Molecular beacon based real-time PCR method for detection of 15 high risk and 5 low risk HPV types

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Abstract: Given the complexity of multiple infections, viral persistence, vaccination and management of therapeutic options, the accurate detection of Human Papillomaviruses is essential in cervical cancer patient management, follow-up and screening. We designed a molecular beacon based one step multiplex real time PCR reaction (MB-RT PCR) to detect 15 high risk and 5 low risk HPV types. The optimized PCR reaction mixture contains 16 forward, 16 reverse primers and 20 type specific molecular beacon (MB) probes targeted to a special sequence of the L1 gene. MBs detecting high risk types are 5'-FAM-3'-DABCYL-labelled, MBs for low risk detection are 5'-TET-3'-DABCYL-labelled, while the internal control is detected by a 5'-FAM-TexasRed-3'-DABCYL wavelength shifting MB. Accordingly, fluorescent data for HPV detection are collected at 530nm for high risk types, 560nm in case of low risk types and the reaction internal control is detected at 610nm on a Roche Lightcycler 2.0 instrument. Analytical and clinical assessment of the system is presented in this work. Specificity analysis detected cross-reactions with non-genital HPV types and with less frequent genital types. The sensitivity for detected types varies between 22-700 copies/reaction. The clinical performance was tested on 161 clinical sample DNAs. The MB-RT PCR results were compared to the typing results obtained by L1F/L1R PCR and hybridization based system we previously developed. The MB-RT PCR system identified HPV DNA with a detection rate of 93.16%. The MB-RT PCR system did not detect the HPV DNA in 11 positive cases, most of these presenting infections with low copy numbers, as hybridization showed. The system detected 5 cross-reactions, which were known from the previous analytical assessment. We propose the MB-RT PCR system as a diagnostic tool since it proved to be a reliable, efficient, and time-saving approach.

Introduction

Persistent infections with HPV can lead to development of malignant lesions, the direct link between HPVs and cervical cancer being well known. Genital HPV types are classified into low-risk and high-risk classes. The accurate HPV testing became a necessity, several studies showing its growing importance in addition to cytology.

We developed a molecular beacon based one step multiplex real-time PCR system which detects 15 high risk (HPV types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) and 5 low risk HPV types (HPV types 6, 11, 42, 43, 44). The optimized PCR reaction mixture contains 16 forward, 16 reverse primers and 20 type specific molecular beacon molecular beacon probes targeted to a special sequence of the L1 gene, where a highly variable sequence is flanked by two conserved sequences, the region targeted by our previous development, the L1F/L1R PCR based system (Jeney et al, J Virol Methods 2007). The first hypervariable region of the L1 ORF was targeted by both systems. The shorter RT amplicon was located within the longer L1F/L1R amplicon. In case of the HPV16 L1 ORF (5526 to 7154 bp.) the L1F/L1R amplicon is positioned from 5609 to 5861 bp., while the RT amplicon from 5724 to 5861 bp. Both systems use the same reverse primer set. The forward primers of the RT system are targeting the same sequences where the L1F/L1R general hybridization probes were designed (Figure 1).

Molecular beacons detecting high risk types are 5'-FAM-3'-DABCYL-labelled, molecular beacons for low risk detection are 5'-TET-3'-DABCYL-labelled, while the internal control added before sample DNA extraction is detected by a 5'-FAM-TexasRed-3'-DABCYL wavelength shifting molecular beacon (Table 1). Accordingly, fluorescent data for HPV detection are collected at 530nm for high risk types, 560nm in case of low risk types and the reaction internal control is detected at 610nm on a Roche LightCycler 2.0 instrument.

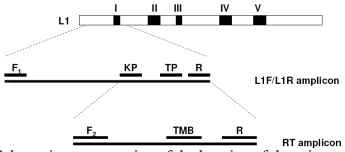

Figure 1. Schematic representation of the location of the primers and probes in the L1F/L1R and the molecular beacon based real-time PCR systems. F1- L1F/L1R forward primers, KP- general hybridization probes, TP- type specific hybridization probes, R- common reverse primers, F2- RT forward primers, TMB- type specific molecular beacons.

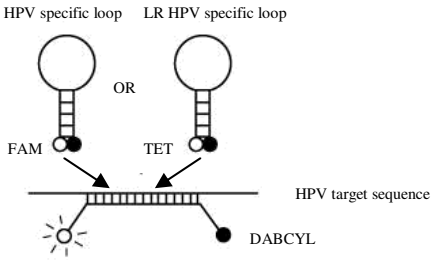

Figure 2. Differently labeled high risk or low risk specific molecular beacons bind to HPV target regions

The method

The total reaction-volume was 22µl, including 7µl of sample DNA. The reaction buffer contained the final concentrations of the following: 91 mM Tris pH8, 4.5 mM MgCl2, 0.008% Ficoll, 0.008% PVP, 0.68 mM DTT, 36 mM KCl, 227 µM of each dNTP, 0.182 µM of each primer (except KP-F/9 0.91 µM), 7.5 units of AmpliTaq Gold (Applied Biosystems, Foster City, CA). Individual molecular beacons were added in a concentration ranging from 0.091 µM to 0.545 µM.

The reaction was carried out in a LightCycler 2.0 PCR thermal cycler (Roche, Basel, Switzerland), with the following parameters: 10 minutes at 95°C, 5 minutes at 55°C, then 35 cycles consisting of 30 seconds at 95°C, 1 minute at 42°C, 30 seconds at 72°C.

Analytical sensitivity and specificity was tested on HPV HPV control plasmids containing the corresponding sequence of the L1 gene. PCR products from clinical samples (in the pCR2.1 Topo vector (Invitrogen, Carlsbad, CA), the length of the subcloned L1F/L1R amplicon varying for the different HPV types) or sequencing verified clinical samples.

Clinical performance was tested on 161 samples presenting positive cytology, collected from STD outpatient clinics. The results obtained by the real-time system were compared to the results obtained by the previously published L1F/L1R system (Jeney et al, J Virol Methods 2007).

HPV 6-44	HPV26-700	HPV52-175
HPV11-44	HPV31-88	HPV56-44
HPV42-22	HPV33-88	HPV58-612
HPV43-44	HPV35-44	HPV59-44
HPV44-22	HPV39-22	HPV66-44
HPV16-44	HPV45-22	HPV68-350
HPV18-44	HPV51-22	

Table 1. The lowest detectable copy numbers are presented for the 20 types

Results

The sensitivity of the system for the 20 detected types is presented in Table 1. The system detected HPV types 42, 44, 39, 45, 51 with the highest sensitivity, and HPV26 was detected at the lowest sensitivity, at 700 copies per sample. Specificity of the system was tested for 45 HPV types (HPV 2a, 3, 6, 7, 10, 11, 13, 16, 18, 26, 27, 29, 30, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 57, 58, 59, 66, 67, 68, 70, 72, 73, 81, 82, 83, 84, 87, 89, 90, 91) . Besides detecting all 20 targeted types, the system showed several cross-reactions, however without compromising overall clinical applicability of the system. HR HPV types 31, 39 cross-reacted in the LR channel. HPV 82 was also positive in the HR channel, but HPV 82 should also be considered a HR type. HPV 3 also cross-reacted in the HR channel, but it is considered less prevalent in cervical pathologies. HPV 70 also cross-reacted in the HR channel, and although being a LR type, it is phylogenetically related to the HR types. HPV types 54 gave signals in the HR channel while HPV 87 and 91 showed both HR-LR cross-reactions. There were a few rare, low risk types which showed cross-reactions in the LR channel: HPV 27, 29, 30, 67, 89. LR cross-reactions are not considered disadvantages of the system. However, the HR cross-reactions need to be addressed in future developments of the system.

Clinical assessment of the system showed a 93.16% detection rate of the system when results were compared to the L1F/L1R system. The real time PCR based system has an estimated sensitivity of 95.45% (63/66) and an estimated specificity of 91.57% (87/95) (p= 4.65 x 10-27, two-tailed Fisher exact probability test) for high risk HPV detection (Table 2).

	RT HR pos	RT HR neg	total
L1F/L1R HR pos	**63**	**8**	**71**
L1F/L1R HR neg	**3**	**87**	**90**
total	**66**	**95**	**161**

Table 2. Comparison of the high risk detection obtained by the L1F/L1R and real-time PCR based systems. The real time PCR based system has an estimated sensitivity of 95.45% (63/66) and an estimated specificity of 91.57% (87/95) (p= 4.65 x 10-27, two-tailed Fisher exact probability test).

Figure 3. Examples of amplification curves obtained on clinical samples, demonstrating typical amplification curves obtained in the three channels with negative, high risk HPV, low risk HPV, mixed high and low risk HPV positive samples. Fluorescence is presented on the Y axis, and cycles on the X axis.