCE

3.2.3.1. DIAGNOSTIC SPECIFICITY, SENSITIVITY AND ACCURACY

The diagnostic specificity is the definition of the probability that the device gives a negative result in the absence of the target marker. It is given by the number of true negative results divided by the total number of samples without the pathogen (which is the sum of the numbers of true negative plus false positive results).
The diagnostic sensitivity is the definition of the probability that the device gives a positive result in the presence of the target marker. It is given by the number of true positive results divided by the total number of samples with the pathogen (which is the sum of the numbers of true positive plus false negative results).
The accuracy of a test represents how close a result comes to the true value. It is the number of the sum of true positive and negative results divided by the number of total results.
To determine diagnostic specificity, sensitivity and accuracy, the 910 samples, previously used for the evaluation of clinical performance were tested.

For the expected results were considered those obtained with HC2: 758 hrHPV negative and 152 hrHPV positive.

For the definition of the diagnostic specificity, among the 758 negative samples, fifteen resulted discordant with the assay object of this study because they resulted hrHPV positive. These samples were subsequently tested with a second reference method (AMPLIQUALITY HPV TYPE EXPRESS v3.0) with the following results:

- Fourteen samples were confirmed positive for hrHPV. They were considered concordant and they were included in the calculations for diagnostic sensitivity.
- One sample resulted positive for lrHPV, but negative for hrHPV and was considered discordant.

For the definition of the diagnostic sensitivity, among the 152 positive samples, thirteen resulted discordant with the assay object of this study because they resulted hrHPV negative. All these samples, tested with the second reference method (AMPLIQUALITY HPV TYPE EXPRESS v3.0), were confirmed hrHPV negative according to the results obtained with the device object of this study and they were included in the calculations for diagnostic specificity. In particular:

- Eight samples were confirmed negative for both hrHPV and lrHPV genotypes

- Five samples resulted negative for hrHPV and positive for lrHPV genotypes.

The obtained results (Table 15) allowed to calculate the diagnostic specificity and the diagnostic sensitivity of the device object of ths study on AriaDx.

		Obtained results		
		Nr Negative samples	**Nr Positive samples**	**Total**
Expected results	**Nr Negative samples**	757	1	758
	Nr Positive samples	0	152	152
	Total	**757**	**153**	**910**

Table 15. Results obtained with the assay object of this study (obtained results) and with HC2 (expected results).

The diagnostic specificity, obtained by dividing the number of true negative results with the total number of samples without the pathogen, resulted of 99.9%.

$$\textbf{DIAGNOSTIC SPECIFICITY}: \frac{757}{757+1} \times 100 = 99.9\%$$

The diagnostic sensitivity, obtained by dividing the number of true positive results with the total number of samples containing the pathogen, resulted of 100%.

$$\textbf{DIAGNOSTIC SENSITIVITY}: \frac{152}{152+0} \times 100 = 100\%$$

The accuracy, calculated by dividing the number of the sum of positive and negative obtained results with the total number of samples, resulted of 99.9%.

$$\textbf{ACCURACY}: \frac{757+152}{757+1+152+0} \times 100 = 99.9\%$$

3.2.4. EVALUATION OF THE EFFECT OF POTENTIALLY INTERFERING SUBSTANCES

It is commonly known that the PCR is sensitive to the action of inhibitory substances that may affect the sensitivity of the assay, or even lead to false negative results. These interfering substances may derive directly from the sample or be introduced during its processing or during the extraction of nucleic acid. The mechanisms with which the interfering substances can alter the DNA amplification are multiple and can act at different levels, for example, by interacting with the nucleic acid or directly/indirectly with the DNA polymerase. The inhibitory substances may be of both inorganic and organic nature. It is commonly known that the collection of the specimens is a crucial point to obtain a suitable sample for molecular analysis, especially for swabs; in fact, the presence of blood or mucus can interfere with the identification of potential pathogens.

Currently, to eliminate interfering substances, the most common strategy consists in the selection of efficient methods of extraction and purification of nucleic acid, in the dilution of the sample or nucleic acid with a resulting dilution of the inhibitory effect and in the careful selection of a resistant DNA polymerase.

An experimental evaluation of the effects of potentially interfering substances on urogenital swabs, the most relevant clinical matrix for the use of the device object of this study, was performed during this validation.

The tested interfering substances (Table 16) consisted of endogenous and exogenous substances that could be present in swabs. The three substances take into consideration were Clotrimazole, that is an antifungal medication used to treat vaginal yeast infections, Povidone-iodine 100 that is an antiseptic used for skin disinfection before and after surgery and blood.

Substances	Matrix	Concentration Tested
Clotrimazole - 2%	Swab	0.5% w/v
Blood	Swab	5% v/v
Povidone-iodine 100	Swab	0.5%

Table 16. List of the interfering substances with the corresponding concentrations tested.

The determination of the effect of possible interfering substances on swabs was experimentally assessed by the comparison of the amplification results between the test

sample and the control sample, where “test sample” is the sample added with the interfering substance and “control sample” is the sample not treated with the interfering substance. The same clinical samples have been used to prepare test samples and control samples.

The evaluation was done using 8 different hrHPV negative samples for each interfering substance selected (Clotrimazole - 2%, Blood and Povidone-iodine 100) and the extraction of test samples and control samples was done in the same run with GENEQUALITY X120 platform.

Extracted DNA have been positivized with positive controls at a concentration (1000 copies/µL of DNA target) that allows for the evaluation of potential interfering effect of the tested substances both with the amplification of the targets and of the BG. This specific concentration was chosen because:

- It is not too much to give significant competition phenomena between targets and BG amplification, which can result in impaired amplification of BG
- It is enough to evaluate also potential interfering effect of the tested substances with the amplification of the target

All the test samples and control samples previously prepared, were tested in triplicate with the assay object of this study an the AriaDx.

In swabs added with povidone-iodine 100, a delayed Ct of BG (approximately 1 Ct) was observed in 3 samples (Figure 14), when compared with the respective controls.

Liquid swabs are not always a homogeneus matrix, therefore, this Ct delay can be ascribed to the different aliquot cellularity and not necessarily to a potential interfering effect of tested substance. This is also confirmed by the fact that no delayed Ct were registered for the target DNA.

All substances tested showed no interference in Real time PCR.

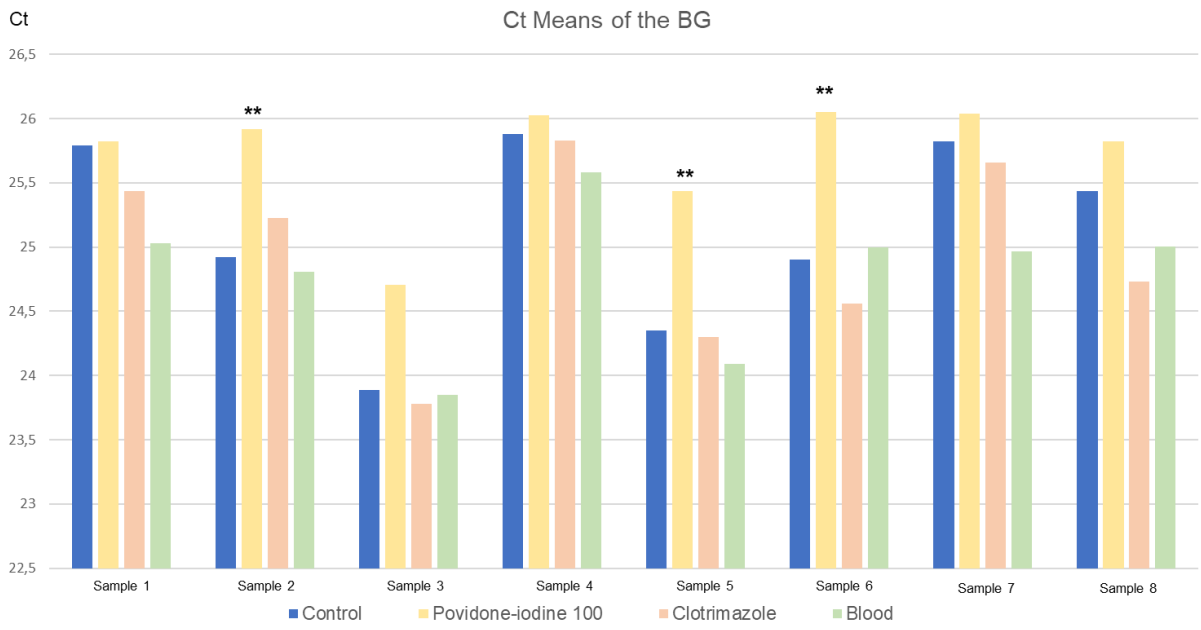

*Figure 14. BG Ct mean values. The results are the mean of the BG Ct of the 8 tested samples with the three added substances. **P < 0.001 vs control samples.*

4. PART II: HIV

4.1. MATERIALS AND METHODS

4.1.1. COLLECTION AND STORAGE OF SAMPLE

For the identification and the quantification of total DNA of HIV-1, the clinical materials mainly used in laboratories consist of PBMC and for this reason, the device has been validated on DNA extracted from PBMC.

4.1.2. PBMC ISOLATION

For the isolation of PBMC, the Ficoll-Hypaque system was used. PBMC were extracted from 10 mL of blood previously collected in BD Vacutainer® CPT™ Ficoll (BD) tubes that were centrifuged for 20 minutes at 1800 *g*. After that, a ring, containing the PBMC, is formed between the Ficoll separatium medium and the plasma (Figure 15).

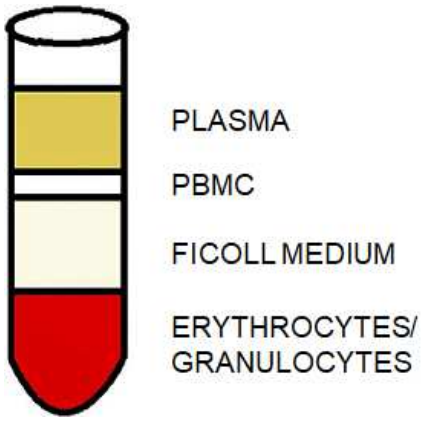

Figure 15.: Diagrammatic representation of the PBMC ring formation (modified from [124]*).*

Then the PBMC were transferred into a tube and later 10 mL of Phosphate-Buffered saline (PBS) solution was added. This tube was centrifuged for 15 minutes at 300 *g*. The pellet was collected and it was resuspended in 1 mL of PBS solution. Then 40 µL of the suspension were added to 20 mL of ISOTON® Diluent (Beckman Coulter) to do the cellular count by using Z1 coulter particle counter (Beckman Coulter). Depending on the results of the cellular count, the PBMC were divied in order to obtain $2x10^6$ in each tube that were subsequently centrifuged for 15 minutes at 300 *g*. Finally, the pellet

from the tubes was resuspended in 200 µL of PBS solution. PBMC were stored for a short time at +2°C/+8°C; for a longer period, they were frozen at -20°C/-30°C.

4.1.3. COMMERCIAL SAMPLES

In order to define the diagnostic performance of the assay, besides the clinical sample, also four of the External Quality Assessment (EQA) controls from the QCMD 2018 HIV DNA panel – Challenge 1, were used.

4.1.4. EXTRACTION METHOD

The DNA was extracted from PBMC with the fully automated QIAcube (Qiagen) instrument by using the QIAamp® DNA Mini kit (QIAGEN), that enable rapid and efficient purification of high-quality genomic DNA. This kit utilizes the selective binding properties of the unique QIAamp silica membrane to isolate pure DNA. After lysis in an optimized buffer and adjustment of DNA binding conditions, the sample was loaded directly onto a spin column. DNA was bound to the silica membrane, and contaminants were totally removed in 2 wash steps. Then the DNA was eluted in small volumes of a low-salt buffer or water.

The assay object of this study was tested for a range of extracted DNA concentrations between 14 and 186 ng/µL, therefore, the determination of the concentration of the extracted DNA must be evaluated by means of a spectrophotometric reading by measuring the absorbance at 260 nm. The concentration of the extracted DNA is an indispensable parameter for the calculation of the copies of HIV-1 DNA present in the clinical sample.

4.1.5. HIV-1 DNA DETECTION ASSAY OBJECT OF THIS STUDY

The HIV-1 DNA detection assay object of this dissertation is an assay for the detection of the DNA of HIV-1, group M, subtypes A, B, C, F, G, AE, AG by Real time PCR amplification, targeting the LTR region and a part of *gag* gene. It allows also the quantification of the number of the viral DNA molecules present in the sample under examination by constructing a standard 4-points curve (from 2×10^2 to 2×10^5 copies of viral DNA per reaction).

The HIV DNA detection assay object of this study is constituted by:

- A ready-to-use PCR mastermix that includes dUTP/UNG system to prevent carry over contamination.
- The Positive Control (PC) that is constituted of HIV-1 DNA fragments containing the gene region of interest and BG fragment. It is used to monitor each Real time PCR run.
- The quantitation standards that consist of serial diluitions of DNA fragments containing the gene region of interest.

4.1.5.1. DESIGN OF THE ASSAY

The choice of the target region on which the design of specific primers and probes was made, was based using a multiple alignment approach on 3500 sequences deposited in the reference database [125]. The LTR region and a parte of the gag gene where chosen because they are the most conserved region, in order to avoid false negative results. Primers and probes design was made by using the tool BIOSEARCH.

The sequence alignments, made to avoid no specific alignment with other regions different from the target sequence, were performed by using the bioinformatics tools:

- MUSCLE (Multiple Sequence Comparison by Log-Expectation)
- BioEdit
- Nucleotide BLAST (Basic Local Alignment Search Tool)

DESIGN OF PROBES

The TaqMan probes used in this assay fullfil these criteria:

- Probe lenght: 13-25 pb
- Melting temperatures (Tm): 68°-70°C
- Amount of GC content: 30-80%
- At the 5'-end must not be present a guanine because its closeness with the fluorophore could lead to a fluorescence decrease.

Sequences of designed probes are not reported, for respecting intellectual proprietary and trade secret.

DESIGN OF PRIMERS

The primers design occured in a region as closed as possible to that of the probes and they fullfil these criteria:

- Amplicon lenght: 80-150 pb
- Primer lenght: 20-30 pb
- Melting temperatures (Tm): 58°-60°C
- Tm of primer *Forward* and *Reverse* very close.
- Amount of GC content: 30-80%
- At the 3'-end the last five nucleotides must not contain more than two GC content.
- Avoid nucleotides repetitions.

Sequences of designed primers are not reported, for respecting intellectual proprietary and trade secret

The sequence specific probes designed for the assay object of this study are marked with FAM and JOE fluorophores, respectively for the detection of the pathogen target nucleic acid and the internal control DNA (the β-globin gene, BG). The target regions and the fluorophores involved in the reaction are shown below (Table 17):

DNA	Target region	Fluorophores
HIV-1 DNA	LTR region and a part of the gag gene	FAM
BG	-	JOE

Table 17. Scheme of the fluorophores associated with target region.

The amplification of the BG gene allows to evaluate the presence in the extract of inhibitors of the PCR reaction and to monitor the extraction process. It is also a valid tool for recognizing any false negative results. This assay has been optimized to favor amplification of the pathogen DNA. Therefore, in the positive samples the amplification signal for the β-globin gene may present a delayed Ct.

To guarantee the correct interpretation of the results, it is necessary to use, in each Real time PCR run, the positive control that is included in the kit and a negative control (water). Each result obtained must be verified by the evaluation of the reaction controls according to the table below (Table 18).

	RESULT	INTERPRETATION
Positive control	Amplification signal simultaneously present in FAM and JOE	Control and PCR worked correctly
	NO amplification signal in FAM and/or JOE	There is some problem with the amplification of the HIV DNA and/or BG, repeat the analysis
Negative control	Amplification signal in FAM and/or JOE	Contamination repeat the analysis
	No amplification signal in any channel	Control and PCR worked correctly

Table 18. Scheme of the correct interpretation of positive and negative control signals.

To determine samples' s results, it is necessary to evaluate first of all, the Ct value of the BG (Table 19). A sample can be considered negative for HIV-1 only when the amplification curve of the internal control has a Ct lower than or equal to 29.

RESULT	INTERPRETATION	
BG	**HIV negative sample**	**HIV positive sample**
Amplification signal with Ct ≤ 29	Suitable sample	Suitable sample
Amplification signal with Ct > 29	Unsuitable sample	Suitable sample*

Table 19. Interpertation of the Ct values of the BG signals.

The evaluation of the FAM fluorescence channel allows to define the presence or absence of pathogen in the sample under examination, and it is also important to determine the quantity of HIV-1 DNA present in the sample. The interpretation results of the target pathogens are listed below (Table 20):

FAM channel	
Result	**Interpretation**
Amplification signal	Positive for HIV DNA
No amplification signal	Negative for HIV DNA

Table 20. Interpretation of the Ct signals of the FAM fluorophore.

To evaluate the HIV DNA quantitation present in a sample, it is necessary to add in the run, the quantification standard. A precise quantification can only be done within the linearity range of the device. In the table below (Table 21) the interpretation of the viral copies/reaction corresponding to the quantification results is summarized.

HIV-1 DNA Quantification result	INTERPRETATION (viral genome copies/reaction)
Quantification result > 10^7 copies/reaction	More than 10^7
10 ≤ Quantification result ≤ 10^7 copies/reaction	Quantity = Quantification result
Quantification result < 10 copies/reaction	Less than 10

Table 21. Interpretation of the viral copies/reaction corresponding to the quantification results

Once the number of HIV DNA copies per reactions has been determined, the number of HIV DNA copies on 10^6 cells can be calculated. To perform this conversion it is necessary to quantify the DNA extracted from the clinical sample.

Considering that 1 µg of DNA corresponds to 1.5 x 10^5 cells [126], it is possible to calculate the number of cells tested for each reaction (Y), applying the following formula:

$$Y = X \times 10 \times 150$$

where:

X is the concentration of the extracted DNA expressed in ng/µL.

The number of HIV DNA copies on 10^6 cells (N) is given by the formula:

$$N = \frac{n \times 10^6}{Y}$$

where:

n is the number of HIV DNA copies per reaction

4.1.6. REAL TIME PCR INSTRUMENT: The Applied Biosystems 7500 Fast Dx

The experiments for the evaluation of assay's performance were done on the instrument Applied Biosystems 7500 Fast Dx Real Time PCR Instrument (ABI 7500 Fast Dx – Applied Biosystems) that allows nucleic acid amplification for *in vitro* diagnostic use. This instrument has five dyes (FAM™/SYBR® Green I, VIC®/JOE™, NED™/ TAMRA™/ Cy3™, ROX™/Texas Red®, and Cy5™ dyes).

These specifications for the thermal profile used for all the experiments are listed below (Table 22):

Step	Repeats	Time	(°C)
1	1	02:00	50.0
2	1	10:00	95.0
3	45	00:15	95.0
		01:00	60.0*

Table 22. Specifications of thermal profile.

* Fluorescence detection step

All the experiments were analyzed in linear scale using the settings described in the following table (Table 23):

INSTRUMENT	CHANNEL	BASELINE	THRESHOLD
ABI 7500 Fast Dx	FAM	3-15	0.2
	JOE	3-15	0.05

Table 23. Settings of baseline and threshold used for the analysis of each run.

The baseline of the Real time PCR reaction refers to the signal level during the initial cycles of PCR in which there is low variation in fluorescent signal. The cycle range in which baseline is calculated should be set carefully to allow accurate determination of the threshold cycle. This range should comprise enough cycles to eliminate the background noise found in the early cycles of amplification but should not include the cycles in which the amplification signal begins to rise above background. The choice of the baseline range is strictly depending on the type of fluorophores and their concentration in the PCR reaction.

The choice of threshold is depending on the kinetics of the reaction that determines the amplification plots (caused by type and concentration of fluorophores, specific design of the assay). The threshold values reported in the table were chosen empirically. They were evaluated and definitively set after the analysis of the performance evaluation study, in order to assure optimal results in terms of diagnostic sensitivity and specificity and analytical sensitivity.

4.1.7. REFERENCE METHODS

Fore the validation of the assay object of this study, two different reference methods were selected:

- GENERIC HIV DNA CELL (Biocentric) that is a research Real time PCR test used for the evaluation of the HIV-1 reservoir. It has been designed on the LTR region and it is able to detect HIV-1 DNA, from subtypes A to H with a detection limit of 40 copies of HIV-1 DNA/1 x 10^6 cells.
- IN-HOUSE ASSAY that has been designed to detect the *pol* gene.

4.1.8. STATISTICAL ANALYSIS

For the statistical analysis, were used the tool MedCalc statistical software (version 16.8.4).

4.2. RESULTS

4.2.1. IDENTIFICATION OF THE CUT OFF Ct VALUE OF THE BG

The kit was designed to use an internal control (BG) that allows detecting inhibition of PCR reaction, monitoring extraction process as well as identifying false-negative samples. The BG is amplified in multiplex with the target pathogen.

The assay has been optimized to favor amplification of the pathogen DNA. Therefore, the amplification signal of the BG as control gene may be delayed in samples positive for HIV-1 DNA. In fact, the low efficiency amplification reaction for the BG gene may be displaced by competition with the high efficiency amplification reaction for HIV-1 DNA. In this case the sample is nevertheless suitable and the positive result of the assay is valid.

In HIV DNA negative samples, the BG amplification is evaluated to exclude problems have occurred during the amplification step (inefficient or absent amplification) or during the extraction step (loss of DNA during extraction or presence of inhibitors in the extracted DNA) which may lead to incorrect results and false negatives.

The analysis of BG amplification includes, first of all, the evaluation of the BG amplification plot, by verifying that the Ct is determined by a fast and regular increase of the fluorescence values and not by peaks or an increase of the background (irregular or high background) and that the curve appears sigmoidal and not rectilinear as it should be.

In case of inefficient amplification, loss of DNA during extraction or presence of inhibitors in the extracted DNA, the BG Ct value is affected.

To determine the cut off Ct value, nucleic acids extraction of BG in a sample certainly not inhibited was performed.

An analysis of the basal Ct value was performed on negative PBMC samples included in this validation study which were defined “suitable” by the reference methods.

The mean Ct values found on the ABI 7500 Fast Dx instrument was 21.7.

Taking into consideration that:

- the PBMC samples included in this validation study were mainly from healthy donors
- the device is intended for the use also with immunocompromised patients who can present a lymphopenic status

A delay over 2 log (6.6 PCR cycles) was considered to be significant when evaluating inhibition. Therefore, according to the above rationale, the Cut Off value for BG evaluation was defined as reported below:

Ct mean (calculated on the Ct mean values) + 6.6 PCR cycles = 21.7 + 6.6 = 28.3 (round Ct off to: 29).

According to these results, a Cut Off value for BG evaluation in PBMC is defined at 29 when performing the analysis on ABI 7500 Fast Dx instrument.

In HIV DNA negative samples, a BG Ct value ≤ 29 excludes problems have occurred during the amplification step (inefficient or absent amplification) or during the extraction step (loss of DNA during extraction or presence of inhibitors in the extracted DNA) which may lead to incorrect results and false negatives.

4.2.2. ANALITICAL PERFORMANCE

4.2.2.1. EVALUATION OF ANALYTICAL SPECIFICITY

The analitycal specificity consists in the evaluation of phenomena of cross-reactivity with DNA belonging to other potentially cross-reactive microorganism to sequence homology, phylogenetic proximity or infecting the same biological district; it is determined by *in silico* analysis of sequence alignments and by experimental testing of samples positive for other pathogens.

The analytical specificity of the assay object of this study is guaranteed by an accurate choice of specific primers and probe, as well as by specific amplification conditions. The primers and probe alignments revealed the absence of non-specific pairing.

Specificity was also checked using human samples positive for pathogens potentially cross-reactive because of:

- the belonging to the same family:
    - *Epstein-Barr virus (EBV),*
    - *Herpes simplex virus 1 (HSV-1),*
    - *Herpes simplex virus 2 (HSV-2),*
    - *Cytomegalovirus (CMV),*
    - *Varicella-zoster virus (VZV),*

- *Human Herpes Virus 6 (HHV 6),*
- *Human Herpes Virus 8 (HHV 8).*

- infecting same districts (bloof, liquor and plasma):
  - *HBV,*
  - *Parvovirus B19,*
  - *JCV,*
  - *BKV,*
  - *Adenovirus.*

A total of 42 samples, contained a high pathogen load were selected to evaluate the potential cross-reactivity. The 4-points standard (from 2 x 10^2 to 2 x 10^5 copies of viral DNA per reaction) was added in the Real time PCR run.

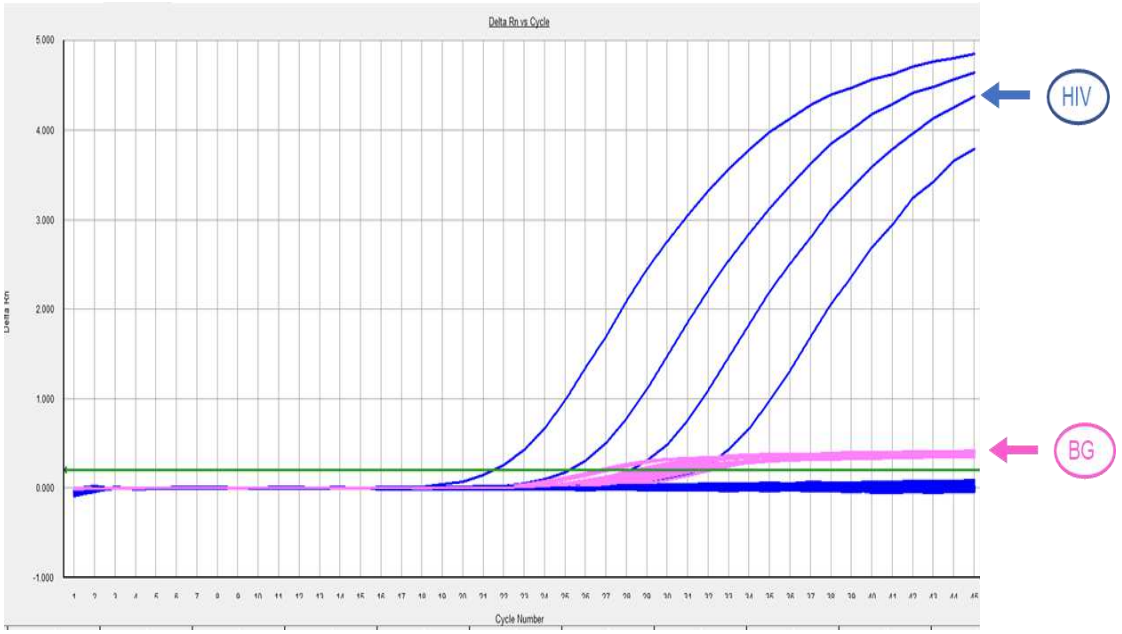

Figure 16. Results of the cross-reactivity PCR Real time run: none of the pathogen tested gave a FAM signal.

All the sample tested were suitable (everyone had a BG signal with a BG Ct value < 29) and none of them was reactive with the device object of this study (Figure 16), in fact, the FAM signals in the run were of the quantification standard previously added.

4.2.2.2. EVALUATION OF ANALYTICAL SENSITIVITY: THE DETECTION LIMIT (LoD)

Analytical sensitivity may be expressed as the limit of detection that consist in the definition of the lowest analyte concentration that can be consistently detected in ≥95% of samples tested by the device.

It was determined using serial dilutions at known concentration of the HIV-1 plasmid, containing one copy of the target region (LTR region and part of *gag* gene).

In particular, the HIV-1 plasmid has been quantified by spectrophotometric analysis and five solutions at the following concentrations (Table 24) were prepared for testing.

QUANTITY	
copies of DNA target/µL	**copies of DNA target/reaction**
1	10
0.5	5
0.25	2.5
0.1	1
0.05	0.5

Table 24. Concentrations of the five diluitions used to define the limit of detection.

As the plasmid BG is amplified in multiplex with the pathogen target, in order to take in consideration also the potential effect of competition between the two targets especially near the limit of the detection of the assay, in each dilution, the plasmid BG was also added to obtain a BG final amount of about 2 x 10^4 copies/reaction.

Three consecutive runs have been performed and for each run were loaded:

- 10 µL of each solution in 8 replicates per run
- 10 µL of the Positive Control, one test per run
- 10 µL of the negative control (H_2O), one test per run

The results (number of positive replicates, Ct mean and Ct SD) of each tested concentration are reported in the table below (Table 25):

Quantity		Observation	Response	Ct	Ct	Positivity
copies/µL	copies/Rx	N. of tested replicates	N. of positive replicates	mean*	SD*	%
0.05	0.5	24	9	37.96	0.64	38
0.1	1	24	7	37.75	0.87	29
0.25	2.5	24	19	37.53	0.99	79
0.5	5	24	23	36.63	0.74	96
1	10	24	24	35.61	0.64	100
		120	**82**			

Table 25. Results (number of positive replicates, Ct mean and Ct SD) for each concentration tested.

*Ct mean and Ct SD refer exclusively to positive replicates

The graphical representation of the probit analysis is illustrated in (Figure 17).

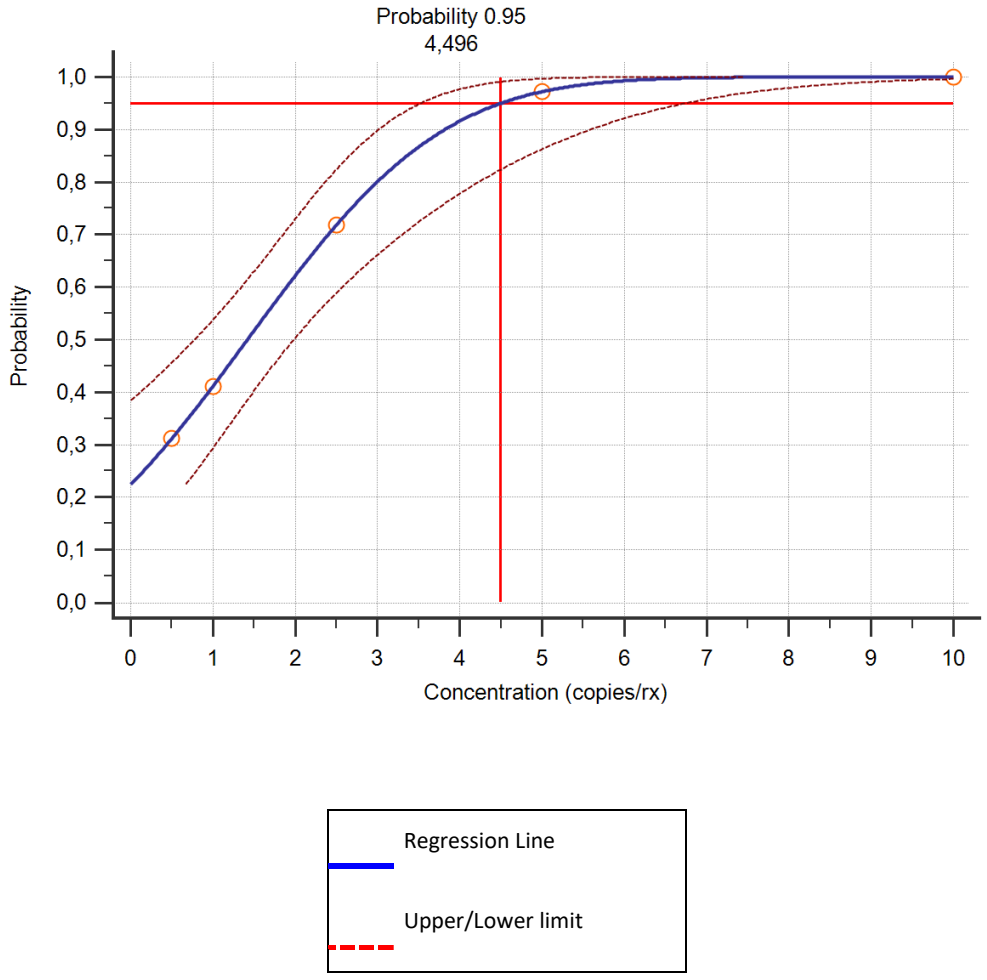

Figure 17. Graphical representation of the probit analysis of the limit of detection.

The analytical sensitivity was calculated by probit analysis using MedCalc statistical software and the results obtained are listed below (Table 26):

Dose*	95% Confidence interval Lower limit	95% Confidence interval Upper limit
4.49 copies/reaction	3.52 copies/reaction	6.73 copies/reaction
0.45 copies/µL	0.35 copies/µL	0.67 copies/µL

Table 26. Values (expressed both in copies/reaction and copies/µL) of analytical sensitivity.

*The copies/µL concentration can be easly converted in copies/reaction by multiplying by 10, because the volume of the tested control loaded was 10 µL.

The limit of sensitivity of the device object of this study (p=0,05) defined on ABI 7500 Fast Dx is 0.45 copies of DNA target/µL, with a 95% confident interval between 0.35 and 0.67 copies of DNA target/µL, corresponding to 4.49 copies of DNA target/reaction, with a 95% confident interval between 3.52 and 6.73 copies of DNA target/reaction.

4.2.2.3. EVALUATION OF ANALYTICAL SENSITIVITY: THE LINEAR RANGE

The linear range of the assay was determined using a panel of dilutions of the quantification standards (10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 5 viral genome copies/reaction), each point amplified in triplicate. The experiments were done in 2 different runs using the same dilution in the same day. As the plasmid BG is amplified in multiplex with the pathogen target, in order to take in consideration also the potential effect of competition between the two targets especially near the limit of the detection of the pathogen target, in each dilution, the plasmid BG was also added to obtain a BG final amount of about 2 x 10^4 copies/reaction.

For each run were loaded:

- 10 µL of each solution in triplicate
- 10 µL of the Positive Control, one test per run
- 10 µL of the negative control (H_2O), one test per run

The results obtained (Table 27) were analyzed using linear regression.

Run	Linear Range	Slope	R^2
1	10^7 – 10 copies/reaction	-3.320	0.994
2	10^7 – 10 copies/reaction	-3.271	0.997

Table 27. Results obtained of the linear range using linear regression.

The analysis of the results by linear regression (R^2 > 0.99) demonstrated that the linear range of the assay object of this study is between 10 and 10^7 viral genome copies / reaction on the ABI 7500 Fast Dx intrument.

The 5 viral genome copies/reaction concentration was out of the linear range (Figure 18), indeed the Ct values of the replicates tested had Ct values too different between them, supporting that at this concentration the assay is not able to correctly quantify the viral genome presence.

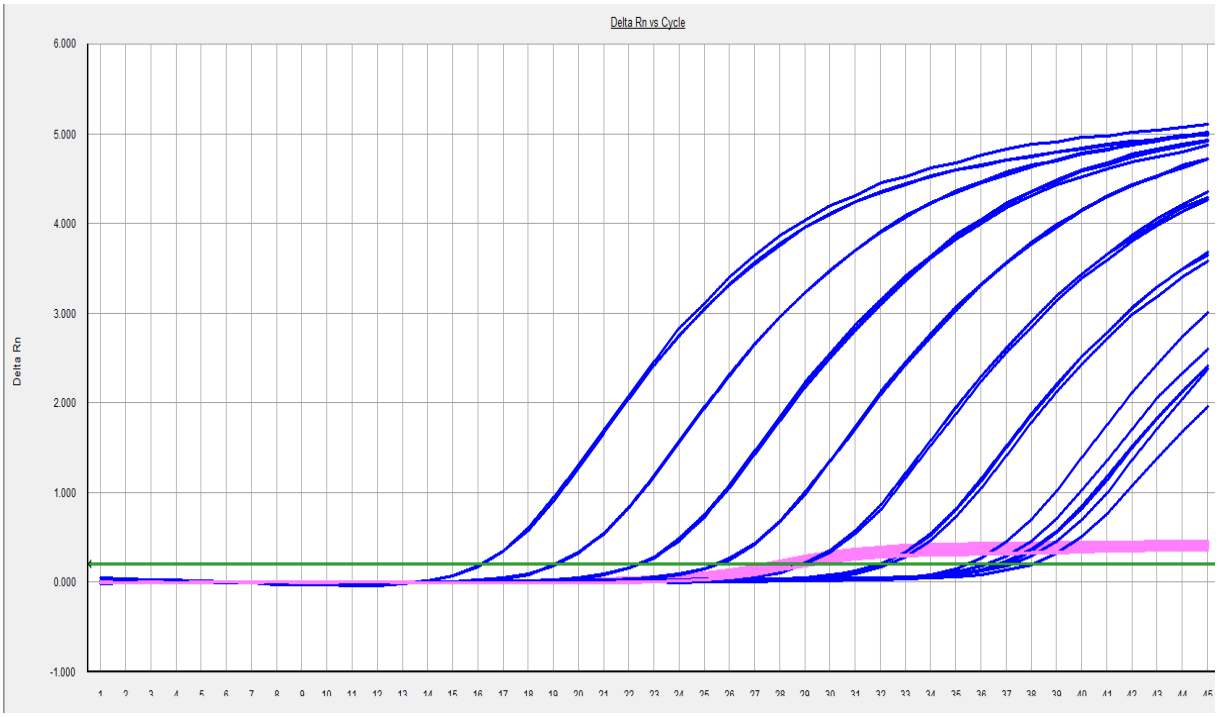

Figure 18. Representation of a PCR Real time session of the diluition tested (10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 5 viral genome copies/reaction) for the definition of the linear range.

4.2.2.4. EVALUATION OF PRECISION

The precision consists in the evaluation of the closeness of the agreement between the results of measurements of the same measurand; it is determined by test replicates of samples at known concentration in the same run (intra-assay variability) or in different days (inter-assay variability). The precision studies for quantitative tests should provide an estimate of the precision of the method at a large portion of the measuring range.
Precision is commonly evaluated by performing a replication experiment to observe the variability in results generated under the normal operating conditions in the laboratory.
Precision of the assay object of this study was tested using three dilutions of the HIV-1 plasmid, containing one copy of the target region (LTR region and part of *gag* gene), at 3 different concentration (high, medium and low concentrations).
In particular, the HIV-1 plasmid has been quantified by spectrophotometric analysis and, three solutions were prepared: one with an analyte concentration at the upper limit of linearity of quantification (ULOQ) and two with low concentration. The three following concentrations were selected for the testing solutions (Table 28).

QUANTITY	
copies of DNA target/µL	**copies of DNA target/reaction**
10^7	10^8
10^3	10^4
5	50

Table 28. The three concentrations (expressed both in copies/µL and corresponding copies/reaction) of the diluitions used for the evaluation of the precision.

As the plasmid BG is amplified in multiplex with the pathogen target, in order to take in consideration also the potential effect of competition between the two targets especially near the limit of the detection of HIV-1, in each dilution, the plasmid BG was also added to obtain a BG final amount of about 2 x 10^4 copies/reaction.

An adequate volume of each solution was prepared the first day and then aliquoted in 20 tubes to avoid thaw/freeze cycles that could alter their concentration, resulting in impaired performance.
Testing was planned in a between-day format, with a total of 20 operating days to perform all the runs, one run per day with different operators.
For each run were loaded:

- 10 µL of each solution in duplicate
- 10 µL of the Positive Control, one test per run
- 10 µL of the negative control (H_2O), one test per run

It has been calculated the estimated within-run Ct standard deviation (Sr), the estimated of total precision Ct standard deviation (S_T) and the total precision percent coefficient of variation (CV_T).

The estimated within-run Ct standard deviation (S_r) has been calculated on the basis of the following formula:

$$Sr = \sqrt{\frac{\sum_{i=1}^{I}(X_{i1} - X_{i2})^2}{2I}}$$

Where:

I = number of days (20)

X_{il} = result for replicate 1 on day i

X_{i2} = result for replicate 2 on day i

The estimated of total precision Ct standard deviation (S_T) has been calculated on the basis of the following formula:

$$S_T = \sqrt{B^2 + \frac{N-1}{N} S_r^2}$$

Where:

N = number of replicats per run

B = standard deviation of daily means

S_r^2 = repeatability variance estimate (standard deviation squared)

The standard deviation of daily means has been calculated on the basis of the following formula:

$$B = \sqrt{\frac{\sum_{i=1}^{I}(\overline{X_{i.}} - \overline{X_{..}})^2}{I-1}}$$

Where:

I = number of days

$\overline{X_{i.}}$ = average replicates on day i

$\overline{X_{..}}$ = average of all results over all days

The total precision percent c*oefficient of variation (CV_T)* has been calculated on the basis of the following formula:

$$CV_T = \frac{S_T}{\overline{Ct}} \times 100$$

Where:

S_T = stimated of total precision Ct standard deviation

$\overline{Ct}$ = Ct mean

	S_r		S_T		CV_T	
	BG	**HIV-1**	**BG**	**HIV-1**	**BG**	**HIV-1**
10^8 copies/reaction	0.246	0.032	0.318	0.061	1.41%	0.42%
10^4 copies/reaction	0.051	0.063	0.107	0.142	0.42%	0.55%
50 copies /reaction	0.037	0.293	0.132	0.325	0.52%	0.97%

Table 29. Values of the estimated within-run Ct standard deviation (Sr), of the estimated of total precision Ct standard deviation (S_T) and of the total precision percent coefficient of variation (CV_T) for each tested concentration.

The three diluitions tested showed a precision percent coefficients of variation (CV_T) of the BG reaction < 2% and of the HIV reaction < 1% (Table 29).

4.2.3. DIAGNOSTIC PERFORMANCE

4.2.3.1. DIAGNOSTIC SPECIFICITY AND SENSITIVITY

The diagnostic specificity consists in the definition of the probability that the device gives a negative result in the absence of the target marker. It is represented by the number of true negative results divided by the total number of samples without the pathogen (which is the sum of the numbers of true negative plus false positive results).
The diagnostic sensitivity consists in the definition of the probability that the device gives a positive result in the presence of the target marker. It is represented by the number of true positive results divided by the total number of samples with the pathogen (which is the sum of the numbers of true positive plus false negative results).

In order to establish the diagnostic specificity and sensitivity of the device, 73 different samples have been analyzed. Of them, 12 were negative and the other 61 were positive HIV-1 samples (Table 30).

Sample Type	Nr samples
Nr Negative expected samples	12
Nr Positive expected samples	61
Total	**73**

Table 30. Number of HIV-1 positive and negative samples used to define diagnostic performance.

These 73 samples used in this study belong from two different categories: 69 were clinical samples and 4 were commercial controls (Table 31).

Sample Type	Nr. Samples
Clinical samples	69
Commercial controls	4
Total	**73**

Table 31. Belonging categories of the samples using in this study.

THE CLINICAL SAMPLES

The 69 clinical samples used to determine the diagnostic performance of the assay, were previously tested with the reference methods:

- **50** samples verified with the reference method GENERIC HIV DNA CELL (Biocentric)
- **19** samples verified with the reference method an in-house assay which detected *pol* gene

THE COMMERCIAL CONTROLS

The 4 commercial controls used to determine the diagnostic performance of the assay were included in the QCMD 2018 HIV DNA panel – Challange 1.

The 73 samples tested with the device object of this study, gave the following results (Table 32):

		Obtained results		Total
		Nr Negative samples	Nr Positive samples	
Expected results	Nr Negative samples	12	0	12
	Nr Positive samples	1	60	61
Total		13	60	73

Table 32. Results obtained on the 73 samples testd with the renferences methods and the assay object of this study.

With the device object of this study,13 samples resulted as HIV-1 negative and 60 resulted as HIV-1 positive.

Particularly, one positive sample according to reference methods resulted negative with the device object of this study.

The obtained results allowed to calculate the diagnostic specificity and the diagnostic sensitivity on ABI 7500 Fast Dx.

The diagnostic specificity, obtained by dividing the number of true negative results with the total number of samples without the pathogen, resulted of 100%.

$$\textbf{DIAGNOSTIC SPECIFICITY}: \frac{\mathbf{12}}{\mathbf{12+0}} \times \mathbf{100} = \mathbf{100}\%$$

The diagnostic sensitivity, obtained by dividing the number of true positive results with the total number of samples containing the pathogen, resulted of 98%.

$$\textbf{DIAGNOSTIC SENSITIVITY}: \frac{\mathbf{60}}{\mathbf{60+1}} \times \mathbf{100} = \mathbf{98}\%$$

The accuracy, calculated by dividing the number of the sum of positive and negative obtained results with the total number of samples, resulted of 99%.

$$\textbf{ACCURACY}: \frac{\mathbf{12+60}}{\mathbf{12+60+1}} \times \mathbf{100} = \mathbf{99}\%$$

5. DISCUSSION

An *in vitro* diagnostic medical device (IVD) is any medical device used *in vitro* for the examination of specimens, including blood and other materials, derived from the human body. IVDs are mainly used to provide information about a physiological or pathological state or regarding a therapeutic treatment.

In European Community (CE) can be legally commercialized, for diagnostic purpose, only devices that are CE-IVD marked, that means they comply with the European In-Vitro Diagnostic Devices Directive (98/79/EC).

The focus of the first part of this study was about the validation of a hrHPV assay based on Real time PCR technology for the primary cervical cancer screening. Early detection of precancerous cervical lesions through screening program is a critical point for reducing cervical cancer incidence and mortality [51]. Indeed, the introduction of well organized cervical cancer screening programs in many countries have gained significant reduction in cervical cancer incidence and mortality [52].

Cervical cancer screening was traditionally made by the detection of cytological abnormalities using Pap test. However recently, European guidelines have introduced the implementation also for HPV DNA testing in primary screening program, usable alone or in combination with cytology [53]. Since HPV infections are very common and usually clear spontaneously, any candidate HPV DNA test must offer an optimal balance between clinical sensitivity and specificity for detection of CIN grade 2 or 3 to minimize excessive follow-up procedures [56]. Therefore, it is necessary to evaluate the clinical performance of any candidate test before its use in organized screening programs according to Meijer's guidelines.

The clinical performance of the hrHPV detection assay object of this study, were evaluated by testing 910 clinical swabs on the AriaDx Real time PCR instrument. The achieved results were subsequently compared with those obtained with the HC2 test, the reference assay already validated. For the definition of the clinical specificity, 837 <CIN2 samples were tested and among them, 80 resulted as positive with HC2 and 82 resulted as positive with the assay object of this study. Anyway, this slight discrepancy, did not affect the definition of the clinical specificity of the assay, that was defined comparable to the reference method.

Regarding the clinical sensitivity, the 73 ≥CIN2 samples tested, have given the same results with both the assays: 71 samples were defined as hrHPV positive and 2 were detected as hrHPV negative. This result was considered good in order to define the clinical specificity, because all the specimens tested were concordant with both the methodics, but it was also an unexpected outcome because we would have thought

the samples to be all hrHPV positive because of their all histological data of ≥CIN2. Indeed, it is now well established the link between cervical cancer and its immediate pre- cancerous lesions arise from persisting cervical infections with hrHPV [56]. This unexpected outcome was subsequently investigated, indeed the 2 hrHPV negative samples were genotyped with AMPLIQUALITY HPV TYPE EXPRESS v3.0. One of them resulted HPV 73 positive, that is a genotype classified by the IARC as a possible carcinogen [127]. Moreover, there are already some scientific evidences that proved that this HPV genotype has been detected in some invasive cervical cancers [128]. The other sample, is confimed as negative also after genotyping and it probably belongs to the rare not HPV related cervical cancers. In literature has been already reported the onset of adenocarcinoma not related with HPV infections [129]. This fact is very rare, indeed, a recent study [130] which used next-generation sequencing to characterize primary cervical cancers, found that only 5% of cervical cancers are HPV negative.

Clinical sensitivity and specificity for this assay on AriaDx resulted adequate.

For the intra-laboratory reproducibility, were tested 536 samples in two rounds. Among them 168 resulted hrHPV positive in the first test and 172 resulted hrHPV positive in the second round, therefore 4 samples were discordant.

This is probably due to the low viral load of the samples, that was at the limit of the sensitivity of the assay. Indeed, the four samples that resulted as negative in the first test, had respectively Ct values of 36.49 (FAM), 39.34 (FAM), 38.52 (CY5) and 38.15 (JOE) in the second round, that are considered as late Ct because of the Ct cut-off value of 40.

However, the percentage of concordance of the results of the two rounds was ascertained as 99.3% (CI 95%: 98.1%-99.8%.).

Regarding the inter-laboratory reproducibility, were tested 534 samples in two different laboratories. Among them 167 resulted hrHPV positive in the first laboratory and 169 resulted hrHPV positive in the second laboratory, therefore 2 samples were discordant. Also in this case, the discordance is probably due to the low viral load of the samples, that was at the limit of the sensitivity of the assay. The two samples that resulted as negative in the first round, had respectively FAM Ct values of 37.29 and 37.19 in the second round, that are considered as late Ct. However, the percentage of concordance of the results of the two rounds was ascertained as 99.6% (CI 95%: 98.7%-100.0%.).

Therefore, with a lower confidence bound higher than 87%, the intra and inter-laboratory reproducibilities on AriaDx are adequate, according to international

guidelines [56].

All these data about clinical performance fulfill Meijer's guidelines criteria, therefore this device was considered suitable for the use in primary cervical cancer screening.

Relatively the analytical performance, to define the device's sensitivity, 41 samples of the 2014 WHO LabNet Proficiency Panel were tested. Each HPV genotype of this panel was tested at the concentrations of 500 and 50 Genome Equivalents per reaction, except for HPV 16 and HPV 18 that were present in the concentrations of 5 and 50 International Units per reaction. Being able to detect at least 50 International Units per reaction for HPV 16 and 18, and at least 500 Genome Equivalents per reaction for the other hrHPVs, the device object of this study is proficient on AriaDx according to WHO criteria.

Regarding the analytical specificity, the evaluation of phenomena of cross-reactivity with DNA belonging to other potentially cross-reactive microorganism has been already defined *in silico* when the assay was designed by the choice of specific primers and probe. For the *in vitro* evaluation, were selected 18 samples containing pathogens potentially cross-reactive because they infect the same HPV districts. Moreover, other 54 samples containing different HPV genotypes not detectable by the assay, were tested.

None of the tested pathogens resulted cross-reactive with the assay object of this study, allowing concluding that this device is specific for the identification of the 14 hrHPV genotypes that it was designed for.

Regarding the definition of diagnostic performance, the 910 samples tested, have given, with the HC2 assay, 758 hrHPV negative and 152 hrHPV positive results.

For the definition of the diagnostic specificity, among the 758 negative samples, fifteen resulted discordant with the assay object of this study because they resulted hrHPV positive, and after the genotyping with the assay AMPLIQUALITY HPV TYPE EXPRESS v3.0, only one sample resulted discordant with the device object of this study. This result can be interpreted with the fact that this sample have a hrHPV viral load so low that cannot be detected by both the references methods. Indeed, the FAM Ct value obtained with the assay object of this study, was 36.98, that is considered as late Ct. In particular, it could be considered as a false negative result for AMPLIQUALITY HPV TYPE EXPRESS v3.0 because this assay is designed on the L1 region of HPV genome, and some studies have shown that assays focused on the L1 region may be associated with a false negative rate due to integration events in the L1 open reading frame [131]. Anyway, the discordant sample result, did not affect the definition of the diagnostic specificity of the assay, that was calculated as 99.9%.

Regarding the diagnostic sensitivity, thirteen hrHPV positive samples according to

HC2 resulted negative with the device object of this study. After the genotyping with AMPLIQUALITY HPV TYPE EXPRESS v3.0, all samples were confirmed negative, according the device of this study, leading to obtain a diagnostic specificity of the 100%.

The obtained results of the analitycal and diagnostic performance, suggest that the assay meets the requirements of the Directive 98/79/CE.

Based on these premises, the assay could be CE-IVD marked in compliance with the European Union diagnostic medical device manufacturing standards.

The assay has been validated by using cervical swabs as samples. Since the starting materials are fundamental to assure a correct result, we have carried out an analysis of eventually interfering substances that may affect the clinical samples. The three substances take into consideration were Clotrimazole - 2%, that is an antifungal medication used to treat vaginal yeast infections, Povidone-iodine 100 that is an antiseptic used for skin disinfection before and after surgery and blood.

The determination of the effects of possible interfering substances was experimentally assessed by the comparison of the amplification results between the test sample (sample added with each selected substance) and the control sample. In case of swabs added with povidone-iodine 100, a delayed Ct of BG (approximately 1 Ct) was observed in 3 samples, when compared with the respective controls. Liquid swabs are not always a homogeneus matrix, therefore, this Ct delay can be ascribed to the different aliquot cellularity and not necessarily to a potential interfering effect of tested substance. This is also confirmed by the fact that no delayed Ct were registered for the target DNA, allowing concluding that the three tested substances show no interference in Real Time PCR experiments, therefore they are not considerd interfering substances.

In the second part of the study the focus was on the development and validation on the ABI 7500 Fast Dx of an *in vitro* device for the identification and quantification of HIV-1, group M, subtypes A, B, C, F, G, AE, AG by Real time PCR amplification.

The quantification of viral DNA is a fundamental parameter for the therapeutic management of HIV-1 positive patients, however its application in the diagnostic routine is delayed by the lack of standardized systems available in commercial formulations.

Recent studies [118] have shown, in patients treated with HAART and in therapeutic success, a direct correlation between the charge of HIV cellular DNA and the number of cells in latency, infected by HIV, which constitute the viral reservoir. Therefore, the size of the reservoir assumes significant importance in the therapeutic management

of the patients. In fact, high levels of HIV DNA have been associated with a more rapid clinical progression of the disease and with treatment failure. The evaluation of HIV DNA levels before and during antiretroviral therapy can be useful to:

- evaluate the suspension of antiretroviral therapy in patients who have been negativized and treated for many years, with consequent relief for patients, reduction in the onset of drug resistance and cost containment;
- assess the effectivness of antiretroviral therapy [132], beacause the amount of HIV DNA determines the response to the therapy itself;
- evaluate a possible change of therapy since the total amount of HIV DNA at the baseline is predictive of the response to therapeutic treatment and therefore can be useful for the personalization of the treatment, as in the case of simplification of therapy [133].

Nowadays, standardized and validated diagnostic assays are poorly available, or difficult to use and the HIV DNA viral measurement systems, available as Real Time PCR methods, are performed according to published protocols, within research laboratories but they are not currently available in commercial formulations approved by the competent authorities. In this context, my PhD project has been collocated: the development of a diagnostic device for the identification and quantification of total HIV DNA capable of satisfying the analytical sensitivity and specificity requirements needed for its use in the diagnostic field.

The design of the assay was carried out by selecting specific primer and probes targeting the LTR and part of *gag* gene region. After the design of the assay, a prototype was produced and was tested in order to verify its analytical and diagnostic performance. The first step of assay's validation was carried out by defining the BG Cut Off value, that is an important parameter to assess if a sample is suitable for the analysis. The BG Cut Off value was obtained by testing HIV-1 DNA negative samples extracted from PBMC and the BG Ct mean obtained was 21.7. A delay over 2 log (6.6 PCR cycles) was considered to be significant when evaluating inhibition. According to these results, a Cut Off value of 29 for BG evaluation in PBMC is defined when performing the analysis. In HIV DNA negative samples, a BG Ct value ≤ 29 excludes problems have occurred during the amplification step (inefficient or absent amplification) or during the extraction step (loss of DNA during extraction or presence of inhibitors in the extracted DNA) which may lead to incorrect results and false negatives.

Relatively the analytical performance, to define the device's specificity, 42 clinical samples containing other pathogens belonging to the same family of HIV or infecting the same distritcs, were tested. None of them reacts with the assay, index that it is

specific for HIV-1 identification. Moreover, the analytical specificity has already been tested *in silico* during the design of the assay, by the selection of specific primers and probes.

The analytical sensitivity was evaluated by defining the detection limit, the linearity range and the precision. The detection limit of the assay, that correspond to the lowest target concentration that can be detected by the assay in ≥95% of samples, was identified by testing five dilutions (0.05, 0.1, 0.25, 0.5 and 1 copies/µL) of the HIV-1 plasmid. Only in the case of the concentration of 1 copies/µL the device was able to correctly detect all the replicates tested, therefore, the limit of sensitivity of the device (p=0,05) was definded as 0.45 copies of DNA target/µL, with a 95% confident interval between 0.35 and 0.67 copies of DNA target/µL.

The linearity range was evaluated by testing other serial diluitions (10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 5 viral genome copies/reaction) of the HIV-1 plasmid. Using the linear regression analysis ($R^2 > 0.99$), was defined that the linear range of the assay object of this study is between 10 and 10^7 viral genome copies/reaction.

Precision is evaluated by performing a replication experiment to observe the variability in results generated under the normal operating conditions in the laboratory. It was evaluated by testing three diluitions (10^7, 10^3 and 5 copies of DNA target/µL), in 20 days with different operators. The three diluitions tested showed a precision percent coefficients of variation (CV_T) of the BG reaction < 2% and of the HIV reaction < 1%. Therefore, analytical sensitivity and specificity for the assay resulted adequate.

Regarding diagnostic performance, 73 samples were tested with the assay object of this study and the reference methods. Among them, 13 resulted as HIV-1 negative and 60 resulted positive with the reference methods. One sample, that was define as HIV-1 positive with the reference method, resulted as negative with the assay object of this study, therefore, this sample was considered discordant. This false negative obtained result was probably due for the low viral load of the sample that could be under the limit of sensitivity of the assay. Anyway, the discordant sample result, did not affect the definition of the diagnostic sensitivity of the assay, that was calculated as 98%. Thus, the diagnostic specificity was determined as 100% because all negative samples were correctly identified by the assay.

The obtained results of the analitycal and diagnostic performance, suggest that the assay is suitable to use in the research field.

Based on these premises, the assay has been marked as RUO (research use only) device, therefore it must have no intended medical purpose or objective.

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