Chapter 6 - HPV detection and typing

Marc Arbyn[1], Laila Sara Arroyo Mühr[2], Johannes Bogers[3], Taeyang Chin[4], Astrid Coppens[3], Kaat Kehoe[3], Elizaveta Padalko[4], Michael Peeters[1], Ana Rita Pereira[3], Mario Poljak[5], Gro Kummeneje Presthus[6], Nina Redzic[3], Kristiane Søreng[6], Davy Vanden Broeck[3], Steven Van Gucht[1]

[1]The Belgian Reference Centre for Human Papillomavirus, Sciensano, Infectious Diseases in Humans, Viral Diseases, Brussels, Belgium

[2]The International HPV Reference Center, Karolinska Institutet, Stockholm, Sweden

[3]The Belgian Reference Centre for Human Papillomavirus, Algemeen Medisch Laboratorium - Sonic Healthcare Benelux, Antwerp, Belgium

[4] The Belgian Reference Centre for Human Papillomavirus, University Hospital Ghent, Laboratory of Medical Microbiology, Ghent, Belgium

[5] The Slovenian Reference Center for Human Papillomavirus, Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Slovenia

[6]The Norwegian HPV Reference Laboratory, Akershus University Hospital, Lørenskog, Norway

The authors are listed in alphabetical order.

*Correspondence: Kristiane Søreng, Kristiane Søreng, Kristiane.Soreng@ahus.no

Contents

6.1 HPV AMPLIFICATION

Amplification of human papillomavirus (HPV) nucleic acids is performed for several reasons; (1) to identify the presence of HPV in a sample, (2) to identify types that are present, i.e., typing, (3) to quantify the amount of HPV and (4) for gene expression studies. Amplification is most often performed directed against deoxyribonucleic acid (DNA) but could also be directed against messenger ribonucleic acid (mRNA) for gene expression studies. In the last years, there has been an increased awareness of applications for HPV typing. With more than 200 different closely related HPV types with sequence similarities, typing can be challenging. It is therefore important to make sure that the HPV types of interest are amplified and identified. Because of this, special concerns need to be taken when designing the assays. There are two main types of amplification: target amplification, such as Polymerase Chain Reaction (PCR) and isothermal amplification, and signal amplification, such as detection of target DNA/probe hybrid with enzyme labeled antibodies or branched chain DNA to amplify signal without changing copy number of target. PCR is the most common method used for HPV amplification today.

6.1.1 Polymerase chain reaction (PCR)

PCR is a technique for *in vitro* replication of a DNA template, using primers, polymerases, and cycles of different temperatures to amplify specific parts of the HPV genome. To identify if HPV is present in the PCR product, additional visualization methods are needed. An example of such method is gel electrophoresis, where a band in the gel reveals the correct amplicon. HPV type identification can also be achieved by sequencing of the amplicon, or by hybridization of the HPV amplicon to a probe attached to a bead or a membrane. In the cases when the PCR product is sequenced, the obtained sequence must be aligned against known HPV reference sequences to identify the HPV-type. This is further discussed in section 6.2.2.

Several types of inhibitors may disturb the PCR amplification, including the DNA present in high concentration. Therefore, the DNA concentration should be considered when running a PCR reaction. Typically, a DNA input of 10-100 ng per 50 µl PCR-reaction is recommended, but this will vary depending on the assay used and the template type. A higher concentration might be needed for high complexity templates, like DNA from clinical samples, compared to low complexity templates, like plasmids. As a control for PCR inhibition and DNA integrity, amplification of an internal control human housekeeping gene, such as β-globin, should be performed in clinical and surveillance samples. The amplicon size of the internal control should be close to the length of the HPV amplicon. Failure to detect the internal control may be due to DNA concentrations that are too low or too high, as well as presence of inhibitors. Repeating the reaction with either less (diluted or reduced volume) or more sample can be tried. The use of positive and negative controls in the PCR reaction is necessary to avoid any false negative or false positive result and is further discussed in section 6.1.5 (also discussed chapter 3.5).

Real-time PCR (qPCR)

Real-time PCR differs from the end-point PCR described above as it visualizes the product of each cycle as the reaction proceeds, i.e. in real-time, rather than just at the end of all cycles. A fluorescent double stranded DNA (dsDNA) binding dye (e.g. SYBR Select (Applied Biosystems))

or sequence-specific fluorescent probes (e.g. TaqMan (Applied Biosystems)) release fluorescence during the amplification process, making it possible to monitor the amplification of the target DNA as the reaction is happening. A standard curve prepared with known copy numbers of the target is used to determine the target concentration in the unknown samples. Probe based real-time PCR can be run with several dyes at a time, making it well suited for multiplexing, allowing detection of multiple HPV types in one reaction.

Reverse transcriptase PCR (RT-PCR)

Reverse transcriptase PCR (RT-PCR) enables the use of mRNA as a template to generate complementary DNA (cDNA) by using the enzyme “Reverse Transcriptase”. RT-PCR serves as a first step for RT-real-time PCR, a common technique used to identify and quantify gene expression.[1] Of particular interest is the expression of the HPV oncogenes *E6* and *E7* as they are important for initiating and maintaining cancer development. If quantification of gene expression is the goal, the expression needs to be normalized towards the expression of a human housekeeping gene e.g. *GAPDH*.

6.1.2 Primers

In a PCR reaction, primers bind to specific sequences of a genome, guiding the polymerase to the site of amplification. While some primers are designed to precisely match and thus amplify one specific HPV type (type specific primers), other so-called consensus primers or general primers, are designed to target several HPV types at once. When amplifying several HPV types using a limited number of primers, the primers are directed against a conserved region of the HPV genome with few mismatched bases. At the same time, for typing, the amplified area should not be too conserved, as it is necessary to distinguish between the different HPV types. An area of HPV typically targeted by consensus primers is the conserved L1 gene. Other areas targeted in HPV PCR reactions are the E6 and E7 genes. The E6 and E7 genes are well suited for HPV detection and typing as their sequence variates more between HPV types. In addition, because of their importance in cancer progression, they are rarely affected by deletions and rearrangements during HPV host integration events.

When designing primers, the sequence and region targeted should be selected to maximize specificity of the assay. Primer length is optimally 15-30 nt, and shorter regions of amplification are more efficient. This is particularly important in situations where the sample DNA may be damaged or fragmented, as in formalin-fixed paraffin-embedded (FFPE) tissue when maximal amplicon size is generally 450 nt. In addition, it is important to ensure that the primers do not bind to off-target sites in HPV or the human genome. Primer-dimer formation and secondary structure should be checked. The melting temperature (Tm) of each primer should be identified using a Tm calculator, and the Tm for both primers should not differ more than 5 °C. There are several free primer tools available online that can be used for checking primer-dimers and melting temperatures.

Examples of primer tools offered from Integrated DNA Technologies and Sigma-Aldrich are found at the websites:

OligoAnalyzer Tool - Primer analysis and Tm Calculator | IDT (idtdna.com)

OligoEvaluator™ - Sequence Analysis

Consensus and degenerate primers

Several consensus primer sets directed against the relatively conserved L1 region of HPV genome are available. The SPF (Short PCR-fragment)[2] primer set amplifies a short fragment of 65 bp and consists of four forward and two reverse primers. Some of the bases are replaced with inosine which can pair with any of the four bases in DNA. In this way, SPF primers can target at least 43 HPV types. The human papillomavirus general primers, GP5+/6+[3] use a fixed pair of primers and a low annealing temperature (40 °C) in the PCR reaction to detect a wide range of HPV types. The newer modified general primers (MGP) target the same ~150 bp region in L1 as GP5+/6+ and consist of five forward and five reverse primers[4]

The first set of consensus primers, MY9/11,[5] used degenerate primers achieved by allowing a mixtures of bases to be incorporated at several position during the synthesis of the primers to increase the number of HPV types that could be amplified. With several degenerate nucleotides in each primer, MY9/11 was a mixture of 25 primers.[6] However, degenerate primers can vary between synthesis batches, making reproduction difficult. The PGMY09/11 primers, consisting of 5 forward and 13 reverse primers[7] were developed to solve this problem. Both MY9/11 and PGMY amplify the same ~450 bp region of L1. The sequences of the primers mentioned here are listed in **Appendix I** (Section 6.5, Tables 6-1 to 6-5). It should be noted that while consensus primers amplify and detect multiple types, identification of specific types requires further analysis of the amplicons, most often type-specific hybridization.

Type specific primers

While consensus primers are made to detect several HPV types, type specific primers are designed to identify one specific HPV type. Numerous type specific HPV primer sets are designed, particularly targeting the high-risk HPV types.[8-10] Type specific primers are often directed against the E6 and E7 genes as these have more sequence variation between HPV types.

An example of a method for HPV typing where type specific primers are utilized is the RIATOL assay, an in-house developed multiplex PCR detecting 18 different HPV-types. The primer and probe sequences used in this assay are shown in **Appendix I** (Section 6.5, Table 6-4 to 6.9) and the principles of the assay are further explained in **Appendix II** (Section 6.6).

6.1.3 Isothermal amplification

An alternative method for target amplification is isothermal amplification. In contrast to PCR methods that require multiple cycles at different temperatures, isothermal amplification occurs with high specificity at a constant temperature. The main advantages of isothermal amplification include reduced equipment costs, as the assay can be performed in a heat block or water bath avoiding the expense of a thermocycler, shortened assays times, and simpler protocols that can be performed by personnel with less training. Several detection methods are established to instantly detect the amplified target directly in the test tube with inexpensive detection equipment. Isothermal HPV assays for cervical cancer screening could be particularly beneficial for low- and middle-income countries seeking to provide early detection of pre-cancerous and cervical cancers to all eligible women.

Several methods for isothermal amplification exist and are reviewed.[11,12] Among the methods applied for HPV detection are Loop-mediated Isothermal Amplifications (LAMP), Recombinase Polymerase Amplification (RPA), and the AmpFire HPV assay, a commercially available LAMP assay, which are further discussed below.

Loop-mediated Isothermal Amplification (LAMP)

LAMP relies on the combination of 4-6 specifically designed primers against the target DNA with *Bst* DNA polymerase, a polymerase with high strand displacement and amplification factor.[13,14] Whereas the *Bst* DNA polymerase amplifies DNA targets, another polymerase, the OmniAmp Pol, has been developed for LAMP of RNA targets.[15] For primer design, several free online LAMP primer design tools are available, such as LAMP primer designing software PrimerExplorer (Eiken Chemical CO), LAMP designer software - OptiGene or the NEB LAMP primer design tool (New England Biolabs).

LAMP has shown to be a highly specific method, with a detection limit of as little as 1 copy per µL and an amplification time of less than 1 hour at 65 °C.[14,16,17] An additional advantage is that the output can be easily visualized by eye using e.g. colorimetric detection directly in the test tube.[18] Recent studies show that LAMP can be used to specifically detect different HPV types both from cell line suspensions as well as liquid-based cytology samples from patients.[19,20]

Despite all the advantages, difficulty in primer design is a challenge for LAMP assays. LAMP primers need to recognize 6-8 regions in the target DNA with specific restrictions to factors such as distance between the primer binding.[21] Moreover, careful design and optimization to avoid primer sets that give slow amplification or false-positive hits is required. Recently, new primer features have been identified, which has improved LAMP sensitivity and specificity[22] Some of the challenges concerning LAMP are nicely reviewed in (Moehling et al., 2021; Yang et al., 2024).[23,24] In addition, identification of multiple individual types in one reaction is restricted.

Recombinase Polymerase amplification (RPA)

RPA is another isothermal amplification alternative, which, in contrast to traditional PCR techniques, does not depend on thermal denaturation and annealing. During RPA, amplification of nucleic acids is performed using a recombinase, single-stranded DNA-binding proteins and a strand-displacing polymerase. This allows isothermal amplification of target DNA at 37-42 °C, within 30-40 minutes.[25] The RPA-method is compatible with multiplexing, making it beneficial for HPV detection.

During the reaction, the recombinase forms a complex with the primers, which scans the double-stranded DNA template for homologous sequences. The primers are inserted at the correct site by the strand-displacement activity of the recombinase and single-stranded DNA-binding proteins stabilize the displaced DNA strand. A subsequent conformational change primer-recombinase complex initiates the amplification by recruiting the strand displacing DNA polymerase. Amplification of the DNA target is achieved by cyclic repetition of this process. [25,26]

The RPA assay has recently been developed for detection of multiple HPV types. Using the PGMY/GP6+primer set targeting the HPV L1 gene (see section 6.1.2) RPA was used to detect 13

high risk (HR)-HPV types in one reaction. [27] Another team developed an RPA assay to cover 20 HR and possibly HR HPV types as well as 14 non-HR HPV types using primers targeting both HPV L1 and E6E7 genes.[28] Unfortunately, while the RPA assay can be multiplexed to detect multiple HPV types, these types are detected as a group and individual HPV types are not identified.

Commercial assays for isothermal amplification

Commercially available HPV assays for isothermal amplification are the AmpFire Screening 16/18/HR and ScreenFire HPV RS (both from Atila Biosystems, Mountan View, CA, USA), detecting 14 and 13 HPV types respectively. HPV can be detected directly from the samples without the need for DNA extraction. The amplified products interact with specific probes, generating fluorescence (FAM/HEX/ROX/CY5 channels), allowing for real-time analysis and typing. ScreenFire is a redesigned version of the AmpFire, using the same biochemistry, but with HPV66 removed from the panel. Whereas AmpFire 16/18/HR provides detection of HPV16, HPV18 and HR-types, ScreenFire has been developed to provide extended HPV typing in the following groups: a) HPV16, b) HPV18/45 c) HPV31/33/35/52/58 and d) HPV39/51/56/59/68. Both AmpFire 16/18/HR and ScreenFire HPV RS have been subjected to validation tests, demonstrating excellent intra- and interlaboratory reproducibility.[29]

AmpFire has also been developed for typing of 15 HR HPV types (AmpFire Genotyping HPV assay, Atila Biosystems) and the AmpFire assays have been evaluated for clinical FFPE tissue samples from the cervix, showing excellent analytic sensitivity and specificity for HPV detection and typing.[30] While endpoint detection could be used, these commercial assays require a real-time PCR system for detection. The assays have been evaluated for primary cervical cancer screening on both self-collected and clinician-taken samples process, and the assay showed similar sensitivity and good specificity when compared to already known screening methods such as Cobas.[31,32]

6.1.4 Signal amplification

Signal amplification techniques can be used as an alternative to target amplification technologies. An advantage of such methods is the reduced risk of contamination through amplicon carryover.[33] Unlike target amplification, signal amplification does not rely on enzymes for amplification, but rather uses probes which hybridize to the target DNA, resulting in the generation of a signal that can be detected for diagnostic purposes. Recently, signal amplification was used to investigate and explore the appropriate intervals for self-taken samples in cervical cancer screening.[34]

An example of a method using signal amplification for HPV detection is Hybrid Capture 2 (HC2) HPV DNA test (Qiagen, Germantown, MD, USA). The assay is based on the hybridization of a labeled RNA probe to the target HPV DNA in the samples. The DNA-RNA hybrids are recognized by antibodies coupled to an enzyme that will react with a chemiluminescent substrate to generate a detectable signal.[35,36] HC2 was the first, and for many years, the only FDA approved commercial HPV assay for use in clinical settings. It has been used for cervical cancer screening worldwide and in many ways set the standards for other clinical HPV assays. However, the HC2-HPV test detects HR types as a group and does not identify individual types. Furthermore, it has shown significant cross-reactivity with a range of non-targeted HPV genotypes.[37]

6.1.5 Positive and negative controls

For all amplification assays, positive and negative controls are required. Several negative controls are important. Water should be included as a sample during extraction, controls for contamination during extraction as well as during the amplification process. Water included as a sample in the PCR set-up controls for contamination during PCR. A human DNA sample negative for HPV, such as human placental DNA, monitors contamination with HPV samples and serves as a positive control for the endogenous DNA control. World Health Organization (WHO) International Standards (IS) for HPV DNA, ordered from the National Institute for Biological Standards and Control (NIBSC), are available for types HPV6, HPV11, HPV16, HPV18, HPV31, HPV33, HPV45, HPV52 and HPV58. These International Standards should not be used in each assay but are designed to allow laboratories to create secondary standards calibrated to IS in International Units (IU). Cell lines are another source of positive controls. The most commonly used cell lines containing HPV DNA are SiHa (1-2 copies of HPV16 per cell)[38] and HeLa (10-50 copies of partially deleted HPV18 per cell but intact L1 and E6E7)[39,40], CaSki (~500 copies of HPV16 per cell)[37], and MS751 (one copy of E6E7, URR and part of E1 of HPV 45 per cell).[40,41]

Alternatives are HPV gene fragments or plasmids with HPV whole genome or genome fragments that can be cloned or purchased from commercial companies.[42] When using plasmids or DNA fragments, the integration site and sequence needs to be verified to be compatible with the assay. For example, integration in L1 could disrupt the amplification region of the L1 consensus assays.

HPV DNA controls are usually prepared in a background of human DNA to mimic clinical samples and minimize loss of target during serial dilutions. Examples of positive and negative controls are listed in **Table 6-1**.

When working with HPV target amplification, thousands of copies of HPV will be made risking amplicon contamination, leading to false positive results. To prevent contamination, the work preparing the master-mix and PCR set up (pre-PCR) must be in a different location from work with amplicons (post-PCR) at different locations. The optimal working environment would be to have a clean room for preparing the master mix, a separate lab with a PCR cabinet for setting up the PCR and a post-PCR room for working with the PCR products.

6.2 EMERGING TECHNOLOGIES

A number of technologies have potential applications in HPV assays, often as options to improve detection and identification of the products of amplification assays. These emerging technologies include Mass Spectrometry (MS), Next Generation Sequencing (NGS) and digital PCR.

Table 6-1 Examples of HPV positive and negative controls

Negative controls	**Article nr**	**Company**
ddH_2O		
Human Genomic DNA	11691112001	Roche
Positive controls		
Plasmids / DNA fragments		
WHO international standard for HPV6	14/256	NIBSC
WHO international standard for HPV11	14/100	NIBSC
WHO international standard for HPV16	06/202	NIBSC
WHO international standard for HPV18	06/206	NIBSC
WHO international standard for HPV31	14/258	NIBSC
WHO international standard for HPV33	14/260	NIBSC
WHO international standard for HPV45	14/104	NIBSC
WHO international standard for HPV52	14/262	NIBSC
WHO international standard for HPV58	14/264	NIBSC
HPV gene fragments/plasmids		IDT/GenScript or others
Cell lines		
SiHa (HPV16)	HTB-35	ATCC, Manassas, VA
HeLa (HPV18)	CCL-2	ATCC, Manassas, VA
Ca-Ski (HPV16)	CRL-1550	ATCC, Manassas, VA
MS751 (HPV45)	HTB-34	ATCC, Manassas, VA

6.2.1 Mass spectrometry (MS)

MS is an analytical technique that determines the molecular weight and structure of a sample. It works by ionizing a sample and sorting the resulting charged particles based on their mass-to-charge ratio (m/z) using an electric and magnetic field. The ionization step can be performed using various methods, such as electron impact, chemical ionization, and laser desorption/ionization (as in MALDI-TOF). The resulting mass spectrum displays the m/z values of the ions as peaks on a graph, allowing the molecular weight and elemental composition of the sample to be determined. Additionally, by comparing the mass spectrum to a reference database, the identity of specific compounds within the sample can be confirmed. Mass spectrometry HPV assays uniquely identify PCR amplicons generated by PCR using MALDI-TOF and comparing the spectrum to reference database.[43] The advantage of MALDI-TOF over other methods of amplicon identification is its high specificity, sensitivity, and ability to multiplex multiple HPV types in a single assay. One MS assay has fulfilled all necessary validation steps to be suitable for screening purposes (Arbyn et al., 2024). In addition to its diagnostic applications, MS can also be used to quantify the amount of HPV DNA present in a sample, allowing for the assessment of viral load which is currently being analysed to gain insight in its predictive value.

6.2.2 Next Generation Sequencing (NGS)

NGS can be considered to be most promising technology for future use, not only for HPV testing but in the diagnostic field as a whole.[44] Although still rather expensive, costs are rapidly decreasing, bringing NGS within reach of being compatible with existing technologies. The essence of NGS comprises reading the sequence of viral nucleotides and mapping it against known databases of sequences. NGS provides the highest level of detail and allows the detection of single nucleotide polymorphisms even within samples of the same (sub) genotype. NGS for HPV can be expected to identify any type in the sample, avoiding restrictions imposed by targeted assays. Most steps and quality assurance procedures needed for NGS are well described by Arroyo Mühr et al.[45]

It should be noted that HPV sequences in clinical samples are naturally by nucleic acid from non-HPV sources (i.e., human, bacterial and other microbes). Therefore, NGS performed directly on the DNA extract without a targeted approach results in few HPV sequences relative to the total reads. Thus, either host genome depletion or viral enrichment is needed prior to library preparation when aiming to identify the whole HPV genome (e.g. (sub)lineage identification) or looking for a high throughput (sequencing depth).

Targeting one or several regions of the HPV genome

For research into specific genomic regions of the HPV genome, amplicon sequencing is advised. This technology is a highly targeted approach, which provides an ultra-deep throughput, enabling researchers to analyze genetic variation and to identify and characterize variants efficiently within these targeted regions.

Amplicon sequencing can be performed by using ready-to-use panels that are optimized and customized for specific targets or genomic content of interest. Both Illumina and Ion Torrent platforms provide these solutions. Another alternative is to design either specific, consensus or degenerate primers to amplify the region(s) of interest, followed by library preparation and sequencing of the PCR products. Both approaches will provide a high throughput, enabling identification and analysis of both point mutations and variations. Examples of such protocols with different platforms can be found in Arroyo Mühr, et al. 2014.[46] However, specimens with poor depth (<100 median depth) and/or low amplicon coverage (<80% coverage) cannot be used for variant calling.

Targeting the whole HPV genome

Whole genome sequencing is recommended for (sub)lineage assignment and it involves sequencing of the whole DNA within a specimen, not only the HPV genome. As already described, HPV positive clinical samples contain not only HPV DNA, but also human DNA as well as genomes from other microorganisms. To obtain a high throughput specifically for the HPV genome, the viral genome needs to be enriched (generally achieved by amplification) prior to proceeding with library preparation and sequencing.

Example of HPV 16 whole genome sequencing:

The HPV 16 whole genome can be sequenced by enriching the viral genome using specific primers that produce overlapping PCR products that cover the entire HPV16 genome (7906 bp). A set comprising of 103 primers (51 forward primers and 52 reverse primers) aiming to amplify the entire HPV 16 genome by producing 47 different overlapping amplicons, ranging in size between 181-375 bp, was fully validated and already implemented by several research groups.[47,48] The sequences of all 103 primers are freely available.[48]

For several platforms, users can have these primers included in "custom panels" (e.g. Ion AmpliSeq HPV16 panel.[48] However, it is also possible to first amplify the HPV DNA manually, and then perform NGS using other platforms (e.g. Illumina), as performed by Arroyo et al.[47] In the latter case, primers need to be divided into separate pools to reduce the occurrence of self-dimers and cross-primer dimers.[47]

Non-targeted detection of HPV through whole genome sequencing

If the purpose is to detect HPV genotypes without using any targeted enrichment, or to discover novel types, non-targeted whole genome sequencing should be performed. Extracted DNA material should be subjected directly to library preparation avoiding specific amplification.

This approach is used for confirmatory testing, when samples that are diagnosed as HSIL or worse are found to be HPV negative when subjected to traditional typing methods. Sequencing is then performed without any targeted enrichment, to detect HPV types that might have "escaped" amplification due to primer mismatching, or due to being distant homologues to the "targeted" sequences.[49]

Once the sequencing reads are obtained, an alignment with an updated HPV database comprising all complete genomes from the different HPV types should be performed. The Papillomavirus Episteme database, found at https://pave.niaid.nih.gov/, is recommended for this purpose. When performing whole genome sequencing, at least 10 reads detected for a specific HPV type together with a coverage of at least 10% of the HPV genome (around 800 bp coverage) should be the minimum applied cut-off. This approach avoids false positivity generated by background noise (e.g., a large number of low complexity reads mapping to just a small region of the genome).

6.2.3 Tagmentation-assisted multiplex PCR enrichment sequencing (TaME-seq)

TaME-seq is a deep sequencing method that enables the detection of HPV genomic variation both at and below the consensus sequence level, as well as HPV-human-chromosome integration events, and involves primer design, laboratory workflow and a bioinformatics pipeline.[40,50] So far, the assay is not approved for diagnostics and is only used for research purposes.

Primer design

A degenerate consensus sequence is obtained by aligning all complete, previously published HPV sequences (National Center for Biotechnology Information (NCBI) and the Papillomavirus Episteme (PAVE) databases). The Primer3 tool[51,52] is used to design the primers, which are manually inspected before a TruSeq adapter is added to the 5' end of each primer. Primer sets for HPV16/18/31/33/45/51/52/58/59 have already been designed and tested.[53,54]

Laboratory workflow

After DNA extraction, tagmentation with Nextera XT DNA Library Preparation Kit (Illumina) fragments the DNA and simultaneously adds adapters to each fragment. The tagmented samples are divided and subjected to two rounds of PCR, followed by size selection, sample pooling and quality/quantity check of the final sequencing library. Sequencing is then conducted on a NovaSeq Illumina sequencer.

Bioinformatics pipeline

The bioinformatics analysis enables the identification of integration breakpoints between HPV and human chromosomes.[50] Due to high sequencing depth at single nucleotide resolution, single nucleotide variants (SNVs) and intra-host single nucleotide variants (iSNVs) can also be detected.[50,55]

Overall, it can be expected that with a higher resolution of details on the viral (sub)genotype, more insights will be gained into the oncogenic potential of sub-typing, hence adding an additional layer to patient management. Self-evidently, when massive amounts of data are produced, proper software infrastructure must follow this evolution, ensuring an important future role for bioinformatics.

6.2.4 Digital PCR (dPCR)

Digital PCR (dPCR) is an innovative technology that can be used for high sensitivity detection of HPV and quantification of viral load.[56,57] Also termed third generation PCR, dPCR uses partitioning of the PCR master mix into thousands of small sub-partitions. Each sub-partition is considered for detection individually, prior to counting positive versus negative sub-partitions. Dilution and sub-partitioning of the sample avoids PCR competition and allows for low copy variants to be detected. Sub-partitioning was originally done in microspheres of inert oil (droplets), but alternative solutions are becoming available without the use of oil. First generation of digital PCRs required high-level technical skills, and assays were expensive. Recently, also here a decline of costs can be noted, paralleled by more ease of use through automation.

With quantitative HPV measurement gaining interest, dPCR will be able to contribute strongly to this evolution through absolute quantification. Virtually all existing primer/probe sets can be transformed into dPCR assays, both in singleplex as multiplex setting, making this technology of interest in future diagnostics.[58,59] An example of how digital PCR can offer added value can be found in follow up of HIV-positive patients, where longitudinal detection of viral load is essential. Furthermore, due to its high sensitivity, dPCR might as well be used to detect circulating HPV in

serum.[60,61] It can be foreseen that in this perspective, digital PCR can take an equivalent position of importance in the HPV diagnostic landscape.

6.3 ASSAY SELECTION

Laboratories undertaking HPV testing have a myriad of commercial and in-house assays to choose from, and decisions about selecting one or more for implementation can be daunting. General principles to assist this selection follow.

6.3.1 Goal of testing

Paramount in assay selection is the goal of testing – clinical (primary cervical cancer screening), surveillance (planning vaccine campaigns or monitoring impact), or research (questions of basic HPV biology and pathogenesis). The application will determine the need for typing, number of types to be detected and or identified, need for sequence data, and approach to evaluating sensitivity and specificity. Importantly, the sensitivity of clinical assays is based on their ability to detect HPV-associated disease (cancer and pre-cancer). Surveillance and research assays rely on analytic sensitivity, how many copies of HPV are detected. For clinical use, review and approval of assays by regulatory agencies is generally required. WHO has prequalified some commercial HPV assays for clinical (in vitro diagnostic) (https://extranet.who.int/prequal/vitro-diagnostics/prequalified-vitro-diagnostics, last accessed October 1, 2024).

6.3.2 Equipment

Equipment needs are another consideration. Does the assay require purchase of new equipment? If so, does the laboratory have required space and power capacity? Can the equipment be used in other assays, or the platform restricted to the HPV assay? What are the costs of equipment maintenance and services and does the laboratory have access to vendors providing service and maintenance on the equipment?

6.3.3 Assay complexity and personnel

HPV assays vary in their complexity. While all require trained laboratory personnel and excellent quality control, some are easier to perform than others. Understanding if the assay requires additional training of existing personnel or hiring more experienced senior laboratorians can influence assay selection. For example, some assay platforms were designed for use with minimal laboratory skills, such as Care HPV or GeneXpert. Some assays require sample processing, usually DNA extraction, prior to testing, while others provide an integrated sample extraction and testing platform (such as cobas or GeneXpert) or tolerate use of unextracted samples (such as AmpFire).

6.3.4 Assay throughput and turn-around time

HPV assays vary greatly in the number of samples accommodated in a run, as well as in the time required for assay completion. Workflow for receipt, processing, testing, and time to reporting differ by assay. Depending on the setting and goals of testing, the laboratory may need to process small batches of samples or establish a high throughput testing method. In general, turn-around time for surveillance and research applications is less critical than for clinical applications.

6.3.5 Data management and reporting

Assays vary in the complexity of interpretation as well as integration with laboratory information systems. For example, NGS assays require bioinformatics and careful review of controls for interpretation. Bioinformatics may be 'packaged', that is available to generate simple reporting of types or may rely on manual processing. Some commercial assays require interpretation of positive or negative results, such as visual interpretation of presence of hybridization bands or application of cut-off values as well as evaluation of positive and negative controls. Most clinical assays provide results that require minimal interpretation and that integrate evaluation of controls. Some assay platforms allow direct (automated) reporting of results into laboratory information systems or databases while other rely on manual entry. Minimizing individual interpretation and manual data entry generally reduces errors.

6.3.6 Cost

While costs are often the first consideration that comes to mind, and in the end is often the bottom line in decisions, all the factors described above must be considered when determining the true cost of the assay. Personnel costs are influenced by required level of experience and number of personnel required to meet required turnaround time. These are in turn determined by assay parameters. Cost of equipment and equipment maintenance need to be factored in. The kind of data management required by the assay, including computers, database, and computational programs influence cost. The cost of required laboratory quality processes, such as competency assessment, QC repeats need to be factored in.

Reagent costs include the HPV test components, costs for any sample preparation, such as extraction, and consumables required for assay performance (pipette tips, lab coats, etc.). Reagent stability affects costs through waste incurred due to inability to fully use them prior to expiration dates. Assay reliability also affects costs as failed assays require repeat testing.

Finally, the cost of waste disposal is easily overlooked but needs to be considered. Does the assay generate toxic or hazardous waste requiring special handling, and if so, what are the costs? What biologic waste is generated and how will this be handled? How much disposable plastics and other materials will be generated and how will laboratory manage these.

6.3.7 In-house versus commercial assays

Development and exploitation of in-house assays for detection of HPV was particularly important in the early years of HPV detection when only a limited number of commercial assays were available.[62] Here, in-house assays are defined as assays that are prepared and conducted by the laboratory. As such, the laboratory has the obligation to validate assay performance.

To setup an in-house assay, a strong initial investment is needed to develop and validate all assay components, such as primer/probe sets. Depending on the degree of automation foreseen in the laboratory to run the in-house assays, the levels of complexity rise, paralleled by the need for more skillful laboratory technicians. An in-house assay may reduce costs through use of already available equipment and lower costs of reagent preparation. In addition, the laboratory may adapt the assay to address their particular question, rather than being forced into predesigned assay outputs.

The validation required for clinical use is extensive, and minimal criteria for use in cervical screening have been defined.[63,64] However, in most cases, additional regulatory approval is necessary, such as US FDA or European CE marking. As a result, most in-house assays are used in surveillance or research.

The use of in-house HPV assays remains highly challenging and as demonstrated by the reports of WHO LabNet,[62] the proportion of in-house assays participating in the proficiency studies is strongly declining. In the 2023 WHO LabNet report an exponential increase of commercial assays was described over time, from 57% in 2011 to 86% in 2023. **Table 6-2** shows a list of the remaining participating in-house assays (with peer-reviewed publication records) published in the WHO LabNet reports 2018-2022(https://www.hpvcenter.se/publications/reports/, last accessed October 1, 2024).

Reasons for this evolution can be of diverse origins, but the most prominent one is the changing regulatory landscape. With the emerging IVDR guidelines becoming effective, operating an in-house assay becomes highly challenging within the European Union (cfr. section 6.5). Furthermore, as outlined above, international standards and criteria for clinical validation have been formulated for HPV assays used in general screening populations, including in-house assays.[63] Only a limited number of assays have been able to sufficiently participate in clinical validation studies, which might be the reason why they are withheld from use in primary screening depending on governmental regulations.

Protocols for a wide variety of in-house assays have been published. A detailed SOP for the RIATOL assay is included in **Appendix II** (Section 6.6) of this chapter.

Commercial assays are not inherently more reliable than in-house assays, but the intent is that the manufacturer will provide assurance of reagent quality and provide data on assay performance metric. However, there are a myriad of commercial assays, at least 264 (and 511 tests variants) inventoried most recently in December 2023.[65] The majority of these assays lack adequate documentation of performance. Laboratories need to be aware of this problem and look to assays that have records of standardized and validated results. While the importance of validation and standardization is most clear in clinical applications, it is also important for surveillance and research. Interpreting study findings requires the ability to understand how assay results compare with prior publications. Assay performance in the WHO HPV proficiency studies can be helpful

when selecting a commercial assay. Good performance in all, or majority, of labs using the assay indicates reliability of assay design as well as reproducibility of results in a variety of settings.

Commercial assays for research and surveillance use vary in assay design, the number of types detected and identified, throughput and reagent costs. In most cases no regulatory review is available, and the laboratory assumes the responsibility for validating assay performance in their hands. The use of defined reference reagents, such as HPV plasmids, is often helpful. The laboratory also assumes the responsibility for verifying pre-analytic requirements for the assay (sample collection, storage conditions, processing).

Clinical assays have stringent requirements for validation because results impact patient care. Screening assays to have an adequate clinical sensitivity in order to safely rule-out patients who are not at increased risk of cervical cancer and sufficient specificity for disease needed treatment to avoid over referral for follow-up. It is important to keep in mind that analytical/technical performance is not identical to clinical performance. Regulatory agencies require manufacturers to provide extensive data on assay performance in screening. The WHO prequalification process for in-vitro diagnostic reagents include HPV tests, and to date 3 manufacturers have gone through this process. **Table 6-3** shows a list of assays which are considered validated for use in screening as of 2024.

Table 6-2. Overview of in-house assays participating in Global HPV genotyping proficiency study (2018-2023) per technique and respective literature references.

Technique	Assays reference	Nr. operating labs
PGMY-CHUV	World Health Organization, 2009 (*Human Papillomavirus Laboratory Manual Immunization, Vaccines and Biologicals*, 2009)[66]	6
Real-time PCR	Moberg et al., 2003 [67]	1
	Flores-Munguia et al., 2004 [68]	1
	Sotlar et al., 2004 [0]	1
	Lindh et al., 2007[9]	3
	Petersen et al., 2007[69]	1
	Broccolo & Cocuzza, 2008[70]	1
	Micalessi et al., 2012[71]	1
PCR Luminex	Wieland et al., 2000[72]	1
	Schmitt et al., 2006[73]	1
	Gheit et al., 2006[74]	1
	Schmitt et al., 2008[75]	1
	Söderlund-Strand et al., 2009[4]	3
	Schmitt et al., 2010[76]	1
Targeted Sequencing NGS	Wagner et al., 2019[77]	1
Reverse line blot*	Van den Brule et al., 2002[78]	1
	Tachezy et al., 2013[79]	1
Enzyme immunoassay (EIA) *	Chouhy et al., 2006[80]	1
	Chouhy et al., 2013[81]	1

*In-house EIA for HPV detection was only used by one laboratory in 2019. Since then no dataset has been generated with this type of assay.

Table 6-3 List of validated HPV assays validated for cervical cancer screening

A. Standard comparator hrHPV DNA tests (validated in population-based randomised trials), used as comparator in validation studies:	
1	A1. Hybrid Capture 2 HPV DNA Test (Qiagen, Gaithersburg, MD, USA)
2	A2. GP5+,6+ PCR-EIA (Diassay, Rijkswijk, the Netherlands)
B. hrHPV DNA tests validated consistently in multiple studies against standard comparator tests:	
3	B1. Alinity m HR HPV Assay (Abbott, Wiesbaden, Germany)
4	B2. Anyplex II HPV HR Detection (Seegene, Seoul, South Korea)
5	B3. Cobas 4800 HPV Test (Roche Molecular System, Pleasanton, CF, USA)
6	B4. HPV-Risk Assay (Self-Screen BV, Amsterdam, The Netherlands)
7	B5. NeuMoDX HPV assay (Qiagen, Ann Arbor, MI, USA)
8	B6. Onclarity HPV Assay (BD Diagnostics, Sparks, MD, USA)
9	B7. PapilloCheck HPV-Screening Test (Greiner Bio-One, Frickenhausen, Germany)
10	B8. RealTime High Risk HPV Test (Abbott, Wiesbaden, Germany)
11	B9. Xpert HPV (Cepheid, Sunnyvale, CA, USA)
C. hrHPV DNA test validated consistently in multiple studies against alternative comparator test:	
12	C1. Cobas 6800 HPV Test (Roche Molecular System, Pleasanton, CF, USA)
D. hrHPV DNA tests evaluated in only one study against standard comparator tests:	
13	D1. CLART HPV4S (GENOMICA SAU, Madrid, Spain)
14	D2. OncoPredict HPV Screening (Hiantis Srl, Milan, Italy)
15	D3. REALQUALITY RQ-HPV Screen (AB ANALITICA, Padua, Italy)
E. hrHPV mRNA test evaluated against standard comparator tests:	
16	E1. APTIMA HPV Assay (Hologic, Bedford, MA, USA)
F. Added since the last international publication of the list of clinically validated HPV tests[82]	
17	F1. OncoPredict HPV QT (Hiantis Srl, Milan, Italy)
18	F2. RIATOL HPV genotyping qPCR assay (AML, Antwerp, Belgium)
19	F3. Allplex HPV HR Detection assay (Seegene, Seoul, South Korea)
20	F4. Vitro HPV Screening Assay (Vitro S. A., Sevilla, Spain)

Adapted from Arbyn et al, 2024[83] and supplemented with info presented by M. Arbyn at EUROGIN 2024 and WHO prequalification website.

6.3.8 Regulatory considerations for laboratory developed tests (LDTs)

When considering in-house developed HPV assays, a topic that must be discussed are the applicable regulatory obligations. Although every region has its own regulatory authority with their own set of regulations, their goal remains the same and is to ensure safety and performance of in vitro diagnostic medical devices (IVDs).

In this section the focus is set on the regulation within the European Union, seeing that this region is currently experiencing the most dynamically changing regulatory environment. The new in vitro diagnostic medical device regulation (IVDR) came into effect on May 26, 2022, and was established in response to several scandals where medical devices reportedly triggered serious adverse effects. This regulation governs the design, manufacturing, performance, and marketing of IVDs ("Directive 98/79/EC of the European Parliament and of the Council of 27 October 1998 on in Vitro Diagnostic Medical Devices," 1998; *Regulation (EU) 2017/746 of the European Parliament and of the Council of 5 April 2017 on in Vitro Diagnostic Medical Devices and Repealing Directive 98/79/EC and Commission Decision 2010/227/ EU*, 2017). Since HPV assays are considered Class C IVDs (*Guidance on Classification Rules for in Vitro Diagnostic Medical Devices under Regulation (EU) 2017/746 - February 2023 Update - MDCG 2020-16 Rev.2*, n.d.), they therefore fall under the new IVDR legislation. Clinical laboratories are mandated to use IVDs which have CE-IVD certification; however, an exemption is made for the in-house developed assays, or the so-called laboratory developed tests (LDTs). In-house assays are exempt from most requirements applicable to CE-marked devices, with exception of certain general safety and performance requirements described in Annex 1, providing they meet certain conditions (*Regulation (EU) 2017/746 of the European Parliament and of the Council of 5 April 2017 on in Vitro Diagnostic Medical Devices and Repealing Directive 98/79/EC and Commission Decision 2010/227/ EU*, 2017). The IVDR conditions that apply to Class C LDTs are quoted in Article 5 as following:

Article 5a: *The devices are not transferred to another legal entity.*

Article 5b: *The devices are manufactured and used in compliance with appropriate quality management systems.*

Article 5c: *The laboratory of the healthcare institution is compliant with standard EN ISO-15189 or where applicable national provisions, including national provisions regarding accreditation.*

Article 5d: *The healthcare institution justifies in its documentation that the specific needs of the patient target group cannot be met or cannot be met at an appropriate level of performance by an equivalent device available on the market.*

Article 5e: *The healthcare institution shall provide its competent authority with information on the use of such devices, including justification for their manufacture, modification and use.*

Article 5f: *The healthcare institution draws up a declaration (containing certain information) which it shall make publicly available.*

Article 5i: *The healthcare institution evaluates the experience gained with the clinical use of the devices and takes all required corrective action.*

Article 5a poses a serious issue for the clinical validation of in-house HPV assays since it is stated that the LDT cannot be transferred to any other legal entity i.e., the assay cannot be performed in another laboratory than the one it is manufactured at. In order to be fully clinically validated according to the Meijer guidelines,[63] an assay must demonstrate a certain degree of inter-laboratory reproducibility i.e., the reproducibility must be assessed in at least two different laboratories. This

is an unresolved issue in the HPV scientific community that must be addressed by the working groups defining the global clinical validation criteria. Another demanding aspect is defined by Article 5d, which states that IVDR exemption can only be granted to an LDT if there is no commercial alternative available on the market. This forces in-house assays to be more innovative than current market standards and is accompanied by its own set of challenges.

In addition to these requirements that in-house developed diagnostic assays must conform with, they will now also be obliged to undergo a conformity assessment process in terms of pre-market evaluation. This process will be conducted by a competent authority and will involve a review of the manufacturer's technical documentation, including design, performance, and clinical evaluation.[84] Furthermore, following the certification by the notified body to place the test on the market, the manufacturer will have to establish a post-market surveillance system to monitor the performance of the device. This will include collecting data on the device's performance, adverse events and executed field safety corrective actions. Manufacturers will also be required to submit annual reports on the device's performance and any adverse events to the notified body.

These additional requirements will, on the one hand increase safety and performance, improve patient outcome by rigorous clinical evaluation, harmonize regulations, increase transparency and traceability, and provide long-term monitoring data of device safety and performance. However, on the other hand, these requirements will also pose significant challenges for manufacturers, including increased costs (e.g., considering additional documentation, auditing, and post-market surveillance efforts), market entry delays, limited variety in available technologies, and need for significant technical expertise and resources.[85] Moreover, the limited availability of competent authorities will also lead to aggravated delays considering that all interested parties with LDTs must submit their documentation and get approved before the May 2024 deadline.

As mentioned above, another major concern from the scientific community regarding the new IVDR is its impact on innovation. Given the strict requirements, public and private laboratories will be less likely to invest finances and resources in new assays for use in clinical practice.[86,87] Inevitably, complying with the new regulations will require manufacturers to allocate significant efforts to the conformity assessment process, post-market surveillance, and clinical evaluation activities, which might divert resources necessary for research and development. Considering the additional costs, smaller companies will also struggle to meet the regulatory requirements and compete with larger companies, which if foreseen impose pivotal limitations on market diversity. Besides, in-house methods often do emerge from fundamental research within academic laboratories, where IVDR will expectedly accelerate tech transfer to larger companies.

Additionally, IVDR imposed market-rigidity will surely affect the capacity of quick-response and adaptability to changes of emerging public health risk, as demonstrated during the recent COVID-19 pandemic where laboratories were forced to modify protocols and switch assays multiple times do to shortage of supplies.[86]

On a positive note, while the LDTs will face more stringent regulations, these regulations will also limit the uncontrolled uprising of novel HPV assays that enter the public health market and will improve quality and reliability of available assays, which in turn will have a positive impact on individual health. While the interpretation of IVDR within the European Union is currently still controversial and under debate, by 2024 it will certainly be one of the most stringent regulations. Other regional regulatory authorities are certainly expected to be triggered and drive the diagnostic

field towards a more robust quality assurance approach that emphasizes the longitudinal monitoring of performance.

Additional IVDR information for commercial assays

It is important to note that CE-IVD certified tests where adjustments are made on intended use or protocol also fall under the LDT classification. For example, if a laboratory performs a CE-IVD test on a sample type or sample transport medium that has not been validated within the CE-IVD validation, this test is considered an LDT. The same applies if certain significant adjustments are made to the protocol e.g., use of a different extraction method then the one described in manufacturer's instructions or adapted incubation times.

The same applies to the software used for analysis of LDTs. The IVDR regulations classify software used in diagnostic assays as a medical device and the classification of the software depends on the intended use, the data it generates, and the risks associated with the software. Therefore, the classification of the software may not necessarily be the same as the classification of the LDT itself.

6.4 REFERENCES

1. Bordigoni A, Motte A, Tissot-Dupont H, Colson P, Desnues C. Development and validation of a multiplex qPCR assay for detection and relative quantification of HPV16 and HPV18 E6 and E7 oncogenes. Sci Rep 2021;11:4039.

2. Kleter B, et al. Novel Short-Fragment PCR Assay for Highly Sensitive Broad-Spectrum Detection of Anogenital Human Papillomaviruses. Am J Pathol 1998;153:1731–179.

3. de Roda Husman AM, Walboomers JM, van den Brule AJ, Meijer CJ, Snijders PJ. The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. J Gen Virol 1995;76:1057–1062.

4. Söderlund-Strand A, Carlson J, Dillner J. Modified General Primer PCR System for Sensitive Detection of Multiple Types of Oncogenic Human Papillomavirus. J Clin Microbiol 2009;47:541–546.

5. Manos MM, Ting Y, Wright DK, Lewis AJ, Broker TR, Wolinsky SM. Use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. Cancer Cell 1989;7:209–214.

6. Qu W, et al. PCR detection of human papillomavirus: Comparison between MY09/MY11 and GP5+/GP6+ primer systems. J Clin Microbiol 1997;35:1304–1310.

7. Gravitt PE, et al. Improved amplification of genital human papillomaviruses. J Clin Microbiol 2000;38:357–361.

8. Karlsen F, et al. Use of multiple PCR primer sets for optimal detection of human papillomavirus. J Clin Microbiol 1996;34:2095–2100.

9. Lindh M, Görander S, Andersson E, Horal P, Mattsby-Balzer I, Ryd W. Real-time Taqman PCR targeting 14 human papilloma virus types. J Clin Virol 2007;40:321–324.

10. Sotlar K, et al. Detection and typing of human papillomavirus by E6 nested multiplex PCR. J Clin Microbiol 2004;42:3176–384.

11. Bartosik M, et al. Advanced technologies towards improved HPV diagnostics. J Med Virol 2024;96:e29409

12. Oliveira BB, Veigas B, Baptista PV. Isothermal Amplification of Nucleic Acids: The Race for the Next "Gold Standard." Front Sens 2021;2.

13. Huang WH, Zhai F, Hong GF. Cloning, Expression, Characterization and Application of Bst DNA Polymerase Large Fragment. Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao 1999;31:379–384.

14. Notomi T, et al. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 2000;28:e63

15. Chander Y, et al. A novel thermostable polymerase for RNA and DNA loop-mediated isothermal amplification (LAMP). Front Microbiol 2014;5.

16. Fujino M, et al. A simple method for the detection of measles virus genome by loop-mediated isothermal amplification (LAMP). J Med Virol 2005;76.:406–413.

17. Zhang H, Xu Y, Fohlerova Z, Chang H, Iliescu C, Neuzil P. LAMP-on-a-chip: Revising microfluidic platforms for loop-mediated DNA amplification. TrAC Trends Anal Chem 2019;113:44–53.

18. Papadakis G, et al. Portable real-time colorimetric LAMP-device for rapid quantitative detection of nucleic acids in crude samples. Sci Rep 2022;12:3775.

19. Lin J, et al, Zhang M. Colorimetric Detection of 23 Human Papillomavirus Genotypes by Loop-Mediated Isothermal Amplification. Clin Lab 2017;63:495–505.

20. Vo DT, Story MD. Facile and direct detection of human papillomavirus (HPV) DNA in cells using loop-mediated isothermal amplification (LAMP). Mol Cell Probes 2021;59:101760.

21. Soroka M, Wasowicz B, Rymaszewska A. Loop-Mediated Isothermal Amplification (LAMP): The Better Sibling of PCR? Cells 2021;10:1931.

22. Huang X, Tang G, Ismail N, Wang X. Developing RT-LAMP assays for rapid diagnosis of SARS-CoV-2 in saliva. EBioMedicine 2022;75:103736.

23. Moehling TJ, Choi G, Dugan LC, Salit M, Meagher RJ. LAMP Diagnostics at the Point-of-Care: Emerging Trends and Perspectives for the Developer Community. Expert Rev Mol Diagn 2021;21:43–61.

24. Yang N, Zhang H, Han X, Liu Z, Lu Y. Advancements and applications of loop-mediated isothermal amplification technology: A comprehensive overview. Front Microbiol 2024;15:1406632.

25. Lobato IM, O'Sullivan CK. Recombinase polymerase amplification: Basics, applications and recent advances. TrAC Trends Anal Chem 2018;98:19–35.

26. Tan M, Liao C, Liang L, Yi X, Zhou Z, Wei G. Recent advances in recombinase polymerase amplification: Principle, advantages, disadvantages and applications. Front Cell Infect Microbiol 2022;12:1019071.

27. Gong J, et al. A simple and rapid diagnostic method for 13 types of high-risk human papillomavirus (HR-HPV) detection using CRISPR-Cas12a technology. Sci Rep 2021;11:12800.

28. Wongsamart R, et al. Multiplex recombinase polymerase amplification for high-risk and low-risk type HPV detection, as potential local use in single tube. Sci Rep 2023;13:829.

29. Hou J, et al. AmpFire HPV and ScreenFire RS HPV validation trial. Am J Clin Pathol 2024;161:535–542.

30. Tang YW, et al. An Isothermal, Multiplex Amplification Assay for Detection and Genotyping of Human Papillomaviruses in Formalin-Fixed, Paraffin-Embedded Tissues. J Mol Diagn 2020;22:419–428.

31. Chung PYJ, et al. Intra- and interlaboratory reproducibility evaluation toward international validation status of the AmpFire assay. J Med Virol 2024;96:e29688.

32. Zhang W, et al. Evaluation of an isothermal amplification HPV detection assay for primary cervical cancer screening. Infect Agents Cancer 2020;15:65.

33. Wang Y. Signal Amplification Methods. In: Clinical Virology Manual 2016. p.167–72.

34. Zhao X, et al. Risk assessment of self-sampling HPV tests based on PCR, signal amplification to guide the appropriate screening intervals: A prospective study in China. J Natl Cancer Cent 2022;2:298–305.

35. Bozzetti M, et al. Comparison between hybrid capture II and polymerase chain reaction results among women at low risk for cervical cancer. Ann Epidemiol 2000;10:466.

36. Lörincz AT. Hybrid Capture TM Method for Detection of Human Papillomavirus DNA in Clinical Specimens: A Tool for Clinical Management of Equivocal Pap Smears and for Population Screening. J Obstet Gynaecol Res 1996;22:629–636.

37. Poljak M, Marin IJ, Seme K, Vince A. Hybrid Capture II HPV Test detects at least 15 human papillomavirus genotypes not included in its current high-risk probe cocktail. J Clin Virol 2002;25:89-97.

38. Mincheva A, Gissmann L, zur Hausen H. Chromosomal integration sites of human papillomavirus DNA in three cervical cancer cell lines mapped by in situ hybridization. Med Microbiol Immunol 1987;176:245–56.

39. Adey A, et al. The haplotype-resolved genome and epigenome of the aneuploid HeLa cancer cell line. Nature 2013;500:207–211.

40. Lagström S, et al. TaME-seq: An efficient sequencing approach for characterisation of HPV genomic variability and chromosomal integration. Sci Rep 2019;9:524.

41. Geisbill J, Osmers U, Dörst M. Detection and characterization of human papillomavirus type 45 DNA in the cervical carcinoma cell line MS751. J Gen Virol 1997;781:655–658.

42. van Baars R, Bosgraaf RP, ter Harmsel BW, Melchers WJG, Quint WGV, Bekkers RLM. Dry Storage and Transport of a Cervicovaginal Self-Sample by Use of the Evalyn Brush, Providing Reliable Human Papillomavirus Detection Combined with Comfort for Women. J Clin Microbiol 2012;50:3937–3943.

43. Cai X, Guan Q, Huan Y, Liu Z, Qi J, Ge S. Development of high-throughput genotyping method of all 18 HR HPV based on the MALDI-TOF MS platform and compared with the Roche Cobas 4800 HPV assay using clinical specimens. BMC Cancer. 2019;19:825.

44. Lomsadze A, Li T, Rajeevan MS, Unger ER, Borodovsky M. Bioinformatics Pipeline for Human Papillomavirus Short Read Genomic Sequences Classification Using Support Vector Machine. Viruses 2020;12:710.

45. Mühr LSA, Guerendiain D, Cuschieri K, Sundström K. Human Papillomavirus Detection by Whole-Genome Next-Generation Sequencing: Importance of Validation and Quality Assurance Procedures. Viruses. 2021;13.

46. Arroyo LS, Smelov V, Bzhalava D, Eklund C, Hultin E, Dillner J. Next generation sequencing for human papillomavirus genotyping. J Clin Virol 2013;58:437–442.

47. Arroyo-Mühr LSA, et al. Human papillomavirus type 16 genomic variation in women with subsequent in situ or invasive cervical cancer: Prospective population-based study. Br J Cancer 2018;119:1163–1168.

48. Cullen M, et al. Deep sequencing of HPV16 genomes: A new high-throughput tool for exploring the carcinogenicity and natural history of HPV16 infection. Papillomavirus Res 2015;1:3–11.

49. Arroyo Mühr LSA, et al. Deep sequencing detects human papillomavirus (HPV) in cervical cancers negative for HPV by PCR. Br J Cancer 2020;123:1790–1795.

50. Hesselberg Løvestad A, et al. TaME-seq2: Tagmentation-assisted multiplex PCR enrichment sequencing for viral genomic profiling. Virology J 2023;20:44.

51. Koressaar T, Remm M. Enhancements and modifications of primer design program Primer3. Bioinformatics 2007;23:1289–1291.

52. Untergasser A, et al. Primer3—New capabilities and interfaces. Nucleic Acids Res 2012;40:e115.

53. Lagström S, et al. HPV16 and HPV18 type-specific APOBEC3 and integration profiles in different diagnostic categories of cervical samples. Tumour Virus Res 2021;12:200221.

54. Løvestad AH, et al. Differences in integration frequencies and APOBEC3 profiles of five high-risk HPV types adheres to phylogeny. Tumour Virus Res 2022;14:200247.

55. Costanzi JM, Stosic MS, Løvestad AH, Ambur OH, Rounge TB, Christiansen IK. Changes in intrahost genetic diversity according to lesion severity in longitudinal HPV16 samples. J Med Virol 2024;96:e29641.

56. Cabel L, et al. HPV ctDNA detection of high-risk HPV types during chemoradiotherapy for locally advanced cervical cancer. ESMO Open 2021;6:100154.

57. Malin K, Louise BM, Gisela H, Mats KG, Gabriella L-L. Optimization of droplet digital PCR assays for the type-specific detection and quantification of five HPV genotypes, including additional data on viral loads of nine different HPV genotypes in cervical carcinomas. J Virol Methods 2021;294:114193.

58. Naegele S, Ruiz-Torres DA, Zhao Y, Goss D, Faden DL. Comparing the Diagnostic Performance of Quantitative PCR, Digital Droplet PCR, and Next-Generation Sequencing Liquid Biopsies for Human Papillomavirus-Associated Cancers. J Mol Diagn 2024;26:179–190.

59. Rotondo JC, et al. Simultaneous Detection and Viral DNA Load Quantification of Different Human Papillomavirus Types in Clinical Specimens by the High Analytical Droplet Digital PCR Method. Front Microbiol 2020;11:591452.

60. Jeannot E, et al. Circulating HPV DNA as a Marker for Early Detection of Relapse in Patients with Cervical Cancer. Clin Cancer Res 2021;27:5869–5877.

61. Rosing F, et al. Quantification of human papillomavirus cell-free DNA from low-volume blood plasma samples by digital PCR. Microbiol Spectrum 2024;12:e0002424.

62. Arroyo Mühr LS, Eklund C, Lagheden C, Forslund O, Robertsson KD, Dillner J. Improving human papillomavirus (HPV) testing in the cervical cancer elimination era: The 2021 HPV LabNet international proficiency study. J Clin Virol 2022;154:105237.

63. Arbyn M, et al. VALGENT: A protocol for clinical validation of human papillomavirus assays. J Clin Virol 2016;76:14–21.

64. Meijer CJLM, et al. Guidelines for human papillomavirus DNA test requirements for primary cervical cancer screening in women 30 years and older. Int J Cancer 2009;124:516–520.

65. Poljak M, Oštrbenk Valenčak A, Cuschieri K, Bohinc KB, Arbyn M. 2023 global inventory of commercial molecular tests for human papillomaviruses (HPV). J Clin Virol 2024;172:105671.

66. Human papillomavirus laboratory manual. Immunization, Vaccines and Biologicals. 2009.

67. Moberg M, Gustavsson I, Gyllensten U. Real-time pcr-based system for simultaneous quantification of human papillomavirus types associated with high risk of cervical cancer. J Clin Microbiol 2003;41:3221–3228v.

68. Flores-Munguia R, Siegel E, Klimecki WT, Giuliano AR. Performance Assessment of Eight High-Throughput PCR Assays for Viral Load Quantitation of Oncogenic HPV Types. J Mol Diagn 2004;6:115–24.

69. Petersen I, et al. Inter-laboratory validation of PCR-based HPV detection in pathology specimens. Virchows Arch 2007;451:701–716.

70. Broccolo F, Cocuzza CE. Automated extraction and quantitation of oncogenic HPV genotypes from cervical samples by a real-time PCR-based system. J Virol Methods 2008;148:48–57.

71. Micalessi IM, Boulet GA, Bogers JJ, Benoy IH, Depuydt CE. High-throughput detection, genotyping and quantification of the human papillomavirus using real-time PCR. Clin Chem Lab Med 2012;50:655–661.

72. Wieland U, et al. Papillomavirus DNA in Basal Cell Carcinomas of Immunocompetent Patients: An Accidental Association? J Investig Dermatol 2000;115:124–128.

73. Schmitt M, Bravo IG, Snijders PJ, Gissmann L, Pawlita M, Waterboer T. Bead-Based Multiplex Genotyping of Human Papillomaviruses. J Clin Microbiol 2006;44:504–512.

74. Gheit T, et al. Development of a Sensitive and Specific Assay Combining Multiplex PCR and DNA Microarray Primer Extension To Detect High-Risk Mucosal Human Papillomavirus Types. J Clin Microbiol 2006;44:2025–2031.

75. Schmitt M, Dondog B, Waterboer T, Pawlita M. Homogeneous Amplification of Genital Human Alpha Papillomaviruses by PCR Using Novel Broad-Spectrum GP5+ and GP6+ Primers. J Clin Microbiol 2008;46:1050–1059.

76. Schmitt M, Dondog B, Waterboer T, Pawlita M, Tommasino M, Gheit T. Abundance of Multiple High-Risk Human Papillomavirus (HPV) Infections Found in Cervical Cells Analyzed by Use of an Ultrasensitive HPV Genotyping Assay. J Clin Microbiol 2010;48:143–149.

77. Wagner S, et al. Development of the TypeSeq Assay for Detection of 51 Human Papillomavirus Genotypes by Next-Generation Sequencing. J Clin Microbiol 2019;57.

78. Van den Brule AJ, Pol R, Fransen-Daalmeijer N, Schouls LM, Meijer CJ, Snijders PJ. GP5+/6+ PCR followed by reverse line blot analysis enables rapid and high-throughput identification of human papillomavirus genotypes. J Clin Microbiol 2002;40:779–787.

79.Tachezy R, Smahelova J, Kaspirkova J, Salakova M. Human Papillomavirus Type-Specific Prevalence in the Cervical Cancer Screening Population of Czech Women. PLoS One 2013;8:e79156.

80. Chouhy D, et al. Development and evaluation of a colorimetric PCR system for the detection and typing of human papillomaviruses. Int J Mol Med 2006;18:995–1003.

81. Chouhy D, et al. Prevalence of human papillomavirus infection in Argentinean women attending two different hospitals prior to the implementation of the National Vaccination Program. J Med Virol 2013;85:655–666.

82. Arbyn M, et al. Chapter 10: HPV testing on cervical cancer specimens: which assay can be used in cervical cancer screening. In: Ayhan A, ed. ESGO Textbook of Gynaecological Oncology. Istanbul: Günes Publishing; 2023: 115-120.

83. Arbyn M, Dhillon SK, Poljak M. Validated HPV tests usable in cervical cancer screening on clinician-collected cervical specimens. HPVWorld.com. 2024;270.

84. Dombrink I, et al. Critical Implications of IVDR for Innovation in Diagnostics: Input From the BioMed Alliance Diagnostics Task Force. HemaSphere 2022;6:e724.

85. Lubbers BR, et al. The New EU Regulation on In Vitro Diagnostic Medical Devices: Implications and Preparatory Actions for Diagnostic Laboratories. HemaSphere 2021;5:e568.

86. Bank PCD, et al. The end of the laboratory developed test as we know it? Recommendations from a national multidisciplinary taskforce of laboratory specialists on the interpretation of the IVDR and its complications. Clin Chem Lab Med 2021;59:491–497.

87. Barberis M. In vitro diagnostic medical device regulation (IVDR): The end of laboratory developed tests (LDT)? Pathologica 2021;113:68–69.

88. Erhart SM, Rivero ER, Bazzo ML, Onofre AS. Comparative evaluation of the GP5+/6+, MY09/11 and PGMY09/11 primer sets for HPV detection by PCR in oral squamous cell carcinomas. Exp Mol Pathol 2016;100:13–16.

89. Depuydt CE, Benoy IH, Beert JFA, Criel AM, Bogers JJ, Arbyn M. Clinical validation of a type-specific real-time quantitative human papillomavirus PCR against the performance of hybrid capture 2 for the purpose of cervical cancer screening. J Clin Microbiol 2012;50:4073–4077.

90. Benoy I, et al. Using the VALGENT-3 framework to assess the clinical and analytical performance of the RIATOL qPCR HPV genotyping assay. J Clin Virol 2019;120:57–62.

91. Larionov A, Krause A, Miller W. A standard curve based method for relative real time PCR data processing. BMC Bioinformatics 2005;6:62.

92. Peirson SN. Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. Nucleic Acids Res 2003;31:73e–773.

93. Svec D, Tichopad A, Novosadova V, Pfaffl MW, Kubista M. How good is a PCR efficiency estimate: Recommendations for precise and robust qPCR efficiency assessments. Biomol Detect Quantif 2015;3:9–16.

94. Best K, Oakes T, Heather JM, Shawe-Taylor J, Chain B. Computational analysis of stochastic heterogeneity in PCR amplification efficiency revealed by single molecule barcoding. Sci Rep 2015;5:14629.

95. Peccoud J, Jacob C. Theoretical uncertainty of measurements using quantitative polymerase chain reaction. Biophys J 1996;71:101–108.

6.5 APPENDIX I: PRIMER SEQUENCES

Table 6-4 SPF primer sequences (Kleter et al., 1998)[2]

Primer	Sequence 5'-3'
SPF1A	GCi CAG GGi CAC AAT AAT GG
SPF1B	GCi CAG GGi CAT AAC AAT GG
SPF1C	GCi CAG GGi CAT AAT AAT GG
SPF1D	GCi CAA GGi CAT AATAAT GG
SPF2B-bio	GTi GTA TCi ACA ACA GTA ACA AA
SPF2D-bio	GTi GTA TCi ACT ACA GTA ACA AA

i = inosine

Table 6-5 GP5+/GP6+ primer sequences (de Roda Husman et al., 1995)[3]

Primer	Sequence 5'-3'
GP5+	TTT GTT ACT TGT GGT AGA TAC TAC
GP6+	GAA AAA TAA ACT GTA AAT CAT ATT C

Table 6-6 MGP primer sequences (Söderlund-Strand et al., 2009)[4]

Primer	Sequence 5'-3'
MGPA	ACG TTG GAT GTT TGT TAC TGT GGT GGA TAC TAC
MGPB	ACG TTG GAT GTT TGT TAC CGT TGT TGA TAC TAC
MGPC	ACG TTG GAT GTT TGT TAC TAA GGT AGA TAC CAC TC
MGPD	ACG TTG GAT GTT TGT TAC TGT TGT GGA TAC AAC
MGP31	ACG TTG GAT GTT TGT TAC TAT GGT AGA TAC CAC AC
MGPG	ACG TTG GAT GGA AAA ATA AAC TGT AAA TCA TAT TCC T
MGPH	ACG TTG GAT GGA AAA ATA AAT TGT AAA TCA TAC TC
MGPI	ACG TTG GAT GGA AAT ATA AAT TGT AAA TCA AAT TC
MGPJ	ACG TTG GAT GGA AAA ATA AAC TGT AAA TCA TAT TC
MGP18	ACG TTG GAT GGA AAA ATA AAC TGC AAA TCA TAT TC

Table 6-7 MY09/MY11 primer sequences (Erhart et al., 2016; Manos et al., 1989)[88]

Primer	**Sequence 5'-3'**
MY09 fwd primer	CGT CCM ARR GGA WAC TGA TC
MY11 rev primer	GCM CAG GGW CAT AAY AAT GG

Degenerate base code: M = A or C, W = A or T, Y = C or T, and R = A or G.

Table 6-8 PGMY09/11 primer sequences (Gravitt et al., 2000)[7]

Primer	**Sequence 5'-3'**
PGMY11-A	GCA CAG GGA CAT AAC AAT GG
PGMY11-B	GCG CAG GGC CAC AAT AAT GG
PGMY11-C	GCA CAG GGA CAT AAT AAT GG
PGMY11-D	GCC CAG GGC CAC AAC AAT GG
PGMY11-E	GCT CAG GGT TTA AAC AAT GG
PGMY09-F	CGT CCC AAA GGA AAC TGA TC
PGMY09-G	CGA CCT AAA GGA AAC TGA TC
PGMY09-H	CGT CCA AAA GGA AAC TGA TC
PGMY09-I	G CCA AGG GGA AAC TGA TC
PGMY09-J	CGT CCC AAA GGA TAC TGA TC
PGMY09-K	CGT CCA AGG GGA TAC TGA TC
PGMY09-L	CGA CCT AAA GGG AAT TGA TC
PGMY09-M	CGA CCT AGT GGA AAT TGA TC
PGMY09-N	CGA CCA AGG GGA TAT TGA TC
PGMY09-P	G CCC AAC GGA AAC TGA TC
PGMY09-Q	CGA CCC AAG GGA AAC TGG TC
PGMY09-R	CGT CCT AAA GGA AAC TGG TC
HMB01	GCG ACC CAA TGC AAA TTG GT

Table 6-9. Overview of the RIATOL multiplex PCR setup with the respective primer and probe sequences, with corresponding fluorophores (VIC, FAM, Cy5 and YAK) and quenchers (MGB and DDQ2).

Multiplex PCR mix	Content	Sequences (5'→3')
Mix 1	FP: HPV 67 L1	CGTCGCAGGCGTAAACG
	RP: HPV 67 L1	ACAGGAGGCAGGTACAC
	Probe: HPV 67 L1	VIC-AGATGTCCGTGTGGCGG-NQF
	FP: HPV 39 E7	CGAGCAATTAGGAGAGTCAGAGG
	RP: HPV 39 E7	TGTGTGACGCTGTGGTTCAT
	Probe: HPV 39 E7	FAM-AACCCGACCATGCAGTT-NFQ
Mix 2	FP: HPV 31 E6	ACGATTCCACAACATAGGAGGA
	RP: HPV 31 E6	TACACTTGGGTTTCAGTACGAGGT
	Probe: HPV 31 E6	VIC-CTCCAACATGCTATGCAACGTCC-NFQ
	FP: HPV 16 E7	AGCTCAGAGGAGGAGGATGAA
	RP: HPV 16 E7	GGTTACAATATTGTAATGGGCTC
	Probe: HPV 16 E7	FAM-CAGCTGGACAAGCAGA-NFQ
Mix 3	FP: HPV 45 E7	CGTCGGGCTGGTAGTTGTG
	RP: HPV 45 E7	ATTGCATTTGGAACCTCAGAATG
	Probe: HPV 45 E7	FAM-ATGACTAACTCCATCTGC-NFQ
	FP: HPV 56 E7	CCAAAGAGGACCTGCGTGTT
	RP: HPV 56 E7	TACTTGATGCGCAGAGTGGG
	Probe: HPV 56 E7	VIC-TACAACAGCTGCTTATGG-NFQ
	FP: HPV 68 E7	ACAACAGCGTCACACAATTCAGT
	RP: HPV 68 E7	CAGTTCTACGTTCCGCAGGTT
	Probe: HPV 68 E7	CY5-ACTGCAACTAGTAGTAGAAGCGTCGCGGG-BBQ
Mix 4	FP: HPV 33 E6	TGTGCGGCGTGTTGGA
	RP: HPV 33 E6	TGGCGTTTTTACACGTCACAGT
	Probe: HPV 33 E6	VIC-CCCGACGTAGAGAAA-NFQ
	FP: HPV 52 E7	GTGTGGACCGGCCAGATG
	RP: HPV 52 E7	CGTCGCAGTGCTATGAATGC
	Probe: HPV 52 E7	FAM-ACAAGCAGAACAAGCC-NFQ
	FP: HPV 58 E6	CCACGGACATTGCATGATTTG
	RP: HPV 58 E6	CTTTTTGCATTCAACGCATTTCA
	Probe: HPV 58 E6	CY5-TGGAGACATCTGTGCATGAAATCGAA-BBQ
Mix 5	FP: HPV 59 E7	TGTGCTACGAGCAATTACCTGACT
	RP: HPV 59 E7	TGATTAACTCCATCTGGTTCATCTTT
	Probe: HPV 59 E7	FAM-CGACTCCGAGAATGA-NFQ

	FP: HPV 35 E6	AAAAACCGCTGTGTCCAGTTG
	RP: HPV 35 E6	CACCTCGGTTTCTCTACGTGTTG
	Probe: HPV 35 E6	CY5-ATTCCATAACATCGGTGGACGGT-BBQ
	FP: HPV 6 E6	GTTCATAAAGCTAAATTGTACGTGGAA
	RP: HPV 6 E6	TGTGAATCTTGTCCGTCCACTT
	Probe: HPV 6 E6	YAK-ACAATATCCTTTAGGGTAACATGTCTTCCATGCATG-EDQ
Mix 6	FP: HPV 51 E7	AAAGCAAAAATTGGTGGACGA
	RP: HPV 51 E7	TGCCAGCAATTAGCGCATT
	Probe: HPV 51 E7	FAM-CATGAAATAGCGGGACGTTGGACG-EDQ
	FP: HPV 53 E6	AACGGTTTCACAAAATTTCACATATG
	RP: HPV 53 E6	TGATTCAGTTGCTGTTGTGTGTCT
	Probe: HPV 53 E6	CY5-ACCGGGTCGTGCCTGACATGC-DDQ2
	FP: HPV 66 E6	GTCCGTTAACACCGGAGGAA
	RP: HPV 66 E6	CCCGGTCCATGCATATGC
	Probe: HPV 66 E6	YAK-AACAATTGCACTGTGAACATAAAAGACGATTTCATT-EDQ
Mix 7	FP: HPV 18 E7	CCGACGAGCCGAACCA
	RP: HPV 18 E7	CTCAATTCTGGCTTCACACTTACAA
	Probe: HPV 18 E7	FAM-AACGTCACACAATGTT-NFQ
	FP: β-Globin	TGCATTTGACTCCTGAGGAGAA
	RP: β-Globin	GGGCCTCACCACCAACTTC
	Probe: β-Globin	VIC-CTGCCGTTACTGCCCT-MGB
Mix 8	FP: HPV 11 E6	GCTTCATAAAACTAAATAACCAGTGGAA
	RP: HPV 11 E6	TCAGGAGGCTGCAGGTCTAGTAC
	Probe: HPV 11 E6	VIC-CGTTGCTTACACTGCTGVIC-NFQ

BBQ: BlackBerry® Quencher 650; DDQ2: Deep Dark Quencher 2; EDQ: Eclipse Dark Quencher; FP: forward primer, RP: reverse primer, NFQ (Nonfluorescent Quencher).

6.6 APPENDIX II: EXAMPLE PROTOCOL FOR IN-HOUSE ASSAY

HPV detection with RIATOL quantitative real-time PCR (qPCR)

Principle of assay

The RIATOL qPCR HPV typing assay is an in-house HPV assay developed at AML, Sonic Healthcare Benelux (Antwerp, Belgium)[71] and used in routine screening for more than 15 years under ISO15189 accreditation. The assay comprises a multiplex real-time PCR that detects and quantifies viral HPV DNA using the multiplex probe amplification technology. RIATOL qPCR is capable of detecting 18 distinct HPV types separately (i.e., HPV6E6, 11E6, 16E7, 18E7, 31E6, 33E6, 35E6, 39E7, 45E7, 51E7, 52E7, 53E6, 56E7, 58E7, 59E7, 66E6, 67L1, and 68E7, see **Table 6-6**), grouped in 8 TaqMan-based multiplex reactions. In addition, human β-globin is used as both an endogenous cellular quality control and as relative normalizer of viral copies detected for HPV-positive samples. Non-inferior accuracy of the RIATOL HPV full-typing qPCR assay for CIN2+ compared to HC2 has previously been demonstrated in two validation studies: under the Meijer criteria with in-house selection of specimens[89] and furthermore within the VALGENT-3 framework.[90]

Equipment

- ThinPrep 5000 Sample Transfer System (STS, Hologic, Bedford, Massachusetts, USA)
- Cervista MTA System (Hologic, Bedford, Massachusetts, USA)
- Janus Liquid Handler Workstation (Perkin Elmer, Waltham, Massachusetts, USA)
- LightCycler 480 thermocycler (Roche Molecular Systems, Pleasanton, California, USA)
- FastFinder Analysis v4.8.1 (UgenTec NV, Hasselt, Belgium)

Reagents

- GenFind DNA Extraction Kit (95-449, Hologic)
- gBlock gene fragments (Integrated DNA Technologies, IDT)
- LightCycler® 480 Probes Master (04902343001, Roche)
- Primers and probes (Multiple suppliers, see Table 6-10).

Preparation of reagents

Preparation of master mix

Preparation of master mixes must be performed in a clean, isolated room by making specific dilutions of the stock solutions of respective primers and probes in LightCycler® 480 Probes Master, according to the **Table 6-10**.

Table 6-10. RIATOL PCR reagent stock and final concentrations used in corresponding multiplex master mixes.

Reagent	Stock concentration	Final concentration	Reagent	Working concentration	Final concentration
FP: HPV 67 L1	10 µM	0.2 µM	Probe: HPV 58 E6	10 µM	0.2 µM
RP: HPV 67 L1	10 µM	0.2 µM	FP: HPV 59 E7	10 µM	0.2 µM
Probe: HPV 67 L1	10 µM	0.2 µM	RP: HPV 59 E7	10 µM	0.2 µM
FP: HPV 39 E7	10 µM	0.2 µM	Probe: HPV 59 E7	10 µM	0.2 µM
RP: HPV 39 E7	10 µM	0.2 µM	FP: HPV 6 E6	10 µM	0.2 µM
Probe: HPV 39 E7	10 µM	0.1 µM	RP: HPV 6 E6	10 µM	0.2 µM
FP: HPV 31 E6	10 µM	0.2 µM	Probe: HPV 6 E6	10 µM	0.2 µM
RP: HPV 31 E6	10 µM	0.2 µM	FP: HPV 35 E6	10 µM	0.2 µM
Probe: HPV 31 E6	10 µM	0.2 µM	RP: HPV 35 E6	10 µM	0.2 µM
FP: HPV 16 E7	10 µM	0.2 µM	Probe: HPV 35 E6	10 µM	0.2 µM
RP: HPV 16 E7	10 µM	0.2 µM	FP: HPV 51 E7	10 µM	0.2 µM
Probe: HPV 16 E7	10 µM	0.2 µM	RP: HPV 51 E7	10 µM	0.2 µM
FP: HPV 45 E7	10 µM	0.2 µM	Probe: HPV 51 E7	10 µM	0.2 µM
RP: HPV 45 E7	10 µM	0.2 µM	FP: HPV 66 E6	10 µM	0.2 µM
Probe: HPV 45 E7	10 µM	0.1 µM	RP: HPV 66 E6	10 µM	0.2 µM
FP: HPV 56 E7	10 µM	0.2 µM	Probe: HPV 66 E6	10 µM	0.2 µM
RP: HPV 56 E7	10 µM	0.2 µM	FP: HPV 53 E6	10 µM	0.9 µM
Probe: HPV 56 E7	10 µM	0.2 µM	RP: HPV 53 E6	10 µM	0.9 µM
FP: HPV 68 E7	10 µM	0.2 µM	Probe: HPV 53 E6	10 µM	0.1 µM
RP: HPV 68 E7	10 µM	0.2 µM	FP: HPV 18 E7	10 µM	0.3 µM
Probe: HPV 68 E7	10 µM	0.3 µM	RP: HPV 18 E7	10 µM	0.3 µM
FP: HPV 52 E7	10 µM	0.2 µM	Probe: HPV 18 E7	10 µM	0.2 µM
RP: HPV 52 E7	10 µM	0.2 µM	FP: β-Globin	10 µM	0.2 µM
Probe: HPV 52 E7	10 µM	0.1 µM	RP: β-Globin	10 µM	0.2 µM
FP: HPV 33 E6	10 µM	0.2 µM	Probe: β-Globin	10 µM	0.1 µM
RP: HPV 33 E6	10 µM	0.2 µM	FP: HPV 11 E6	10 µM	0.2 µM
Probe: HPV 33 E6	10 µM	0.1 µM	RP: HPV 11 E6	10 µM	0.2 µM
FP: HPV 58 E6	10 µM	0.2 µM	Probe: HPV 11 E6	10 µM	0.2 µM
RP: HPV 58 E6	10 µM	0.2 µM	Probe: HPV 58 E6	10 µM	0.2 µM

Stock solutions of PCR reagents should be protected from light and kept at -20 ^{o}C for no longer than 12 months. Stock solutions should be aliquoted appropriately to avoid repeated freeze-thaw cycles. Prepared master mixes should as well be protected from light and kept at 4 °C for no longer than 24 hours.

Preparation of calibration curves

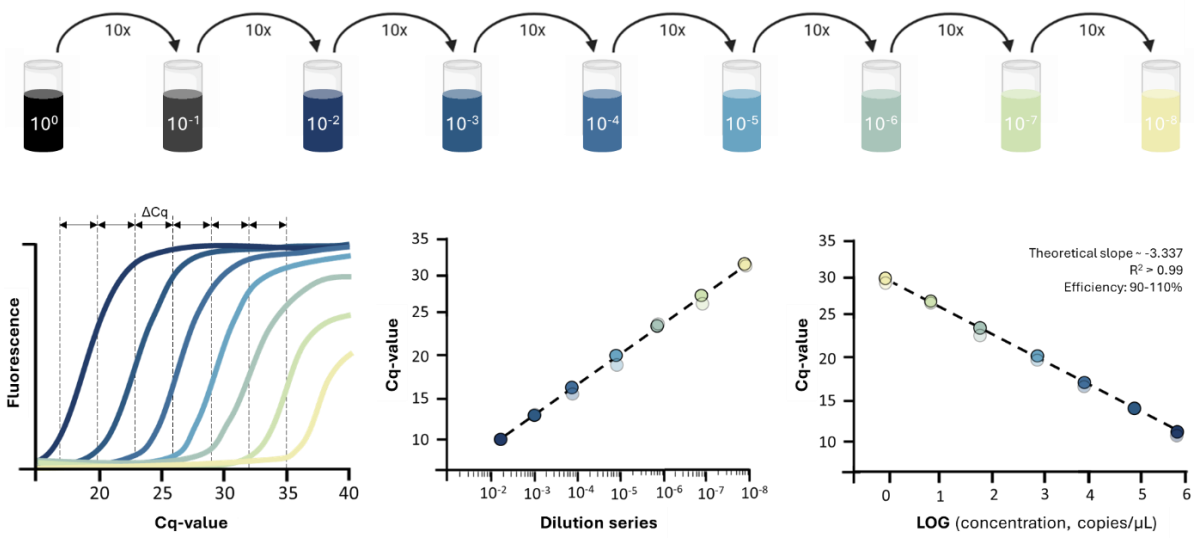
In qPCR assays, standard curves are often generated to achieve absolute quantification of results and confirm the efficiency of the qPCR experiment.[91] To develop standard curves, artificial templates or purified PCR products from which theoretical concentration have been accurately quantified by independent analysis, are evaluated in serial dilutions (**Figure 6-1**).[92] Construction of double stranded gBlocks based on synthetic gene fragments offers valuable design sequence freedom especially beneficial in multiplex assays where various control amplicon sequences need to be combined into single constructs, therefore reducing expenses and experimental variability.

Fundamentally, the dynamic range for qPCR must comprises at least 7-8 data points spanning across different orders of magnitude (10-fold dilutions) in order to calibrate the mass concentration for each experimental target. The resulting Cq values and the number of log DNA copies per µL, are used to plot the standard curve on the y and x axes, respectively. Precision of results is

assessed by running each standard in triplicate, and non-template negative controls must be included to detect reagent contaminants and background amplification.

Besides the efficiency and specificity of the reaction, a standard curve dilution experiment is needed to determine the limit of quantification (LOQ).[93] Stochastic effect might occur naturally in the real-time PCR reaction since a DNA molecule will be either replicated or not replicated at each cycle with a probability below 1.[94,95] Thus, for samples where only few target molecules are present, i.e., high dilution factors standards, triplicates might yield significant different results. Only dilution series within the linear range, i.e., with all data points following the regression line ($R^2 > 0.99$), should be considered. The calibration equation of each specific target is documented and applied in every experimental run to transform Cq values in DNA or viral concentration for each sample.

Figure 6-1 Serial dilutions of standard concentrations for preparation of calibration curves.



During a qPCR reaction the amount of product at each cycle is measured in a logarithmic relation with [N0 × (1 + k)i], where N0 represents the number of molecules in the original sample, k the amplification efficiency and i the cycle number at the time of reaction. The cycle quantification (Cq) of each reaction is precisely determined when the fluorescence yield exhibits an exponential behavior, just prior to reaching the PCR reaction saturation, i.e., depletion of primers and/or nucleotides. The slope of the dilution curve defines the amplification efficiency (k = 10-1/slope). Decisions are made based on the interpolated copy number and all dilutions are measured in triplicate. The mean, standard deviation (SD) and/or coefficient of variance (CV) is calculated from the interpolated copy number per sample and the goodness of fit of the standard curve is measured by its regression coefficient R2.

Procedure

Sample reception and nucleic acid extraction

Upon reception, 2 mL of each sample are transferred into a 96-deep well plate by the STS liquid handling robot for further batch processing. DNA extraction is then conducted in the automated Cervista MTA system, according to the manufacturer's instruction. The quality of resulting DNA is remarkably high with a 260/280 nm absorbance ratio of approximately 1.8 – 2.0, assuring intact molecules with low molecular shearing, free of contamination with proteins, RNA or polysaccharides. See quality control procedures below for more details.

PCR reaction

The 384-well PCR plates are prepared by the Janus Liquid Handler robot (Perkin Elmer), by mixing 2 µL of extracted DNA or specific controls with 4 µL of each master mix solution. In total each sample and control are tested with 8 triplex- or duplex-master mixes, as previously described in **Table 6-10**.

qPCR amplification is performed on a LightCycler 480 instrument (Roche) as illustrated in **Table 6-11**, which allows the use of ultra-low reaction volumes due to its remarkable design.

Table 6-11 qPCR RIATOL multiplex thermocycler program

Program name	Cycles	Target (°C)	Acquisition Mode	Hold (mm:ss)	Ramp (°C/s)
Pre-incubation	1	95	None	10:00	4.80
Temp adjustment	1	60	None	02:00	2.50
Amplification	45	95	None	00:10	4.80
		60	Single	00:30	2.50
Cooling	1	40	None	00:10	2.50

Total approximate duration per run: 1h 10 min.

Interpretation of results

At the end of an amplification run, the qPCR run file is automatically exported to the FastFinder (UgenTec) online-platform for analysis, review and authorization of results by trained technicians. A core-script is initially developed through an AI-based results-driven fine-tuning of analytical Cq-cutoffs, baseline corrections, and cross- and/or prevalence-driven contamination detection. Predetermined instrument- and target-specific standard curves are employed by the software to convert Cq values into DNA and viral concentration units, expressed in ng/µL and copies/µL respectively. Furthermore, the platform also features a quality control module allowing instant awareness of failed checks making immediate technical counteraction possible.

To achieve a valid result, (1) the negative control of each run must be negative for all targets; (2) every target-specific positive control must be individually detected and may not deviate more than 3-SD from the average Cq value determined; and (3) the internal cellular control gene (β-globin) must be detected at a concentration of at least 0.12 ng/μL per sample. In addition, all decision-making actions are audit-trailed by the software, making the FastFinder semi-automatic analysis algorithm a tremendous addition to the RIATOL qPCR, comprising an all-in-one highly accurate clinical grade platform for high throughput HPV quantification.

Quality control procedures

The quality control procedures are described in **Table 6-12**

Table 6-12 Control procedures and troubleshooting

Control procedure	Protocol	Troubleshooting
New lot quality control	Perform a test run by combining the new lot of a reagent with existing lots of known quality. Test the gBlock gene fragments serial dilutions in quadruplicate and ensure that the resulting Cq values do not deviate more than 3-SD from the average Cq value.	Repeat the experiment after ensuring the stability of the gBlock dilutions. If the issues persist, the lot is unsuitable for routine use and a new lot should be obtained.
Positive controls	Add controls (gBlock gene fragments) for each HPV type and β-globin gene to each 384-well PCR plate. FastFinder module monitors positive controls, which must not deviate > 3-SD from average Cq. Boundaries allow 5% CV and are instrument specific. Average Cq recalculated every six months or with new positive control lot.	If a specific positive control is not detected but a positive sample for that HPV type is present, the run is valid for further analysis. If there are no positive controls or positive samples, the run is invalid and troubleshooting is necessary, such as preparing new master mix, positive controls, or checking instrument functionality.
Negative control	In each sample well plate, add 2 ml of cellular material-free ThinPrep solution randomly allocated. The FastFinder quality control module continuously monitors the negative control. The negative control must test negative for all detected HPV types and β-globin.	If any targets are detected consecutively, thoroughly decontaminate the entire workflow. Decontaminate all the robots surface, detectors, pipette arms, waste bin, and barcode scanner using bleach, de-mineralized water, and ethanol.
Invalids	DNA concentration of a valid sample must be ≥ 0.12 ng/μL.	Invalid samples should be retested once. If a second test also yields an invalid result, the sample is considered inadequate, and a new sample request should be made. The weekly ratio of invalid samples must be below 2% of the total processed samples.

Contamination control	Once a month, run a water plate with the complete workflow. All plate positions must test negative for all detected HPV types and β-globin.	If any targets are detected, perform a thorough decontamination of the complete workflow. Decontaminate all the robots surface, detectors, pipette arms, waste bin, and barcode scanner using bleach, de-mineralized water, and ethanol. Repeat the contamination control procedure.
Performance control	Once a week for ten weeks, retest known positive samples in the complete workflow. There should be no qualitatively discrepant results for specific HPV types, and the CV on Cq-level should not exceed 15%. This process is continuously repeated.	If qualitatively discrepant results are observed or the CV is >15%, a thorough root cause analysis must be performed.