Amplification of Human papillomavirus genome using multiply primed Rolling-Circle Amplification

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Abbreviations

bp base pairs
BSA Bovine serum albumin
CIN Cervical Intraepithelial Neoplasia
DNA Deoxyribonucleic acid
dNTPs Deoxyribonucleoside thiophosphate
E1-E7 Early genes
EGF Epithelial growth factor
HIV Human immunodeficiency virus
HMBS Homo sapiens hydroxymethylbilane synthase
HPV Human papillomavirus
kb kilobase pairs
L1/L2 Late genes
LCR Long control region
M DNA Molecular size marker
nt nucleotide
PAP Papanicolaou
PCR Polymerase chain reaction
PV Papillomavirus
RCA Rolling-circle amplification
ssDNA single-stranded DNA
STD Sexually transmitted disease
Abstract

It is well established that cervical cancer is caused by infection by human papillomavirus (HPV). It is of great interest to amplify these HPV genomes for further analysis, for instance to identify new types of HPV in infected patient material. For amplification, many recent studies have used a technique employed by an amplification kit called TempliPhi100, where multiply primed RCA (RCA) is performed using random primers. One problem due to the random, unspecific primers ability to bind to DNA of different origins is amplification of background human genomic DNA when analysing patient material. Therefore, in this study we have designed specific primers for conserved regions of the HPV genome in order to reduce the background. We then evaluated how the multiply primed RCA was affected when using random primers compared to our specific HPV primers with a quantitative real-time PCR assay (*hpVIR*) for detection and quantification of high-risk HPV types. The findings in this study clearly showed an increased amplification efficiency using specific HPV primers compared to random primers when using a HPV plasmid template with copy numbers as low as 2000. Moreover, we were able to reduce the background generated by human genomic DNA when amplification was performed with our specific HPV primers. Our findings shows that multiply primed RCA performed better from HPV infected patient samples when using the HPV-specific primers.
1. Introduction

1.1 Cervical cancer

It is now well established that persistent infection with human papillomavirus (HPV) is a major risk factor for the development of cervical cancer in women. This virus is present in almost 99% of all cervical cancer cases all over the world[1]. Globally, an estimated incidence of 493,000 cases and 274,000 deaths were reported in year 2002. Of these cervical cancer cases, approximately 80% occur in developing countries[2]. The infection, caused by certain HPV types, is a sexually transmitted disease (STD) and probably the most prevalent one in the world[3]. The prevalence of HPV among women worldwide has been predicted to be 10% with the highest rate seen among young sexually active women[3]. Interestingly, it is shown that 70% of the affected women will clear the infection within one year[4]. If the infection persists for more than 2 years, the probability to resolve the infection is reduced leading to an increased risk of developing cervical cancer. Furthermore, there is an increased risk in relation to number of sex partners and several other co-factors have been established to be involved in the development of cervical cancer.

The virus infects strictly epithelial cells of the genital female tract (cervix uterine) which leads to the development of cervical intraepithelial neoplasia (CIN) and cancer. CIN is a noninvasive premalignant lesion that occurs during persistent infections and is classified based on the severity of the lesion. CIN I corresponds to a low-grade lesion (mild dysplasia) where 1% of the cases progress to invasive cervical cancer. High-grade lesions include CIN II (moderate dysplasia) and CIN III (severe dysplasia). The likelihood of progression to invasive cervical cancer for CIN II and CIN III is 5% and 15%, respectively[5].

1.2 Cervical cancer screening

Cellular changes are detected by Papanicolaous-staining of cervical smears (Pap-smear), which is used in the gynecological screening program of women. For this, the cells of the cervix are collected with a cytobrush and then spread on a glass slide. Subsequently, the cells are stained and examined by microscopy to detect abnormalities and lesions.

However, this cytological examination has been criticized due to its high false negative rate and low sensitivity. Many women do not show any cytological abnormalities even though they are infected by HPV. Furthermore, menopausal women with detectable abnormalities do not necessarily need to be HPV infected and can in fact be due to hormonal reasons[6]. Therefore, have HPV-testing with the higher specificity been introduced into the screening program at Uppsala Academic Hospital as a complement to cytology. The method used is based on a real-time PCR method named hpVIR[7, 8].

As mentioned, the frequency of cervical cancer is high in developing countries such as Africa and part of Asia. This is partly due to a poor, or lack of, screening program but also due to a high incidence of human immunodeficiency virus (HIV) infected women. HIV infection causes a weakness of the immune system which facilitates the acquirement of HPV infections[9]. These women can therefore carry several different HPV types, many of which have yet not been identified, consequently making this an interesting field for research.

1.3 Human papillomavirus

The papillomavirus (PV), belonging to the Papillomaviridae family, are species-specific and have been found both in humans and animals. The first PV was detected in 1933, when it was shown to cause warts in rabbits. Furthermore, they observed that this virus could induce the formation of cancer. It was then, in 1970, that HPV was established to be involved in the development of cervical cancer and today, HPV is present in almost 99% of all cases[10]. HPV is a large group, involving over 200 different types and are very tissue-specific which causes different symptoms. Some types infect cutaneous tissue while others cause persistent infection in mucosal tissue that can lead to development of cervical cancer. Nevertheless, most infections caused by HPV are in fact asymptomatic. The cutaneous HPV types infect epithelial cells of the skin which consecutively leads to the development of warts. This is most commonly caused by HPV type 1-4 infections of keratine-rich surfaces leading to a benign proliferation of skin cells of hands and feet[3]. Furthermore, the mucosal HPV types are classified into a high-risk group including HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82, and a low-risk group including HPV types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72 and 80. The high-risk types are strongly associated with the development of cervical cancer which in turn is confirmed by the fact that HPV16 and HPV18 are found in over 70% of all cases worldwide[11]. Infections with the low-risk type are normally not involved in the development of the disease and instead associated with benign mucosal cell proliferation. These benign abnormalities are seen as angiogenital warts (condyloma acuminatum) and laryngeal papillomas, commonly related to HPV type 6 and type 11 infections[3].

Moreover, the PV types are divided into several genus groups. The mucosal HPV types infecting the genital tract (high-risk and low-risk) are grouped in the genus alpha-papillomavirus. Furthermore, the cutaneous HPV types are grouped in the alpha, beta, gamma, mu and nu genus. To decide how the HPV types are grouped and especially, to
determine a new type, the degree of the sequence homology is analyzed for the L1 gene. If the gene between two types differs more than 10%, they are considered as two separate types whereas types varying between 2-10% are considered to be subtypes and finally, a HPV-variant is determined if the difference is considered to be less than 2%.

1.3.1 Structure and replication of HPV

The papillomavirus is a small DNA virus of approximately 50 to 55 nm in diameter. It has no envelope and its circular genome of double stranded DNA is protected by an icosahedral capsid. The icosahedron is composed of two structural proteins encoded by the late L1 and L2 genes. The genome has approximately 8000 base pairs and encodes in addition to the structural genes, seven or eight early genes depending on virus type. All the genes are situated on one strand of the double stranded genome. The long control region (LCR) is a regulatory region for the replication and transcription of the viral genome. Primarily, the early genes are transcribed by using the host cell polymerase. In order to do so, the viral promoter must be similar to the host cells which allow the binding of host cell transcription factors and the polymerase. E1 and E2 are transcribed in the host cell nucleus subsequently leading to the initiation of the replication. In detail, E1-protein is bound to LCR trough a linkage made of the E2-protein. E1 has a DNA helicase activity which allows the separation of the DNA strands starting from the origin of replication located in LCR. Subsequently, the genome is replicated by using the host DNA-polymerase[3].

The oncogenical transformation of host cells is achieved by the transcription of viral oncogenes E5, E6 and E7. E5 encodes a membrane protein that binds to EGF-receptor (epidermal growth factor) subsequently activating a mitotic signal which leads to cell proliferation but also inhibiting apoptosis. Furthermore, the E7-protein has the ability to interact with pRB (phosphorylated Retinoblastoma) hence down-regulating its tumor suppressing activity, which leads to proliferation of host cells. Finally, the protein encoded by E6 degradates p53 (a tumor suppressor protein) which results in induced DNA synthesis but also activation of the telomerase activity for elongation of telomeres. Following an increased replication of the viral genome, expression of the late genes L1 and L2 is activated. These genes encode for structural protein needed for the assembly of the capsid for release of the virus particle. During this process of cell proliferation, the virus is translocated upwards from the primary infected basal cells to the outer layer of the squamous epithelia (stratum corneum), where the cells are terminally differentiated and the virus particles released (Figure 1.)[3].

![Figure 2. Genome of human papillomavirus.](image)

The virus has a double-stranded circular DNA genome of approximately 8000 base pairs composed of seven or eight early genes (E) depending on virus type, and two late genes (L).
1.4 Multiply primed Rolling-Circle Amplification

For the analysis of clinical samples, several techniques have been developed to be used for whole genome amplification of microorganisms. These techniques are also used for phylogenetics and evolutionary studies of microbes such as HPV[12]. The most common method used for amplification is the polymerase chain reaction (PCR). This frequently used technique is dependent on prior sequence knowledge in order to determine the specific primers. Furthermore, the generated products are relatively short which creates difficulties in amplifying whole genomes. However, PCR has been used for detection and amplification of HPV in clinical samples. For this, degenerated specific primers are used targeting regions conserved in several HPV types. However, as mentioned, amplified products are short, about 200 base pairs[13].

In order to be able to study longer DNA sequences, a new technique for amplification of entire circular genomes has been introduced. This method, known as rolling circle amplification (RCA), is primarily associated with the discovery of new virus types. RCA has been developed by imitating a natural replication process of the genome seen in viruses and plasmids, known as rolling-circle replication. Unlike PCR, this amplification is isothermal, i.e. no temperature cycling is needed, and the amplification is carried out at a single temperature of 30 ºC[12]. For RCA, an enzyme known as phi29 DNA polymerase is used which is an essential enzyme for rolling-circle amplification. This enzyme posses several unique features such as a synthetic 5´-to-3´ polymerization reaction and a degradative 3´-to-5´ ssDNA exonuclease reaction. Most importantly, this enzyme has a strand displacement activity that allows the complementary strand to be displaced during replication[14]. In general, the multiply primed RCA is initiated by the annealing of primers to a single-stranded circularized genome creating several ‘start points’ for replication (Figure 3a). These primers, composed of both forward and reverse (primer pairs), are protected from the exonuclease activity of the enzyme as it degrades the 3´-end of single-stranded DNA. This is done by adding a thiophosphate modification between the last two nucleotides on the 3´-end of the primer[15]. Thereafter, phi29 DNA polymerase starts to synthesize the complementary strand in a 3´-to-5´ direction (Figure 3b). When the enzyme reaches the 5´-end of a primer located further downstream on the genome, the strand displacement activity takes place and the elongated primer is displaced (Figure 3c). The displacement of the complementary strand thus generates a new template for the annealing of new primers hence a new elongation step. The enzyme continues with the synthesis for several rounds elongating the template continuously due to the displacement. Finally, a RCA product of concatemeric, high-molecular weight, linearized double stranded DNA is obtained (Figure 3d).

![Figure 3. Illustration of the multiply primed RCA.](image-url)
The multiply primed RCA is a very efficient reaction due to the properties of the phi29 DNA polymerase. In addition to the already mentioned features, this enzyme is highly processive and studies have shown that it is capable of adding over 70,000 bases per binding event to the template. Furthermore, they showed that the enzyme is very stable and do not disassociate from the template throughout elongation, hence leading to enormous amount of product in during a long reaction time[14].

Currently, this method for multiply primed RCA can be supplied as an amplification kit named TempliPhi100. This simple kit contains three reaction tubes and one positive control, and is easily applied according to accompanied instructions (GE Healthcare, Illustra TempliPhi Amplification Kit). It is noteworthy, that the primers used in this kit are random hexamers (6 nucleotides in length) and present as a set of 4096 (4^6) primers[16]. These primers bind to matching position in the circularized genome generating multiple start points for replication. Consequently, the use of random primers avoids the need to have prior knowledge of the sequence. Due to this, multiply primed RCA using random primers has been extensively used in the discovering of new virus types[17]. For instance, several new papillomavirus types (13 novel types) have been identified based on this technique[12, 18]. Rector et al. was able to successfully perform amplification with TempliPhi100 on biopsies from a porcupine, and subsequently digest the HPV DNA with a restriction enzyme for visualization on a gel. Furthermore, the obtained product was sequenced and classified as a new papillomavirus (EdPV-1)[19]. However, Rector et al. optimized the TempliPhi100 method by adding extra dNTPs during amplification of HPV. The multiply primed RCA proceeds rapidly until the amount of dNTPs is depleted. This is important to consider when amplifying large genomes such as the HPV genome. Rector et al. observed that when adding 450 µM extra dNTPs, a significant increase of amplification efficiency was obtained as compared to amplification performed according to manufactures instructions[20].

As described earlier, HIV positive women are found to carry multiple HPV infections, of which many have yet to be identified. Therefore, the use of random primers during amplification of patient materials with TempliPhi100 can be of great value. However, patient materials contain both viral genome and human genomic DNA. This clearly raises one problem due to the ability of random, unspecific, primers to bind to DNA of different origins hence generating a background of amplified human genomic DNA. Consequently, it is of great interest to reduce this background when using HPV infected patient material.

### 1.5 Real-Time Quantitative Polymerase Chain Reaction

This quantitative PCR, hpVIR, has since 2007 been used in clinical practise for the detection of individual or grouped high-risk HPV types in patient samples in Uppsala county. The technique is based on detecting a fluorescent signal obtained during amplification when using specific primers and fluorescent probes for the HPV types. The probe is designed to match the target DNA sequence located between the primers, and carries two fluorophores namely a reporter dye (R) and a quencher dye (Q). The reporter does not emit any light as long as it remains proximate to the quencher and instead, the quencher emits light of a specific wavelength (Figure 4a). However, when the reporter is separated from the quencher, as occurs during elongation of the primer, the emission is changed to a reporter specific wavelength. The emitted reporter light is recorded for each elongation which in turns generates a picture representing the accumulation of PCR products (Figure 4b). Based on these recordings, a cycle threshold (Ct) value is calculated after extrapolating the emitted reporter light. The Ct
representing the cycle the reporter light reaches above the threshold which reveals the initial amount of DNA in each sample.

For analysis of patient samples with *hpVIR*, four separate reactions are used. One reaction uses primers and probes specific for detection of human genomic DNA. The other three reactions detect high-risk HPV types. For instance, one reaction uses primers and probes for detection of HPV 33, 35, 39, 52 and 58\cite{7, 21}.

![Figure 4. Illustration of the formation of reporter light furthermore, how the recorded light is increased with time. (a) Schematic image of the reporter-light formation occurring when the reporter dye (R) is separated from the quencher (Q) during elongation. (b) Illustration of the increased reporter-light, accumulated for each cycle. The Ct-value corresponds to what cycle the reporter-light intensity reaches a threshold value, subsequently used for determination of the initial amount of DNA in the sample.](image)

1.6 Aim

The aim of this study was to apply multiply primed RCA for amplification of human papillomavirus genome. This was achieved by using TempliPhi100 kit, where multiply primed RCA was performed with random hexamers primers. Moreover, a new method was developed by designing a set of specific primers for HPV genome, subsequently used for multiply primed RCA. Furthermore, we wanted to compare the performance of using specific HPV primers against random primers during amplification of HPV in the presence of human genomic DNA. To evaluate how the amplification efficiency was influenced when using these two types of primers, samples were analyzed by *hpVIR*, a Quantitative Real-Time PCR assay.
2. Materials and methods

2.1 DNA samples and patient material

As a source of HPV DNA, a plasmid with a complete integrated HPV35 genome was used. The plasmid, pT713delta, contains an insert of approximately 7851 bp and holds a total size of 9212 bp.

DNA samples from patients infected with HPV were kindly provided from a study by Gustavsson et al where DNA was extracted from cervical cells attached to the sampling brush (cytobrush). These samples were detected by Gustavsson et al. to contain single infections of HPV16. HPV16 has a total size of approximately 7904 bp.

Table 1. Characteristics of samples from patients included in the study.

<table>
<thead>
<tr>
<th>Sample</th>
<th>HPV type</th>
<th>Number of HPV copies (1)</th>
<th>Number of HMBS copies (2)</th>
<th>Titer (3)</th>
<th>Origin of DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>62377</td>
<td>4998</td>
<td>12.480</td>
<td>Cervical cells on brush</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>43149</td>
<td>43243</td>
<td>0.998</td>
<td>Cervical cells on brush</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>262</td>
<td>36082</td>
<td>0.007</td>
<td>Cervical cells on brush</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>18166</td>
<td>26868</td>
<td>0.676</td>
<td>Cervical cells on brush</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>361</td>
<td>13342</td>
<td>0.027</td>
<td>Cervical cells on brush</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>441300</td>
<td>11251</td>
<td>39.223</td>
<td>Cervical cells on brush</td>
</tr>
<tr>
<td>7</td>
<td>16</td>
<td>117413</td>
<td>20112</td>
<td>5.838</td>
<td>Cervical cells on brush</td>
</tr>
</tbody>
</table>

(1) Number of HPV16 copies in each sample was quantified by hpVIR.
(2) Homo sapiens hydroxymethylbilane synthase (HMBS), a human single copy gene. Also quantified by hpVIR.
(3) Number of HPV16 copies normalized with respect to the amount of human DNA. This was calculated by dividing number of HPV16 copies with number of HMBS copies.

2.2 Dilution of HPV35 plasmid

The DNA-concentration of the HPV35 plasmid was determined by using spectrophotometry with a NanoDrop® ND-1000 (NanoDop Technologies). A series of dilutions were performed to generate a decreasing amount of plasmid DNA to be used in the experiment. The total concentration of the original HPV35 plasmid sample was calculated to 205 ng/μl. Two replicates per sample were measured. A dilution series was performed with following concentrations 100 ng/μl, 50 ng/μl, 25 ng/μl, 12.5 ng/μl and 6.3 ng/μl.

Furthermore, a dilution series of HPV35 plasmid sample was prepared based on copy numbers ranging from 2x10^9 copies/μl to 2x10^4 copies/μl.
2.3 Multiply primed RCA performed with TempliPhi100

TempliPhi100 amplification kit was supplied by GE Healthcare (Amersham Bioscience, UK), and used according to manufactures recommendation. Using a twofold amount of reagent for each sample, 2 µl plasmid DNA was mixed with 10 µl Sample buffer (containing random primers) and denatured at 95 °C, according to Table 2, subsequently cooled until further use. 1 µl of a positive control, pUC 19, was also denatured and used as a quality control for this reaction. A mastermix was thereafter prepared for each sample containing 10 µl Reaction buffer (containing dNTPs and salts) and 0.4 µl Enzyme mix (containing the enzyme and random primers in glycerol). Additionally, a modified protocol of the TempliPhi100 was used in some experiments by adding 450 µM extra dNTPs to the mastermix. Finally, the cooled denatured sample was added to the mastermix and incubated over night in 30 °C at 4h and/or 18h. Afterwards, the enzyme was inactivated at 65 °C for 10 min, and subsequently incubated at 4 °C until further use according to Table 2. Furthermore, for analysis by hpVIR[21], RCA with TempliPhi100 was performed by mixing 2 µl plasmid DNA, 1 µl of human genomic DNA (10 ng/µl) and 5 µl Sample buffer for each reaction. After denaturation of DNA, 5µl of the mastermix containing 5 µl Reaction buffer and 0.2 µl Enzyme mix, was added to the denatured sample and amplified as described earlier.

Table 2. Isothermal DNA amplification-program used for RCA reactions:

<table>
<thead>
<tr>
<th>Step</th>
<th>T (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>3 min</td>
</tr>
<tr>
<td>Cooling</td>
<td>4</td>
<td>∞</td>
</tr>
<tr>
<td>Amplification</td>
<td>30</td>
<td>4 h / 18h</td>
</tr>
<tr>
<td>Enzyme inactivation</td>
<td>65</td>
<td>10 min</td>
</tr>
<tr>
<td>Cooling</td>
<td>4</td>
<td>∞</td>
</tr>
</tbody>
</table>

2.4 Multiply primed RCA performed with HPV specific primers

2.4.1 Designing new primers

A set of specific primers was designed based on alignments of several HPV genomes, mainly high-risk types found in a HPV sequence database (http://hpv-web.lanl.gov/, version 1997). By analyzing partly conserved regions, primers (both forward and reverse) of 10 to 12 nucleotides were designed, located across the entire HPV genome. Although the L1 gene is the most conserved gene, partly conserved regions were found through out the entire genome and both consensus and degenerated primers were designed. The primers was also protected by a thiophosphate modification at the 3’end (between the last two nucleotides, NNNNNNNNN*N*N) to increase the stability since the phi29 DNA polymerase digest single stranded DNA at the 3´end (3´-to-5´ exonucleolytic activity).
### Table 3. Designed specific primers for HPV genome.

<table>
<thead>
<tr>
<th>Primer name(^1)</th>
<th>Sequence(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E6 412F</td>
<td>5’-AATAAGGT<em>G</em>Y-3’</td>
</tr>
<tr>
<td>E7 644R</td>
<td>5’-AATTTGTC<em>Y</em>T-3’</td>
</tr>
<tr>
<td>E1 1257R</td>
<td>5’-GCCATATCCY<em>C</em>T-3’</td>
</tr>
<tr>
<td>E1 1881F</td>
<td>5’-CCAGARTG<em>G</em>A-3’</td>
</tr>
<tr>
<td>E1 2085R</td>
<td>5’-TGTCCTYCAC<em>A</em>T-3’</td>
</tr>
<tr>
<td>E1 2329F</td>
<td>5’-TTKGAATG<em>A</em>G-3’</td>
</tr>
<tr>
<td>E1 2374F</td>
<td>5’-TATGTAAY<em>T</em>C-3’</td>
</tr>
<tr>
<td>E1 2484R</td>
<td>5’-GYTTTCCA<em>T</em>C-3’</td>
</tr>
<tr>
<td>E4 3356F</td>
<td>5’-TCTGTRTCY<em>A</em>G-3’</td>
</tr>
<tr>
<td>E4 3367R</td>
<td>5’-AGCAGTGGY<em>T</em>A-3’</td>
</tr>
<tr>
<td>E2 2773R</td>
<td>5’-GCATRTAAA<em>C</em>G-3’</td>
</tr>
<tr>
<td>E2 2933F</td>
<td>5’-CAGGTGTTG<em>C</em>G-3’</td>
</tr>
<tr>
<td>L2 4824F</td>
<td>5’-GAAGAAATW<em>C</em>C-3’</td>
</tr>
<tr>
<td>L2 4991R</td>
<td>5’-GCAGGATTYT<em>C</em>A-3’</td>
</tr>
<tr>
<td>L2 5102R</td>
<td>5’-GCTGGCCTY<em>T</em>G-3’</td>
</tr>
<tr>
<td>L2 5617F</td>
<td>5’-CCGTAACG<em>T</em>G-3’</td>
</tr>
<tr>
<td>L2 5639F</td>
<td>5’-TTTGCAGA<em>T</em>G-3’</td>
</tr>
<tr>
<td>L1 6236F</td>
<td>5’-GATGGGTGAYA<em>T</em>G-3’</td>
</tr>
<tr>
<td>L1 6327R</td>
<td>5’-ATAATCWGGA<em>T</em>A-3’</td>
</tr>
<tr>
<td>L1 6364F</td>
<td>5’-GATCCATAT<em>G</em>G-3’</td>
</tr>
<tr>
<td>L1 6597R</td>
<td>5’-CAAATYCCA<em>T</em>T-3’</td>
</tr>
<tr>
<td>L1 6646F</td>
<td>5’-GTAGATACY<em>A</em>C-3’</td>
</tr>
<tr>
<td>L1 6763R</td>
<td>5’-CACAARTG<em>A</em>A-3’</td>
</tr>
</tbody>
</table>

\(^1\) Primers were named by the location on the gene, number of 3’ nucleotide of the HPV16 genome and position of the primer (F, forward; R, reverse).

\(^2\) Degenerated primers were used (Y = T or C; R = A or G; K = G or T; W = T or A)

#### 2.4.2 Performing multiply primed RCA with designed HPV primers

A primer mix was prepared by pooling 5 µl of each of the 23 primers to a final concentration of 100 µM. The denaturation of 2 µl HPV35 plasmid was performed in a mix of 1 µl phi29 DNA polymerase reaction buffer (10X), 1 µl primermix and 8 µl dH₂O. Samples were subsequently denatured as outlined in Table 2. For amplification of each sample, a mastermix of 1 µl phi29 DNA polymerase reaction buffer (10X), 0.2 µl bovine serum albumin (BSA, 1 ng/ µl), 1.5 µl dNTPs (100 mM), 1 µl primermix, 4.3 µl dH₂O and 2 µl phi29 DNA polymerase (10 U/ µl) was prepared and added to the denatured sample. Samples were incubated at 30 °C as outlined in Table 2. Furthermore, a new protocol was tested by adding 1 µl of human genomic DNA (10 ng/ µl) together with 2 µl plasmid DNA to mimic the complex DNA environment seen in samples from patients and submitted to RCA as performed earlier. In addition to this, a protocol where 3 ng/ µl of human genomic DNA added together with 2.5 µl plasmid DNA was also tested.
2.4.3 Using HPV infected DNA-samples from patients for multiply primed RCA with designed HPV primers

The new protocol using designed primers was furthermore tested on DNA samples from patients as outlined in Table 1. These samples were infected with HPV16 and DNA was extracted from cells collected by the cytobrush. For this protocol, 2 µl of DNA was used as template in the RCA as described previously.

2.5 Restriction enzyme analysis

BamH1 is known to cut HPV35 plasmid and HPV16 at a single site. For each reaction, a 20 µl digestion mix was prepared containing 2 µl enzyme (10U), 3 µl 10X enzyme buffer and dH2O, subsequently placed on ice until further use. Thereafter, 10 µl of the amplified RCA product was added to digest by incubation at 37 °C for 16 h. Furthermore, a modified protocol was tested by digesting 2 µl of non-amplified HPV35 plasmid with 1 µl BamH1 enzyme and 2 µl BamH1 buffer. The reactions were stopped after 2 hours in order to add 2 µl extra enzyme to the RCA products and 1 µl for the non-amplified plasmid. This was performed to ensure proper digestion. After additional 2 hours, the reactions were stopped and a fraction of the products was used for the electrophoresis. The remaining amount was digested over night and then stored at -20 °C for future use.

2.6 Gel electrophoresis

The digested products were analyzed by electrophoresis on a 0.8% agarose gel in 0.5X Tris-Borate-EDTA buffer (TBE). Of each digested sample, 2 µl was mixed with loading dye and dH2O to a final volume of 10 µl. For the non-amplified HPV35 plasmid, 4 µl of the digested sample was used. The agarose gel was run initially at 30 V to ensure that the products consisting of high molecular weight DNA would not remain in the well and separation was performed properly. After 30 min the voltage was increased to 120 V and samples were run for a further 1.5 hours. The separated products were visualized by ethidium bromide (EtBr, 0.01 µg/ml) and analyzed under UV-light and compared to a 1 kb molecular size ladder (GeneRuler, Fermentas)

2.7 Quantitative real-time polymerase chain reaction, hpVIR

To detect and quantify the RCA products, a real-time quantitative PCR assay named hpVIR [21] was used. This HPV typing assay detects 12 high-risk HPV types by using primers and fluorescently labelled probes specific for individual or groups of HPV types. The typing assay is composed of four reactions, in which three detects high-risk HPV types and one detects human genomic DNA (HMBS). Since the RCA products contained very high amounts of DNA, the samples had to be diluted to be applicable for quantification with the hpVIR system. In this study, RCA samples were diluted 100 times (data not shown). For each reaction 3 µl RCA product was added to a mastermix of 25 µl, containing 2 x Taqman universal mastermix, 3.12 ng bovine serum albumin (BSA), 5 µM of each primer and probe. The thermal cycling was performed as described in Table 4. Fold enrichment of HPV35 plasmid was calculated by dividing number of HPV35 plasmid copies after RCA by the number of HPV35 plasmid copies used as templates in the RCA. Furthermore, to determine the relative enrichment of HPV35 plasmid compared to human genomic DNA, the fold amplification of HPV35 plasmid was divided by the fold amplification of human genomic DNA.
Table 4. Real-time quantitative PCR program.

<table>
<thead>
<tr>
<th>Step</th>
<th>T (°C)</th>
<th>Time</th>
<th>Cycles$^{(1)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>10 min</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>15 sec</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>57</td>
<td>1 min</td>
<td>40</td>
</tr>
</tbody>
</table>

$^{(1)}$ A two-step cycle consisting of step 2 and 3.
3. Results

3.1 Rolling-circle amplification of HPV plasmid using TempliPhi100

HPV35 plasmid was amplified using TempliPhi100. However we also tested an alternative protocol by adding an additional amount of dNTPs (450 µM). For this reaction, varying concentrations of HPV35 plasmid were used as input material for the multiply primed RCA. Also a positive control, pUC19 with a total size of 2.7 kb, was amplified. The samples were incubated either during 4 hours or 18 hours and, subsequently submitted to a restriction enzyme digestion. A non-amplified HPV35 plasmid of 400 ng was also digested. The digested products were visualized on agarose gel to verify that the RCA was successful and a product of expected size was obtained (about 9.2 kb). Interestingly, samples processed using extra dNTPs generated more RCA products as seen on the agarose gel compared to samples with no extra dNTPs. This is clearly observed in Figure 5 (B), where samples were incubated a longer time. It is well known that phi29 DNA polymerase is a very stable enzyme, therefore enabling the generation of a high amount of amplification product with a longer incubation time. Furthermore, the results indicate that when using TempliPhi100 according to manufactures instructions, dNTPs are depleted during amplification probably due to the large HPV genome. However, when adding extra dNTPs, the amplification proceeds with sufficient dNTPs to generate high amount of RCA products.
3.2 Rolling-circle amplification of HPV plasmid using own design

HPV35 plasmid was amplified by RCA using the HPV-specific primers. For this, 2 µl of varying concentrations of HPV35 plasmid were used as template for the multiply primed RCA. Also, 2.5 µl of HPV35 plasmid, quantified based on copy numbers instead of concentrations, were used for the RCA. This was performed to make the reactions representative since viral load in HPV infected patient samples is determined based on numbers of HPV copies. The samples were incubated either for 4 or 18 hours. Thereafter, 10 µl of each amplified product was submitted to a restriction enzyme digestion with BamH1. 200 ng of a non-amplified HPV35 plasmid was also digested. The digested products were visualized on an agarose gel to verify that the RCA was successful and a product of expected size was obtained (about 9.2 kb). In order to determine the results of using HPV-specific primers, the same set of samples tested with TempliPhi100, were applied to our protocol using HPV-specific primers. The results showed an increase in band intensity using HPV-specific primers as compared to random primers suggesting a more efficient reaction (Figure 6).
To mimic the complex environment of both human and viral DNA present in patient samples, a new protocol was tested by addition of human genomic DNA (10 ng/μl) to each reaction. Interestingly, when adding human genomic DNA to the plasmid samples as previously used the intensity of the bands was reduced although still visible (Figure 7). This suggests that the multiply primed RCA using specific HPV primers is still efficient even though human genomic DNA is added as a competitive agent during the reaction.

**Figure 6. Agarose gel separation of RCA product of HPV35 plasmid using specific HPV primers.** RCA of HPV35 plasmid was performed with specific primers according to designed protocol. Amplification products were digested with BamH1 and visualized on 0.8% agarose gel and compared to a 1 kb molecular size ladder. Lane 1-4 represents reactions incubated at 30 °C for 4 hours. The concentrations of HPV35 plasmid used as template in the RCA are marked above corresponding reaction. Lane 5-8 consequently corresponds to same reactions as marked for lane 1-4 but incubated for 18 h. Here we clearly see how the reaction is enhanced if the incubation time is increased. Lane 11 shows 200 ng of a non-amplified HPV35 plasmid used for restriction enzyme digestion. ng, nanogram. Lane M, DNA molecular size marker.
Figure 7. Agarose gel separation of RCA product of HPV35 plasmid and human genomic DNA using specific HPV primers.
In order to mimic the complex environment seen in HPV infected samples from patients, 10 ng/µl of human genomic DNA was added to same samples shown in Figure 6. Lane 1-4 represents reactions submitted to multiply primed RCA for 4 hours. The concentrations of HPV35 plasmid used as template in the RCA are marked above corresponding reaction. Lane 5-8 consequently corresponds to same reactions as marked for lane 1-4 but incubated during 18 h. Here we noticed a minor reduction of band intensity but overall, bands were still visible for all reactions. ng, nanogram. Lane M, DNA molecular size marker.

Figure 8. Agarose gel separation of RCA product of HPV35 plasmid using specific HPV primers.
HPV35 plasmid was quantified based on copy numbers and a serie of samples with varying copies of HPV35 plasmid were used for RCA. Furthermore, 3 ng of human genomic DNA was added to each
sample. The copy number of HPV35 used in each RCA reaction is marked above corresponding sample. Incubation of samples occurred during 18 hours only. A distinct band could be visualised on agarose gel for RCA reactions even when using copy numbers of HPV35 plasmid as low as $5 \times 10^3$, as represented by lane 4. Lane M, DNA molecular size marker.

### 3.3 Rolling-circle amplification of HPV infected samples from patients using own design

To test if the designed protocol using specific primers was applicable to HPV infected patient samples, 2 µl of extracted DNA from HPV16 infected clinical samples were used as template for the multiply primed RCA. These samples were of varying HPV16 titer as shown in Table 1. RCA was performed as described earlier with 18 hours incubation only. The amplified products were visualized on an agarose gel and showed the expected size (about 7.9 kb).

![Figure 9. Agarose gel separation of RCA product from HPV16 positive patients using specific HPV primers.](image)

Samples from patient earlier typed positive for HPV16 were also successfully amplified using multiply primed RCA with specific primers. Nevertheless, samples with extremely low HPV16 titer did not generate any visual band on the agarose gel as seen in lane 3. Interestingly three bands could be observed as seen in lane 4, however no further analysis was made for this sample. Reactions were performed as previously described. Lane M, DNA molecular size marker.

### 3.4 Using Real-Time Quantitative polymerase chain reaction (hpVIR) for detection and quantification of RCA amplified HPV genome

To determine the amplification efficiency for TempliPhi100 and the new protocol using specific HPV primers, HPV quantities were analyzed by a real-time PCR assay, hpVIR,[21]. This was done by amplifying HPV35 plasmid of determined copy numbers, both with TempliPhi100 and the new primer design. To evaluate the specificity of the RCA reaction, 10 ng of human genomic DNA was added to each plasmid sample and 2 µl of was used as template and incubated at 18 hours. 3 µl of the RCA product was thereafter used as in the hpVIR assay. The first protocol used to determine the amplification efficiency was based on
RCA templates with low copy numbers of HPV35 plasmid, Figure 10 (in green). Since no significant amplification efficiency could be detected for these samples, with exception of 4000 copies, a new RCA was performed with higher copy numbers of HPV35 plasmid (Figure 11). RCA products amplified with specific HPV primers (in red) showed notably higher HPV35 amounts compared to those where random primers (in blue) were used, as seen in Figure 12. Furthermore, to evaluate how the reaction was altered in the presence of human genomic DNA, the relative enrichment of HPV35 plasmid compared to human genomic DNA was calculated (Figure 13). A very high increase of both fold enrichment and relative enrichment was observed in samples submitted to the new design based on specific HPV primers as compared to samples using the TempliPhi100 random primer protocol. A high relative enrichment of HPV35 plasmid suggests a more specific reaction, as can be seen when using the new design compared to TempliPhi100 (Figure 13).

**Figure 10.** RCA products obtained using TempliPhi100 and the new design.
Samples with copy numbers between 200-4000 of HPV35 plasmid were used as input for RCA. Thereafter, samples were submitted to hpVIR, to quantify the obtained RCA products. Green staples represents the amount HPV35 plasmid used as input in RCA. Red and blue staples represents obtained RCA products using new design respectively TempliPhi100. No major increase in numbers of HPV35 plasmid copies after RCA were detected except for sample containing 4000 copies as input for RCA.
Figure 11. RCA products obtained using TempliPhi100 and new design.
Samples with copy numbers between 2000-11000 of HPV35 plasmid were used as input for RCA. Thereafter, samples were submitted to hpVIR, to quantify obtained RCA product. Green staples represents amounts of HPV35 plasmid used as input in RCA. Red and blue staples represents obtained RCA products using new design respectively TempliPhi100. As seen for all samples, a greater quantity of RCA product was obtained when using new design with HPV specific primers.

Figure 12. Fold enrichment of HPV35 plasmid samples using multiply primed RCA.
Amplification efficiency was determined by dividing HPV35 plasmid copy numbers obtained after RCA with HPV35 plasmid copy numbers used as template for RCA. Red staples represents fold enrichment for samples submitted to new design using specific HPV primers. Blue staples represents fold enrichment using TempliPhi100. A significant increase in fold amplification was obtained when using specific HPV primers for all copy numbers of HPV35 used as input in the RCA.
Relative enrichment was determined by dividing fold amplification of HPV35 plasmid with fold amplification of human genomic DNA. Red staples represents relative enrichment of HPV35 plasmid using new design with HPV specific primers. Blue staples represents relative enrichment of HPV35 plasmid using TempliPhi100.
4. Discussion

4.1 Evaluating multiply primed RCA-products obtained with TempliPhi100 and the new design through electrophoresis

The focus of this project was to compare the result of using specific HPV primers with random primers for amplification performed by multiply primed RCA. TempliPhi100-kit was used for amplification of HPV genome using random primers. Furthermore, a new method was developed by designing specific primers for HPV subsequently used for amplification by multiply primed RCA.

Amplified products were digested with a single cutting enzyme of both HPV35 plasmid and HPV16 to generate a fragment of correct size, 9.2 kb and 7.9 kb respectively. Products were successfully visualized and analyzed on agarose gels. However, RCA-products are known to be of a complex nature i.e. concatemeric high molecular weight linear double stranded DNA, and extremely high quantities can be obtained. Interestingly, as seen on all agarose gel pictures (Figures 5-9), a fraction of the product amplified by multiply primed RCA remained in the wells. This was not observed for non-amplified samples of HPV35 plasmid (Figure 5 B, 6 and 7). A possible explanation for this is that the digestion requires more restriction enzyme due to the complex nature and great quantities obtained after amplification.

Amplification with TempliPhi100 according to manufactures instruction was successful either when incubating for 4 hours or 18 hours. However, a longer incubation time generated a high amount of RCA product represented by thicker bands (Figure 5 B). This result has previously been shown by Rector et al. and furthermore, they tested an additional reaction by adding extra dNTPs (450 µM). In my study, addition of extra dNTPs (450 µM) to the RCA was also tested and as seen in Figure 5, the RCA was enhanced.

However, in this study we wanted to compare the significance of using random primers with TempliPhi100 according to manufactures instructions, against the designed specific HPV primers. Consequently, the same amount of input for amplification performed with TempliPhi100 was used for amplification with new design (Figure 6). The result shows an increase in band intensity when using specific HPV primers rather than random primers suggesting a more efficient multiply primed RCA.

As previously mentioned, a problem that occurs when using random primers for analysing patient material is the generating background of amplified human genomic DNA. This occurs due to the ability of the unspecific random primers to bind to DNA of different origins, both viral and human. To mimic this complex environment of both human and viral DNA observed in patient materials, human genomic DNA was added to each samples containing HPV35 plasmid subsequently amplified with new design. Interestingly, the band intensity and band thickness was slightly diminished for all samples. The UV-exposure in Figure 7 was shorter compared to agarose gel picture seen in Figure 6, resulting in reduced band intensity although this does not reflect the amount of product on gel. However, a distinct band of expected size was still visualized in all samples submitted to multiply primed RCA using specific HPV primers. These results suggest that the amplification of HPV35 plasmid is still effective although performed in the presence of human genomic DNA.

The sample concentration was determined by spectrophotometry (ng/ reaction) and the copy numbers of HPV plasmid was thereafter calculated to be able to determine the copy number
interval still visible on an agarose gel. A distinct band could be observed when using as low as 5x10³ HPV plasmid copies for amplification even though human genomic DNA of 900 copies (3 ng/µl) was added to the reaction (Figure 8). Subsequently, HPV16 positive samples from patients were submitted to the RCA using specific HPV primers (Figure 9). These samples were of varying viral titers, i.e. a high virus titer corresponding to high quantities of HPV16 and low quantities of human genomic DNA, in contrast to low virus titre corresponding to low quantities of HPV16 and high quantities of human genomic DNA. Here we observed a successful amplification and fragments of expected size were visualized for all samples, except sample no 3 (Figure 9) which contained the lowest virus titre.

4.2 Using hpVIR to compare the amplification when using specific HPV primers and random primers (TempliPhi100) during multiply primed RCA

Fold enrichment of HPV35 plasmid was drastically increased for all samples amplified with specific primers compared to amplification with TempliPhi100. Amplification enrichment was as high as 1 x 10⁶ fold using specific HPV primers compared to 10.9 fold using random primers with TempliPhi100 (Figure 12). It is important to consider that for samples containing very low numbers of HPV neither using the new design or TempliPhi100 will yield a sufficient amplification with RCA. This seems to apply for copy numbers ranging up to around 2000 (Figure 10). Interestingly, when using samples ranging from 2000-4000 copies and higher, a drastic increase of HPV copies obtained after RCA was only detected in samples submitted to the new design with specific HPV primers (Figure 11). Since no samples above 11 000 copies of HPV35 plasmid were submitted to RCA, there is a possibility that same drastic increase in amplification can be seen for TempliPhi100 when using samples of higher copy numbers than demonstrated in this study. Previously, it has been shown that an increase of amplification efficiency of 172 fold can be obtained when samples of 60 000 HPV16 copies are amplified with TempliPhi100[20]. RCA is an isothermal reaction, where samples are incubated at 30 ºC. This rather low temperature increases the risk of mismatches during the amplification hence reducing the specificity of the primers, both random primer and specific primer for HPV. Nevertheless, our specific primers are longer, about 10-12 nucleotides, compared to primers used in TempliPhi100 of 6 nucleotides, which increases the specificity during amplification.

Moreover, it is important to evaluate how human genomic DNA affects the multiply primed RCA of HPV using specific primers and random primers. This is of great importance since amplification of human genomic DNA generates a background in addition to amplified HPV DNA. Therefore, the relative enrichment of HPV35 plasmid compared to human genomic DNA was determined both for the new design and TempliPhi100. Here we observed a significantly higher degree of relative enrichment of HPV35 plasmid for all samples amplified using specific HPV primers (Figure 13). The relative enrichment of HPV35 plasmid was as high as 4 x 10⁴ when using specific HPV primers, but only a 7.5 fold was obtained when amplifying same sample with TempliPhi100. Consequently, the background generated by human genomic DNA was significantly reduced by using specific HPV primers rather than using random primers.

Furthermore, a set of HPV positive samples from patients amplified with the new design using specific HPV primers, were also analyzed and HPV typed with the hpVIR assay (data not shown). Here we could successfully identify correct HPV type for corresponding sample. Furthermore, quantification of HPV amounts could not be performed correctly since the majority of the amplified samples contained an overload of RCA product not applicable to the
hpVIR assay. Interestingly we observed that samples not visible on agarose gel were successfully quantified by hpVIR, in contrast to samples that were visible on agarose gel that contained an overload of RCA-product. However, hpVIR is a useful technique for detection and quantification of RCA-products even though present in a complex formation.

In summary, our results show that multiply primed RCA using specific primers is a suitable technique for amplifying HPV genome. Furthermore, the background generated by human genomic DNA is significantly reduced which makes this new design a prominent method for amplifying HPV infected patient samples.

4.3 Future perspective

It is established that several subtypes of HPV16 exist[22]. Furthermore, it has been observed that some HPV16 variants are more prone to develop cervical cancer during infection compared to other HPV16 variants[23]. This can either be explained immunologically, where the immune system of the patient can eliminate the virus, or that some HPV16 variants are less cancerogenic. Therefore, it is of great interest to compare women infected with HPV16, which developed cancer with women who cleared their infections. This can easily be tested using multiply primed RCA with our designed HPV primers. Subsequently, RCA products can be sequenced to determine the different HPV16 variants.

As mentioned, multiply primed RCA using specific primers for HPV, is a promising method for detection of new HPV types. However, further investigation is required when RCA-products are submitted to restriction enzyme digestion for analysis on agarose gel. Since there is no prior knowledge about the sequence, difficulties may occur in choosing a proper single-cutting enzyme for the reaction occur. Therefore, a study is being performed where all single cutting enzymes for all HPV-species groups included in the alpha genus are being mapped. By doing so, possible restriction enzymes specific for each species group can be detected and hopefully, undiscovered HPV types belonging to one group can be submitted to these restriction enzymes. Furthermore, samples can be submitted to restriction enzyme digestion prior amplification. RCA requires circular DNA, so by linearizing known HPV types only unknown HPV types can be amplified since they remain circularized. Nevertheless, there is always a risk that the unknown type happens to carry the same restriction site and will also be linearized before submitted to RCA. After amplification, samples of unknown HPV types must be sequenced. As previously mentioned, RCA products (if not digested) are of a complex nature and in addition, several RCA products for several HPV types might be present in the sample. Sequencing of this type of material is complex, hence further investigation and optimization is needed for this step.
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6. References


