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www.genoid.hu
info@genoid.hu

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A real-time PCR based high-throughput screening system for high-risk HPV detection

Attila Sebe[1,2], Borbála Szenthe[1], Anett Kovács[1], Miklós Nyíri[1], Csaba Jeney[1,3], Tibor Takács[1]

1-GenoID Ltd., Budapest, Hungary, 2-Semmelweis University, Dept. of Pathophysiology, Budapest, Hungary, 3-Semmelweis University, Dept. of Microbiology, Budapest, Hungary

Background and aims: HPV screening is an emerging concept, which may fundamentally influence cervical cancer screening. Various high-risk HPV detection systems were previously designed, however without addressing the need for an automated, robotized system that is also suitable technically for high-throughput screening.
Method: A molecular beacon based one step multiplex real-time PCR (MB-RT PCR) system was designed for high-throughput detection of 14 high-risk HPVs (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) on the ABI7900HT instrument, in 96 or 384 well plate format. Detection is achieved in 3 channels. Recognizing the demand for the identification of HPV vaccine types, HPV 16 and 18 are detected together in one channel. The other high-risk types are detected in group in a second channel. The artificial internal control added before DNA preparation in a controlled quantity is detected in a third channel. The clinical performance was tested on 139 liquid based cytology specimens. The MB-RT PCR was compared to Full Spectrum HPV Amplification and Detection System (GenoID, IVD-CE mark) results.
Results: The MB-RT PCR system identified correctly high-risk HPV DNA positivity/negativity with a detection rate of 97.12%, according to the above mentioned grouping. The analytical sensitivity was ~100 DNA copies/reaction for each detected type.
Conclusion: We propose the MB-RT PCR as a high-throughput primary HPV-based cervical screening tool.

INTRODUCTION

Persistent infections with high-risk HPV can lead to development of malignant lesions, the direct link between HR HPVs and cervical cancer being well known. The accurate HPV testing became a necessity, several studies showing its growing importance in addition to cytology. HPV screening is an emerging concept, which may fundamentally influence cervical cancer screening. Various high-risk HPV detection systems were previously designed, however without addressing the need for an automated, robotized system that is also suitable technically for high-throughput screening.

We developed a molecular beacon based one step multiplex real-time PCR system which detects 14 high-risk HPV types in two separate groups: group A- HPV 16, 18, group B- HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68. Real time PCR based HPV detection has several advantages over other detection methods: one tube- one step format, time and cost efficiency, better contamination control. The system was first described for LightCycler2.0 (Takács et al, J Virol Methods 2008), but later optimized for ABI7900HT. The optimized PCR reaction mixture contains an optimized set of primers and 14 type specific 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. The reverse primer set used in the RT amplification system is a selection of specific primers from the previous system. The forward primers of the RT system are targeting the same sequences where the L1F/L1R general hybridization probes were designed (Figures 1-2).

The amplified product is detected by measuring the fluorescence of the labeled molecular beacons. An Internal Control (IC) added before sample DNA extraction is also detected by a specific molecular beacon to ensure proper DNA isolation procedure and PCR. There are 3 different dyes labeling the type specific molecular beacons: molecular beacons detecting the 12 high-risk types are 5'-TET labeled, HPV 16 and 18 are detected by 5'-JOE labeled molecular beacons, while the Internal Control is detected by a 5'-FAM labeled molecular beacon. HPV 16 18, high-risk and internal control amplification is detected in separate detection channels (Table 1.)

ABI7900HT allows performing real time HPV testing in 96 and 384 well format, and its combination with robotic tools for DNA extraction and PCR setup would ensure a potent and efficient high-throughput screening tool.

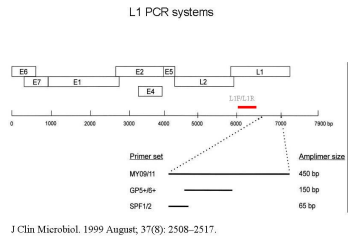

Figure 1. Schematic representation of the locations of the different general primer sets (L1F/L1R, MY 09/11, GP5+/6+, and SPF) on the HPV genome

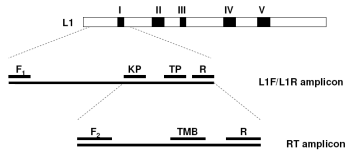

Figure 2. Schematic representation of the location of the primers and probes in the L1F/L1R and the molecular beacon based real-time PCR systems. F1- L1F/L1R forward primers, KP- general hybridization probes, TP- type specific hybridization probes, R- common reverse primers, F2- RT forward primers, TMB- type specific molecular beacons.

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, primer concentration ranging from 150 nM to 240 nM, 7.5 units of AmpliTaq Gold (Applied Biosystems, Foster City, CA). Individual molecular beacons were added in a concentration ranging from 68,2 nM to 682 nM. The reaction was carried out in an ABI7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA), with the following parameters: 10 minutes at 95°C, 5 minutes at 55°C, then 25 cycles consisting of 15 seconds at 95°C, 20 seconds at 30°C, 15 seconds at 95°C, 60 seconds at 50°C, 30 seconds at 72°C (detection at 50 °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 212 samples in PreservCyt, DNA being prepared by AmpliLute Liquid Media Extraction Kit (Roche, Mannheim, Germany). 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).

Target	Detector	Detection Channel
HPV 16, 18	16 18	JOE
HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	HR	TET
Internal Control	HPVRTIC	FAM

Table 1. Detection channels and targets on ABI7900HT

	RT HR pos	RT HR neg	total
L1F/L1R HR pos	67	4	71
L1F/L1R HR neg	7	134	141
total	74	138	212

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 90.54% (67/74) and an estimated specificity of 97.1% (134/138)

RESULTS

The analytical sensitivity of the system for the 14 detected types is ranging between 70-700 copies/reaction. Specificity of the system was tested for 44 HPV types (HPV 2a, 3, 6, 7, 10, 11, 13, 16, 18, 26, 27, 28, 29, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 57, 58, 59, 66, 67, 68, 70, 72, 74, 81, 82, 83, 87, 89, 90, 91) , without detecting any cross reactions.

Clinical assessment of the system showed a 98.2% detection rate when results were compared to the L1F/L1R system. There were only 4 positive samples by the L1F/L1R system, where the real time PCR system yielded a false negative result. There were 7 samples where the real time PCR system yielded positive results, while the L1F/L1R system was negative. From these samples, 6 samples contained HPV types non classified as HR or LR. The presence of these types inhibited the amplification of the HR types from the samples, while the real time PCR amplification is more specific, containing only primers for HR amplification- therefore it can detect HR HPVs more efficiently. The real time PCR based system has an estimated sensitivity of 90.54% (67/74) and an estimated specificity of 97.1% (134/138) for high-risk HPV detection (Table 2).

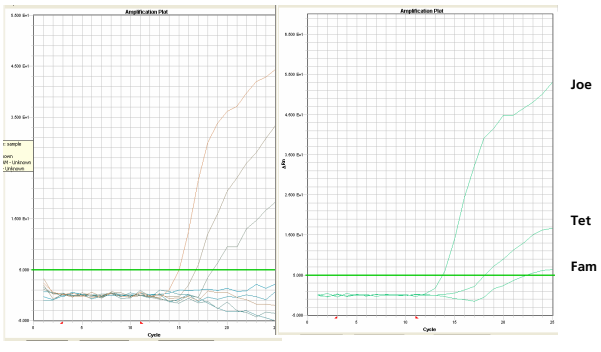

Figure 3. Positive and negative clinical samples in the HR TET detector, data obtained by ABI7900HT.

Figure 4. A clinical sample positive in the three channels, HPV 16/18, HR HPV and IC positive.

CONCLUSION

The MB based real time PCR is an efficient one step high-risk HPV screening approach. ABI7900HT allows performing real time HPV testing in 96 and 384 well format, and its combination with robotic tools for DNA extraction and PCR setup would ensure a potent and efficient high-throughput screening tool.