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

Tibor Takács [a,1], Csaba Jeney [a,b,*,1], Laura Kovács [a], Johanna Mózes [a], Márta Benczik [a], Attila Sebe [c]

[a] GenoID Ltd., Pf. 672, 1399 Budapest, Hungary
[b] Department of Microbiology, Semmelweis University, Nagyvárad tér 4., 10. em, 1089 Budapest, Hungary
[c] Department of Pathophysiology, Semmelweis University, Nagyvárad tér 4., 18. em, 1089 Budapest, Hungary

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Abstract

Detection of HPV infections requires a robust time-effective single-step method for efficient screening. A molecular beacon-based one-step multiplex real-time PCR system was developed to detect 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). 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. Accordingly, fluorescent data for HPV detection are collected at 530 nm for high-risk types, 560 nm in case of low-risk types and the reaction internal control is detected at 610 nm on a Roche LightCycler 2.0 instrument. The sensitivity for detected types varies between 22 and 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 the L1F/L1R PCR and hybridization-based system described previously, and the concordance rate between the two systems was 89.44%. The favorable characteristics shown by this multiplex single-step real-time HPV detection system make this promising approach worthy for further development and application for clinical screening.
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Keywords: Molecular beacon; Multiplex real-time PCR; HPV detection

1. Introduction

Persistent infections with HPV can lead to development of malignant lesions, the direct link between HPVs and cervical cancer being well known (Walboomers et al., 1999; Kjaer et al., 2002). Over 100 HPV types were identified so far (de Villiers et al., 2004), about 40 of these being considered genital HPV types and classified into low-risk, intermediate-risk and high-risk classes (Munoz et al., 2003). Accurate HPV testing became a necessity, several studies showing its growing importance in addition to cytology, for example in detecting cervical intraepithelial neoplasia grade II or higher (CIN2+) lesions (Cuzick et al., 2006).

Nucleic acid-based methods (direct probe and signal amplification methods, PCR-based techniques) are the most common and most sensitive detection approaches. PCR-based systems include mostly a second post-PCR detection and typing technique, such as solid phase hybridization in the case of the Roche AMPLICOR HPV test. Limitations of these methods were either related to the inherent complexity of the systems or the time span of the respective techniques.

Regarding efficiency, accuracy, simplicity, cost- and time-effectiveness real-time PCR techniques are the most adequate tools to detect pathogens, presenting major advantages over other systems. Real-time PCR eliminates post-PCR processing of PCR products; it is an easier methodical approach, than a combination of PCR with a detection method. As a one-step method, it is more reliable and faster, providing improved contamination control. Most of the real-time PCR-based HPV detection systems described previously were for quantitation of viral loads (Tucker et al., 2001; Gravitt et al., 2003), or were developed for single-type detection of HPVs. The complexity

* Corresponding author at: Department of Microbiology, Semmelweis University, 1089 Budapest, Nagyvárad tér 4., 10. em., Hungary. Tel.: +36 70 4543223; fax: +36 1 4650127.
E-mail address: csjeney@gmail.com (C. Jeney).
[1] These authors contributed equally to this article.

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of HPV detection and typing requires multiplex approaches. Several investigators described real-time-based multiplex systems, where a limited number of types were detected in parallel reactions. In the system described by Moberg et al. (2003) samples are tested in two reaction tubes to detect viral types or groups of viral types in each reaction using three fluorophores per reaction. This system detects 10 HPV types. The system based on self-probing fluorescent primers (Scorpions) is also a two-step approach presenting first a general detection of HPV followed by a second, type-specific reaction (Hart et al., 2001). The system described by Szuhai et al. (2001) is also based on a two-step strategy: the SybrGreen-based general detection is followed by the molecular beacon-based type-specific detection of seven HPV types. The PreTect HPV-Proofer system detects HPV E6/E7 mRNA based on a real-time multiplex NASBA with molecular beacon probes (Molden et al., 2007).

Molecular beacons allow multiplex detection of PCR products, generating a signal only in the presence of the target and remaining dark in its absence. Stem-loop hairpin probes were shown to have better specificity in gene detection compared to linear oligonucleotide probes (Roberts and Crothers, 1991). Molecular beacons show higher sensitivity and signal-to-background ratio than TaqMan probes. Shared-stem molecular beacons were also found to yield better performances than TaqMan probes (Wang et al., 2005). Therefore, molecular beacons were chosen over other probes to design a new real-time HPV detection system, which allows single-tube multiplexing and sensitive simultaneous detection.

In this report, the development of a molecular beacon-based one-step multiplex real-time PCR system is described 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. 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. Accordingly, fluorescent data for HPV detection are collected at 530 nm for high-risk types, 560 nm in case of low-risk types and the reaction internal control is detected at 610 nm on a Roche LightCycler 2.0 instrument. The assessment of analytical characteristics and clinical applicability is presented in this paper.

2. Materials and methods

2.1. HPV plasmids and HPV real-time internal control

HPV control plasmids were prepared by subcloning 244–262 bp PCR products from clinical samples into the pCR2.1 Topo vector (Invitrogen, Carlsbad, CA), the length of the subcloned L1F/L1R amplicon varying for the different HPV types. Plasmids contained subgenomic fragments of the following HPV genomes (GenBank accession numbers are shown): HPV 2a, NC_001352; HPV 3, NC_001588; HPV 6, NC_000904; HPV 7, NC_001795; HPV 10, NC_001576; HPV 11, M14119; HPV 13, NC_001349; HPV 16, K02718; HPV 18, NC_001357; HPV 26, NC_001583; HPV 27, NC_001584; HPV 29, NC_001685; HPV 30, NC_001585; HPV 31, NC_001527; HPV 33, NC_001528; HPV 34, NC_001587; HPV 35, NC_001529; HPV 39, NC_001535; HPV 40, NC_001589; HPV 42, NC_001534; HPV 43, NC_005349; HPV 44, NC_001689; HPV 45, NC_001590; HPV 51, NC_001533; HPV 52, NC_001592; HPV 53, NC_001593; HPV 54, NC_001676; HPV 56, NC_001594; HPV 57, NC_001353; HPV 58, NC_001443; HPV 59, NC_001635; HPV 66, NC_001695; HPV 67, D21208; HPV 68, X67161; HPV 72, X94164; HPV 73, X94165. Sequencing-verified clinical samples were used in case of other types (HPV types 70, 81, 82, 83, 84, 87, 89, 90, 91).

The internal control (IC) of the HPV real-time PCR is a 140-bp long artificial DNA sequence cloned into the pCR 2.1. Topo vector (Invitrogen, Carlsbad, CA). The sequence contained KP-F/16 forward and L1C2 reverse primer-binding regions and the internal control hybridization probe-binding region. The IC was added to the lysis solution prior to clinical sample preparation (concentration: 4.8 ng/ml). Thus it was present during PCR, amplified and detected by the specific internal control probe.

Table 1
The list of the 16 forward primers

1	KP-F/1	5′-CGCACCAACATATTTTATT-3′
2	KP-F/2	5′-CGCACAAGCATCTATTATTA-3′
3	KP-F/3	5′-CGCACAAGCATATTTTATC-3′
4	KP-F/4	5′-CGCACCAGTATATTTTATCA-3′
5	KP-F/5	5′-CGCACAAGCATTTACTATCA-3′
6	KP-F/6	5′-CGCACCAACTACTTTTACC-3′
7	KP-F/7	5′-CGTACCAGTATTTTCTACCAC-3′
8	KP-F/8	5′-CGCACAGGCATATATTACT-3′
9	KP-F/9	5′-CGCACCAACATATATTATCA-3′
10	KP-F/10	5′-CGTACCAACCTGTACTATTATG-3′
11	KP-F/11	5′-GCACCAACTTATTTTACCAT-3′
12	KP-F/12	5′-ACCAACCTCTTTTATTATGG-3′
13	KP-F/13	5′-AGCACAAATATATATTATTATGG-3′
14	KP-F/14	5′-CGCACCGGATATATTACT-3′
15	KP-F/15	5′-CGCACAAATATTTATTATTATGC-3′
16	KP-F/16	5′-CGGACGAATGTTTATTACC-3′

2.2. Primers

HPLC-purified oligonucleotide primers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). The list of 16 forward and 16 reverse primers is presented in Tables 1 and 2.

2.3. Molecular beacons

Molecular beacon probes were designed following the guidelines published by Tyagi and Kramer (1996). Melting profiles of the probes and secondary structure prediction were assessed by Mfold (Version 3.2) following the guidelines described by

Table 2
The list of the 16 reverse primers

17	L1C2	5′-TACCCTAAATACTCTGTATTG-3′
18	L1R2	5′-TACCCTAAATACCCTATATTG-3′
19	R1	5′-AATTCTAAAAACTCTGTACTG-3′
20	R45	5′-TACTCTAAATACTCTGTATTG-3′
21	R11	5′-TACCTTAAACACTCTATATTG-3′
22	R16	5′-TATTCTAAATACCCTGTATTG-3′
23	R42	5′-AACTCTAAATACTCTGTACTG-3′
24	R44	5′-CATCTTAAAAACCCTATATTG-3′
25	R03	5′-AACCCTAAACACCCTGTATTG-3′
26	R04	5′-AACGCGAAAAACCCTATATTG-3′
27	R05	5′-TACCCTAAAGACCCTATACTG-3′
28	R06	5′-AACTCTAAATACCCTATACTG-3′
29	R07	5′-AACGTGAAATACACGATATTG-3′
30	R08	5′-CACACGGAACACCCTGTACTG-3′
31	R54	5′-CACCCTAAACACCCTATATTG-3′
32	R85	5′-AACCCGAAACACTCGATACTG-3′

Zuker (2003), located at the following Internet site: http://www.bioinfo.rpi.edu/applications/mfold/cgi-bin/dna-form1.cgi.

General structure of the probes is: 5′–stem–HPV complementary sequence–stem'–3′. The loop contains the specific HPV complementary region. Stem and stem' are the sequences formed by the complementary base pairs which form a double-helix stem structure in the solution. These sequences are preferably palindrome. There are four or five bases at each end of the probe. The stem nucleotides preferably comprise C–G pairs. However, variances to this rule were applied in some cases. There are 10 conventional molecular beacons and 11 shared-stem molecular beacons in the system. The specific probes were selected to have a maximum sequence difference with all other HPV types. Optimal loop melting temperature (T_m) was between 52 and 56 °C, while the stem T_m was between 48 and 52 °C.

The quencher molecule linked to the 3′ end was DABCYL, while fluorophores were linked to the 5′ end. For detection of low-risk types, probes were TET labelled. FAM was used in case of high-risk probes. The internal control probe contained a wavelength-shifting label consisting of fluorescein and TEXASRED.

HPLC-purified molecular beacons were synthesized by Integrated DNA Technologies (IDT, Coralville, IA), except the internal control probe (Sigma–Proligo, Boulder, CO). The list of the 20 type-specific probes and one internal control probe is presented in Table 3. The alignment of the type-specific molecular beacons within the amplicons is presented in Table 4.

2.4. Molecular beacon-based real-time PCR

Components were purchased from Sigma (St. Louis, MO), unless otherwise stated.

The total reaction volume was 22 μl, including 7 μl of sample DNA. The reaction buffer contained the final concentrations of the following: 91 mM Tris pH 8, 4.5 mM $MgCl_2$, 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 at a concentration ranging from 0.091 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 min at 95 °C, 5 min at 55 °C, then 35 cycles consisting of 30 s at 95 °C, 1 min at 42 °C, 30 s at 72 °C.

The fluorescent data for HPV detection were collected as follows: high-risk types at 530 nm, low-risk types at 560 nm, reaction internal control at 610 nm. Fluorescent data were collected only in the last 20 cycles. Results were analyzed by the LightCycler Software 4.05.

2.5. Color compensation

Color compensation was carried out using three colors (FAM-, TET-, or wavelength-shifting color pair FAM-TEXASRED-labelled oligo dT) following the instructions stated in the LightCycler 2.0 Instrument Operator's Manual.

2.6. Analytical sensitivity and specificity

Sensitivity and specificity were assessed using plasmids containing specific HPV sequences or sequence-verified clinical samples. Sensitivity of the 20 probes was calculated to DNA copy numbers. The specificity of the system was assessed 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).

2.7. Clinical assessment of the system

Clinical samples with positive cytology were collected from sexually transmitted disease outpatient clinics and were tested by the L1F/L1R multiplex PCR and hybridization-based detection and typing system, positive samples being typed for general low-risk (detecting HPV types 6, 11, 42, 43, 44) and 14 high-risk targets (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) (Jeney et al., 2007). DNA was prepared from 161 samples following the DNA extraction method previously described (Jeney et al., 2007). Results yielded by the L1F/L1R system were compared to the results obtained by the real-time system on the same samples and the concordance of high/low-risk positivity was assessed.

Following this clinical assessment further samples were assessed by the real-time system. A first set of 25 samples was detected as low risk by the L1F/L1R system. The second set of 50 samples were detected as HPV positive during general detection, but not found positive by the type-specific detection for HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 or LR.

2.8. Sequencing

PCR products amplified from clinical samples were treated with the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany). These templates were then sequenced using the BigDye Terminator Cycle Sequencing

Table 3
The list of the 21 molecular beacons

HPV 6/B5	5′-TET-CGCGAAAACAGTTGTGCCAAAGGTGTCAGCGCG-DABCYL-3′
HPV 11B3/2	5′-TET-GCCGAAAACAGTTGTACCAAAGGTGTCTGCGGC-DABCYL-3′
HPV 42B1	5′-TET-CCTCCCAAAAAGGCCAAATAAGACAGGAGG-DABCYL-3′
HPV 43B6	5′-TET-CCCGCCCCCTTAAAAATTCCTCTCGGG-DABCYL-3′
HPV 44/55B1	5′-TET-CCTCCATACGACCAGCAAACAAGACGGAGG-DABCYL-3′
HPV 16B5v.2	5′-FAM-CCTGCCAATAACAAAATATTAGTTCCTAAAGCAGG-DABCYL-3′
HPV 18B8	5′-FAM-CCGGTATCCTGCTTATTGCCACCCCGG-DABCYL-3′
HPV 26B1	5′-FAM-AGCGTACCTAAAACTGGCCAAAAGCGCT-DABCYL-3′
HPV 31B5	5′-FAM-CGGCCATACCTAAATCTGACAATCCGCCG-DABCYL-3′
HPV 33B7	5′-FAM-GCCGTTTTTTAGCGTTAGTAGGATTTTTCGGC-DABCYL-3′
HPV 35B3	5′-FAM-CCGGCTATTAGCTGTGGGTCACCGG-DABCYL-3′
HPV 39B4	5′-FAM-CCGGTATGAATGGTGGTCGCAAGCCGG-DABCYL-3′
HPV 45B3	5′-FAM-CCGGGCTGTTCCTAAGGTATCCGCCGG-DABCYL-3′
HPV 51B2	5′-FAM-CGGCAGCACGCGTTGAGGTTTTAGCCG-DABCYL-3′
HPV 52B9	5′-FAM-CCGGAAAAACACCAGTAGTG**C**TAATGCCGG-DABCYL-3′
HPV 56B3	5′-FAM-CCGGCCAAAACAAACATTCCCAACCGG-DABCYL-3′
HPV 58B2	5′-FAM-CGGCTTCCATCAAAAGTCCCAATAACGCCG-DABCYL-3′
HPV 59B3	5′-FAM-CGGCATCCATATTTTAAAGTACCTAAAGGCCG-DABCYL-3′
HPV 66B1	5′-FAM-CGAGGCAAATCTGGTACCAAAACAAACCTCG-DABCYL-3′
HPV 68B2	5′-FAM-CGGCTTAAGGTTCCTATGTCTGGGGGCCG-DABCYL-3′
HPV-ICB4	5′-(TEXASRED)TTTTTT(FLUORESCEIN)DT)CGGCCCCATAGACAGTTTATACAGATCAGCCG-DABCYL-3′

The mismatch in the sequence of the HPV 52B9 molecular beacon is marked.

Kit v3.1 (Applied Biosystems). Sequencing reaction products were run on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). DNA sequences were aligned with the public database on NCBI homepage using BLAST algorithm. Sequencing was performed with the reverse primer set.

2.9. Statistical methods

The two-tailed Fisher exact probability test (http://faculty.vassar.edu/lowry/tab2x2.html) was used to analyze contingency tables comparing the results obtained by the L1F/L1R system and the real-time PCR-based system.

3. Results

3.1. Development of the molecular beacon-based one-step multiplex real-time HPV detection system

The L1F/L1R primer-based HPV detection and typing system (Jeney et al., 2007) featured a multiplex PCR reaction followed by a post-PCR solid phase hybridization step to detect specific PCR products. The main characteristics of the amplification system served as a starting point in developing the real-time PCR-based HPV detection system.

The L1F/L1R amplification system targets a special hypervariable region of the L1 gene, where a highly variable sequence is flanked by conserved regions in the case of all HPV types. This region is situated in the first hypervariable region of the L1 gene (Olcese et al., 2004). The real-time detection system was set to detect 15 high-risk and 5 low-risk HPV types. The 16 forward and 16 reverse primers also target the same hypervariable region of the L1 gene. The reverse primers were the same as in the L1F/L1R amplification system; the forward primers were redesigned, and targeted the sequences which were used for general hybridization in the previous development (Fig. 1) (Jeney et al., 2007). This multiplex amplification system amplifies a similar number of types as the L1F/L1R system, however, only 20 type-specific molecular beacons were designed, therefore 20 types are detected with the current system, a number that can be extended upon introduction of new probes.

To increase probe binding, shared stems were used in some cases (HPV 16, 31, 35, 39, 44, 45, 51, 59, 66, 68, IC). The majority of molecular beacons contained four base-stems. The molecular beacons containing four base-stems have significantly higher hybridization on-rate constant than molecular beacons with five or six base-stems, therefore these adapt better to reaction dynamics (Tsourkas et al., 2003). In some cases five base-stems were used, which do not influence reaction dynamics (HPV 16, 26, 42, 44, 66). In the case of HPV 52, where the

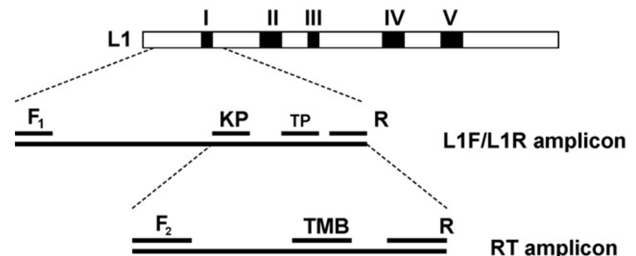

Fig. 1. Schematic representation of the location of the primers and probes in the L1F/L1R and the molecular beacon-based real-time PCR systems. The first hypervariable region of the L1 ORF was targeted by both systems. The shorter RT amplicon was located within the longer L1F/L1R amplicon. In case of the HPV 16 L1 ORF (5526–7154 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. 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.

Table 4
The alignment of the type-specific molecular beacons

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HPV6    AGACTTCTTGCAGTGGGTCATCCTTATTTTTCCATAAAACGGGCTAACA---------------AAACTGTTGTGCCAAAGGTGTCAGGATATCAATACAGGGT
HPV11   AGACTCCTTGCTGTGGGACATCCATATTACTCTATCAAAAAAGTTAACA---------------AAACAGTTGTACCAAAGGTGTCTGGATATCAATATAGAGT
HPV44   AGACTTCTTGCTGTGGGCAACCCTTATTTTGCCATACGACCAGCAAACA---------------AGACACTTGTGCCTAAGGTTTCGGGATTTCAATATAGGGT
HPV42   AGGCTATTGGTTGTTGGTCACCCTTATTACTCTATTACAAAAAGGCCAA------------ATAAGACATCTATCCCCAAAGTGTCTGGTTTACAGTACAGAGT
HPV43   CGTTTGCTTGCAGTGGGTCACCCATATTTCCCCCTTAAAAATTCCTCTG------------GTAAAATAACTGTACCTAAGGTTTCTGGTTATCAATACAGAGT
HPV33   AGACTTCTTGCTGTTGGCCATCCATATTTTTCTATTAAAAATCCTACTA------ACGCTAAAAAATTATTGGTACCCAAAGTATCAGGCTTGCAATATAGGGT
HPV58   AGACTTTTGGCTGTTGGCAATCCATATTTTTCCATCAAAAGTCCCAATA------ACAATAAAAAAGTATTAGTTCCCAAGGTATCAGGCTTACAGTATAGGGT
HPV52   CGATTACTAACAGTAGGACATCCCTATTTTTCTATTAAAAACACCAGTAGTGGTAATGGTAAAAAAGTTTTAGTTCCCAAGGTGTCTGGCCTGCAATACAGGGT
HPV16   AGACTACTTGCAGTTGGACATCCCTATTTTCCTATTAAAAAACCTAACA---------ATAACAAAATATTAGTTCCTAAAGTATCAGGATTACAATACAGGGT
HPV35   AGGCTATTAGCTGTGGGTCACCCATACTATGCTATTAAAAAACAAGATT---------CTAATAAAATAGCAGTACCCAAGGTATCTGGTTTGCAATACAGAGT
HPV31   AGGCTGCTTACAGTAGGCCATCCATATTATTCCATACCTAAATCTGACA------ATCCTAAAAAAATAGTTGTACCAAAGGTGTCAGGATTACAATATAGGGT
HPV56   CGATTGCTTGCCGTAGGACATCCCTATTACTCTGTGACTAAGGACAATAC-------------CAAAACAAACATTCCAAAGTTAGTGCATATCAATATAGGGT
HPV66   AGGTTGCTTGCTGTTGGCCATCCTTATTACTCTGTTTCCAAATCTGGTAC------------CAAAACAAACATCCCTAAAGTTAGTGCATATCAGTATAGAGT
HPV18   AGATTATTAACTGTTGGTAATCCATATTTTAGGGTT---CCTGCAGGTGGTG------GCAATAAGCAGGATATTCCTAAGGTTTCTGCATACCAATATAGAGT
HPV45   CGATTATTAACTGTAGGCAATCCATATTTTAGGGTTGTACCTAATGGTGCAG------GTAATAAACAGGCTGTTCCTAAGGTATCCGCATATCAGTATAGGGT
HPV39   AGATTATTAACAGTAGGACATCCATATTTTAAAGTG---GGTATGAATGGTG------GTCGCAAGCAGGACATTCCAAAGGTGTCTGCATATCAATATAGGGT
HPV68   AGGTTATTAACTGTAGGCCATCCATATTTTAAGGTT---CCTATGTCTGGGG------GCCGCAAGCAGGGCATTCCTAAGGTGTCTGCATATCAATACAGAGT
HPV26   CGTTTATTAACATTAGGACATCCATATTTTTCCATA---CCTAAAACTGGC--------CAAAAGGCCG-AAATTCCTAAGGTATCTGCCTATCAGTACAGGGT
HPV51   AGACTAATAACATTAGGACATCCCTATTTTCCAATA---CCTAAAACCTCA--------ACGCGTGCTG-CTATTCCTAAAGTATCTGCATTTCAATACAGGGT
HPV59   AGACTTCTTACAGTTGGACATCCATATTTTAAAGTA---CCTAAAGGTGGTA------ATGGTAGACAGGATGTTCCTAAGGTGTCTGCATATCAATACAGAGT
         *  *  *     * **  * ** ** *      *                                      * ** ** **    *     ** ** ** **
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The boxes emphasize the specific probes. "–" stands for gaps in the alignment and "*" marks the consensus.

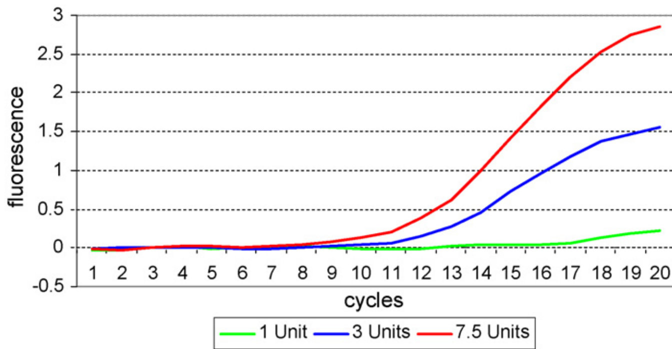

Fig. 2. Increased AmpliTaq Gold enzyme volume improved threshold cycle and signal intensity. Each sample contained 7000 copies/reaction HPV 6 plasmid.

design conditions were very strict, one mismatch had to be introduced to prevent secondary structure formation in the molecular beacon, without compromising its optimal function.

The internal control was added at sample preparation in such an optimal concentration (450 copies/μl sample), which did not compete with the specific HPV PCR products, yet ensured detectable signals. When samples display strong high-risk or low-risk positivity, the IC signal may disappear.

The reaction buffer was optimized in order to improve amplification efficiency and hybridization specificity by including several components. Amplification was enhanced by the introduction of dithiothreitol (Ralser et al., 2006). Molecular crowding had also a stimulating effect on the reaction, by introducing Ficoll (Zimmerman and Harrison, 1985) and PVP (James, 1999) to the reaction buffer.

The optimization of the PCR program required the balance between specificity and signal intensity. Shorter annealing increases specificity, while longer annealing improves signal level, therefore annealing was optimal when a 60 s cycle at 42 °C was applied.

The AmpliTaq Gold enzyme quantity was also optimized in order to meet the requirements of a multiplex system. As shown in Fig. 2, the higher AmpliTaq Gold enzyme volume significantly improved threshold cycle and signal intensity.

The results of the reaction were evaluated in the three channels and samples were detected as negative, high-risk or low-risk positive, or as mixed high-risk–low-risk positive. This approach resulted in a simple one-step–one-tube system suitable for everyday clinical practice. Examples of amplification curves obtained on clinical samples are presented in Fig. 3 demonstrating typical amplification curves obtained in the three channels (530, 560, 610 nm) with negative, high-risk, low-risk, high- and low-risk positive samples.

In some cases where the LightCycler software yielded uncertain results, the results were reassessed and considered positive when the respective sigmoid curves showed significant deflection from the NTC curve.

3.2. Analytical sensitivity and specificity of the system

The sensitivity of the system for the 20 targeted types was assessed by using plasmids with known concentrations in dilution series. The sensitivity was calculated based on the last positive dilution in the series. Most of the types were detected at lower copy numbers than 50 copies per sample. The system detected HPV types 42, 44, 39, 45, 51 with the highest sensitivity, and HPV 26 was detected at the lowest sensitivity, at 700 copies per sample. The sensitivity of the system for the 20 detected types is presented in Table 5. Typical amplification curves obtained on a HPV 6 dilution series is presented in Fig. 4.

Table 5
The lowest detectable copy numbers are presented for the 20 detected types

HPV type	Lowest detectable copy number/reaction
HPV 6	44
HPV 11	44
HPV 42	22
HPV 43	44
HPV 44	22
HPV 16	44
HPV 18	44
HPV 26	700
HPV 31	88
HPV 33	88
HPV 35	44
HPV 39	22
HPV 45	22
HPV 51	22
HPV 52	175
HPV 56	44
HPV 58	612
HPV 59	44
HPV 66	44
HPV 68	350

Specificity 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) on plasmids encoding for these types; in some cases sequencing-verified clinical samples were used, as stated in Section 2. Besides detecting all 20 targeted types, the system showed a few cross-reactions, however, without compromising overall clinical applicability of the system. High-risk HPV types 31, 39 cross-reacted in the low-risk channel. HPV 82, HPV 3, HPV 70, HPV 54 generated signals in the HR channel while HPV 87 and 91 showed both high-risk–low-risk cross-reactions. There were a few rare, low-risk types which showed cross-reactions in the low-risk channel: HPV 27, 29, 30, 67, 89. low-risk cross-reactions are not considered to be the disadvantages of the system. However, the high-risk cross-reactions need to be addressed in future developments of the system.

3.3. Clinical evaluation

In order to assess clinical performance of the molecular beacon-based real-time PCR HPV detection system, clinical samples were tested. The results were compared to the detection and typing results obtained by the L1F/L1R primer-based multiplex PCR and hybridization-based HPV detection and typing system (Jeney et al., 2007). For this evaluation, 161 random clinical samples were assessed by both systems. Samples assessed by the L1F/L1R system were typed for 14 high-risk types (HPV

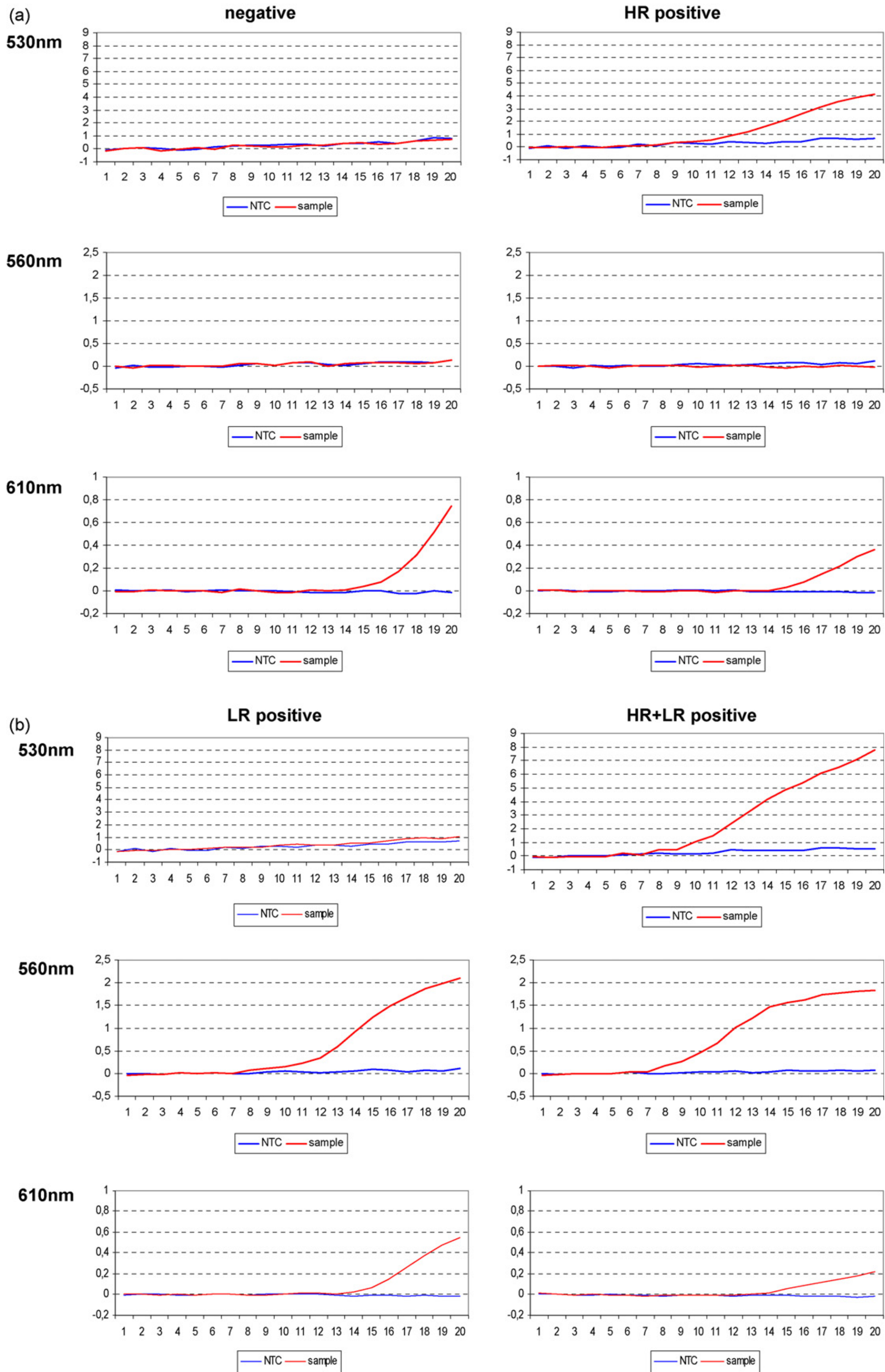

Fig. 3. Examples of amplification curves obtained on clinical samples, demonstrating typical amplification curves obtained in the three channels with negative, high-risk, low-risk, high- and low-risk positive samples. The monotone ascending background in the 530 nm channel in case of negative and low-risk samples was the consequence of the high concentration of the molecular beacons used for high-risk detection. Since these signals were not specific sigmoid amplification curves, evaluation was not influenced by their presence. Fluorescence is presented on the *Y* axis, and cycles on the *X* axis.

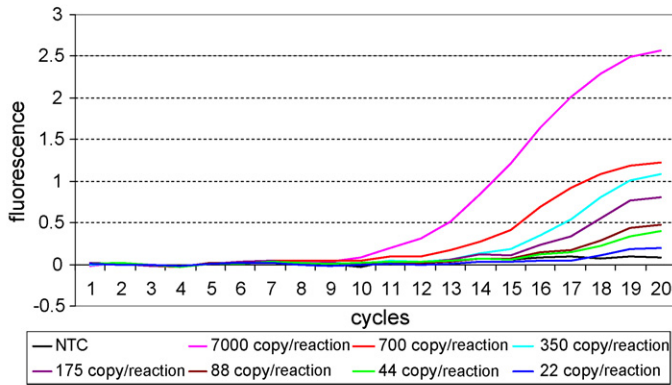

Fig. 4. Sensitivity was analyzed using dilution series of plasmids with known concentrations. This is exemplified by the HPV 6 dilution series. The copy number/reaction is stated in the legend.

16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) and for general low-risk positivity, HPV types 6, 11, 42, 43, 44 being detected only as low-risk types. The L1F/L1R system detected 80 positive cases and there were 71 negative samples. Ten further samples were HPV positive, but for types not included in the current typing pool, classified as "not included" (NI).

When comparing the results obtained by the two systems, 144 of the 161 samples were detected correctly by the real-time system, results showing a concordance of 89.44% with the detection results given by the L1F/L1R system. From these 144 samples correctly detected, the real-time system detected 50 samples containing high-risk HPV types, 6 samples showed low-risk positivity, 11 cases were for both high-risk and low-risk positive, while 77 cases proved to be negative. There were 10 discordant samples (6.83%) where the real-time system did not detect the correct result. Six of these samples presented very low hybridization signals by the L1F/L1R system, and since the sample DNA volume used by the real-time system is 30% less, probably the HPV DNA levels in those samples were below the detection sensitivities of the types in question. Seven further cases yielded discordant results (4.34%), probably as the consequence of known cross-reactions of the real-time system. In these cases a high-risk type (HPV 31) was considered high-risk–low-risk positive, or NI samples were considered either high risk or low risk by the real-time system. The estimated specificity and sensitivity of the system were analyzed for high-risk detection (Table 6).

Table 6
Comparison of the high-risk detection obtained by the L1F/L1R and real-time PCR-based systems

	RT HR positive	RT HR negative	Total
L1F/L1R HR positive	63	8	71
L1F/L1R HR negative	3	87	90
Total	66	95	161

The real-time PCR-based system has an estimated sensitivity of 95.45% (63/66) and an estimated specificity of 91.57% (87/95) ($p = 4.65 \times 10^{-27}$, two-tailed Fisher exact probability test).

In order to assess further the clinical relevance of cross-reactions, two further pools of samples were assessed. The first pool included 25 samples which were typed as low-risk positive by the L1F/L1R system. Two of these samples were found to give signals to the high-risk channel and were further analyzed by sequencing. One of the samples proved to be HPV 89; however, the other sample could not be analyzed by sequencing, since it proved to be a mixed infection.

The second pool included 50 samples previously detected as NI types. The real-time analyses revealed HR signals in seven cases. Sequencing identified these samples as containing HPV 70, 82, 87, 90, 91, two samples containing HPV 54. As discussed previously, HPV 82 can be also considered a high-risk type. Further, the low-risk HPV 70 is also phylogenetically related to high-risk HPV types. HPV types 54, 87 and 91 were also shown to cross-react during the analytical assessment of the system. Finally, analytical properties for HPV 90 were assessed on a confirmed clinical sample. It is probable, that the sample assessed in this latter pool may contain higher copy numbers than the sample used during the analytical assessment, indicating the need to test the system further for these types.

4. Discussion

Several studies showed that the accurate detection of genital HPV DNAs increased the sensitivity of the screening for cervical malignancies. In comparison with cytology HPV DNA testing was found more sensitive and equally specific for screening of atypical cells of undetermined significance (ASCUS) samples, and it sensitively predicted the recurrence of cervical intraepithelial neoplasia in women treated for high-grade cervical intraepithelial neoplasia (Arbyn et al., 2005). Compared to cytology, the HPV testing was found more sensitive for prevalent CIN2 (Koliopoulos et al., 2007). Recently, based on a study involving 10,154 women, compared with cytology, HPV testing had greater sensitivity for the detection of cervical intraepithelial neoplasia (Mayrand et al., 2007). Moreover, the addition of an HPV test to the Pap test in screening for cervical cancer reduced the incidence of grade 2 or 3 cervical intraepithelial neoplasia or cancer detected by subsequent screening examinations (Naucler et al., 2007).

Most of the PCR assays are based on degenerated and/or consensus primers that allow the detection of a large spectrum of types. After the amplification process, these techniques imply a second post-PCR technique for detection of PCR products. Real-time PCR approaches present several advantages over conventional PCR-based methods. Besides eliminating the post-PCR detection steps, real-time PCR is a sensitive target amplification method (Heid et al., 1996).

Previous real-time HPV detection systems were designed with two main strategies. The amplification with molecular beacon primers enables type-independent prescreening for the presence of HPV in samples (Jordens et al., 2000). Others designed systems which distinguished different HPV types along several single-type or group detection guidelines, and different probes. Previous developments showed the feasibility of multiplex combination of HPV specific probes. HPV molecu-

lar beacon probes proved to be highly specific and well suited for application in a multiplexed detection system (Szuhai et al., 2001). Moreover, molecular beacons were found more sensitive, than TaqMan probes (Wang et al., 2005; Tapp et al., 2000).

In the previous development, a multiplex PCR- and hybridization-based system was designed to detect 46 HPVs, targeted to a special region of the L1 gene (Jeney et al., 2007). In this region, a highly variable sequence is flanked by two highly conserved regions, a special region which allowed the design of highly type-specific primers and hybridization probes. The same region was targeted by the present development, where the primers and molecular beacon probes are highly specific to the included HPV types. The reaction presents all the advantages of real-time PCRs. It is a multiplex, one-step HPV detection system, which detects 15 high-risk and 5 low-risk HPV types. High-risk and low-risk HPV types are detected in different emission channels, the included reaction internal control being detected in a third channel. This setup involves both the accurate detection of HPVs and the quality control of the reaction by the implementation of the internal control. The real-time PCR strategy eliminates the post-PCR handling of samples, which reduces contamination risks. Samples are detected as negative or containing high-risk, low-risk, or high-risk and low-risk HPV DNAs. While the detection and typing with the previous L1F/L1R PCR- and hybridization-based system required 8 h for detection and typing, the major advantage of the real-time PCR system is its time effectiveness, results being obtained in only 3 h.

Several authors raised concerns about cross-reactions and cross-hybridizations in the systems based on multiplex detection of HPVs due to the inherent complexity given by the high degree of sequence homology (Hubbard, 2003). The system described features a carefully selected target region of the HPV sequence, which allows a highly specific detection. However, as shown during the specificity analysis, the system presented a few minor cross-reactions with low-risk HPV types. These types have less relevance to cervical pathology. The limitation of the system is the cross-reaction of some low-risk types to the high-risk channel. Although the primers and probes were carefully selected to discriminate between the types targeted by the system, several less frequent types showed cross-reactions, leading to the need to design more specific molecular beacon probes. This problem will be addressed in future developments of the system. HPV 82 was positive in the high-risk channel, but HPV 82 should also be considered a high-risk type (Munoz et al., 2003). HPV 3 also cross-reacted in the high-risk channel, but it is considered less important in cervical pathologies. HPV 70 generated high-risk signal, and although being a low-risk type, it is phylogenetically related to the high-risk types (Munoz et al., 2003). HPV 54 was positive in the high-risk channel while HPV 87 and 91 showed both high-risk–low-risk cross-reactions.

As far as the discrepancies between the L1F/L1R and the real-time system, these could be explained by the sample volume used in both systems. The L1F/L1R system amplifies from 10 μl of sample, while the real-time system requires only 7 μl of sample, 30% less than the L1F/L1R system. From the 10 samples showing discrepancies between the two systems, 6 presented very low hybridization signals obtained by the L1F/L1R system. It is conceivable that the reduced sample quantity biased these detections, the HPV DNA levels being below the sensitivity levels in those cases.

The molecular beacon-based real-time PCR HPV detection system presents several features which qualify it as powerful tool for HPV diagnostics. This novel method is an adequate tool for cervical screening programs, and has the advantages of a rapid, time-effective single-step approach.

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