

PreTectTM HPV-Proofer: Real-time detection and typing of E6/E7 mRNA from carcinogenic human papillomaviruses

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Received 14 October 2006; received in revised form 29 January 2007; accepted 31 January 2007

Available online 26 March 2007

Abstract

Monitoring human papillomavirus (HPV) E6/E7 mRNA expression may provide an accurate and informative diagnostic approach for detection of oncogene activity related to the development of severe dysplasia or cervical carcinoma. A multiplex nucleic acid sequence based amplification (NASBA) assay, utilizing molecular beacon probes for real-time detection was developed for the identification of E6/E7 mRNA from HPV types 16, 18, 31, 33 and 45. The assay is called PreTectTM HPV-Proofer and this report describes the development and the analytical performance of the assay.

The reproducibility of PreTectTM HPV-Proofer with regard to a positive result was found to be between 96 and 100%, depending on HPV type. The melting temperature for the different molecular beacons was in the range of 48–55 °C, indicating conformational stability, i.e. the molecular beacons will not get activated by the 41 °C annealing temperature, but will be activated by the annealing to the target itself. The limit of detection for HPV 16 was ten SiHa or CaSki cells and for HPV 18 one HeLa cell. No cross reactivity was observed with E6/E7 mRNA from the other tested HPV types. mRNA from cervical cells was also successfully amplified after more than one year of storage.

In conclusion, the PreTectTM HPV-Proofer assay, individually identifying E6/E7 mRNA expression from five carcinogenic HPV types, is a reproducible assay that may serve as a valuable tool in monitoring HPV infections producing proteins with a transforming potential.

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Keywords: PreTectTM HPV-Proofer; mRNA; Cervical cancer; HPV; E6/E7; Screening

1. Introduction

Carcinogenic human papillomavirus (HPV) infection is considered to be the etiological agent for cervical squamous cell carcinoma (SCC). HPV DNA has been detected in as much as 99.7% of SCCs, with the most common HPV types identified as HPV 16, 18, 31, 33 and 45 (Bosch et al., 1995; Clifford et al., 2003; Munoz et al., 2003; Walboomers et al., 1999). Therefore, in order to increase the sensitivity for detection of severe dysplasias or SCC, suggestions have been made to include HPV detection and typing in screening programs (Meijer et al., 1992). However, the prevalence of HPV infections is high compared to the number of women who will develop SCC, especially in younger women, and therefore DNA tests will suffer from low specificity.

Currently available HPV DNA tests are generally targeting the conserved L1 region. The oncogenic effect, however, is dependent on the E6 and E7 genes of the high-risk HPV types, and their continuous expression seems to be necessary for transformation and maintenance of a neoplastic or dysplastic phenotype of the cells (Hawley-Nelson et al., 1989; Jeon et al., 1995; von Knebel Doeberitz et al., 1994; zur Hausen and de Villiers, 1994). Thus, detection of E6/E7 transcripts of high-risk HPV types may serve as a risk evaluation factor for the development of cervical neoplasia (Falcinelli et al., 1992; Hsu et al., 1993; Sotlar et al., 1998). In addition, a repeated monitoring of a specific HPV type gives an indication of a persistent infection, which has been shown to be a predictor of high-grade lesions (Bosch et al., 2002; Ho et al., 1995; Kjaer et al., 2002).

The nucleic acid sequence based amplification (NASBA) reaction is a method that amplifies single stranded nucleic acids or RNA equivalents (e.g., viral genomic RNA, mRNA or rRNA) even in a background of double stranded DNA. By exclusively detecting mRNA, it is possible to monitor biological activity

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based on gene expression. The NASBA method has been used in a wide variety of different diagnostic assays, detecting both bacterial and viral RNA (el-Galil et al., 2005; Bruisten et al., 1993; Greijer et al., 2002; Lanciotti and Kerst, 2001; Loens et al., 2005; van der Vliet et al., 1993; van Gemen et al., 1993). Real-time multiplex NASBA with molecular beacons is a one-tube amplification and detection method and the risk of carry-over contaminations is thereby minimized.

The technical performance of the diagnostic kit PreTect™ HPV-Proofer developed by NorChip AS, Norway is described. This assay is based on real-time multiplex NASBA and has the advantage of detecting HPV type specific E6/E7 mRNA from the carcinogenic HPV types 16, 18, 31, 33 and 45. Two primer- and probe-sets are included in each reaction vessel. PreTect™ HPV-Proofer can therefore be used in monitoring a persistent viral infection. Also, due to the carcinogenic capacity of the E6/E7 gene products, detection of these transcripts identifies the HPV infections with a transforming potential.

2. Materials and methods

2.1. Cell lines

HeLa (containing HPV 18), SiHa and CaSki (containing HPV 16) cell-lines were obtained from the American Type Culture Collection, USA. SiHa and HeLa cell-lines were maintained in Dulbecco's modified Eagles medium (DMEM); CaSki cells were maintained in RPMI-medium. The media were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 25 µg/ml gentamicin. The cells were incubated at 37 °C in a 5% CO₂ atmosphere. The cells were trypsinated, counted using Bürkers chamber, and lysed in NASBA lysis buffer (NucliSens, BioMérieux, Boxtel, Netherlands). SiHa cells contain one to two copies of integrated HPV 16 DNA; CaSki cells contain 500–600 copies of HPV 16 DNA; HeLa cells contain approximately 10–50 copies of HPV 18 DNA per cell (Syrrjanen et al., 1988). The HPV negative cell line MA-11 (Rye et al., 1996) was used as background in the limit of detection (LOD) study.

2.2. Clinical samples

The clinical samples used in the testing for genetic specificity have been described elsewhere (Molden et al., 2005).

2.3. Primers and probes

The target area for primer design was the E6/E7 region from HPV types 16, 18, 31, 33 and 45. All primers and probes were designed to detect the full-length transcripts. A full-length transcript is defined as any un-spliced transcript covering at least the E6/E7 region and may also be fused to human sequences. In the verification and performance evaluation of the assay, two different types of probes were used for detection, an ECL probe and a molecular beacon probe.

Detection of mRNA from the human U1 small nuclear ribonucleoprotein (snRNP) specific protein A (U1A) is used to monitor sample mRNA integrity. Two differently labelled

molecular beacon probes are used in each multiplex reaction. Fluorescein (FAM) is used as fluorophore for the detection of HPV 16, 31, and 33; Texas Red (TxR) is used for the detection of U1A, HPV 18, and HPV 45. All of the molecular beacons contain the non-fluorescent quencher Dabsyl CPG (1-dimethoxytrityloxy-3-[O-(N-4'-sulfonyl-4-(dimethylamino)-azobenzene)-3-aminopropyl]-propyl-2-O-succinoyl-long chain alkylamino-CPG).

2.4. Positive controls of assay performance

Positive controls were designed to monitor the integrity of primers, probes and NASBA reagents. These positive controls comprise artificial target sequences covering a somewhat short stretch of the native transcript target sequence. The molecular beacons cannot directly bind to these oligos, but during the NASBA reaction they are amplified and converted to templates for the molecular beacons. As a supplement to the cell line derived templates, these oligos were also used to investigate the sensitivity of the assay.

2.5. Nucleic acids isolation

The isolation of DNA and RNA was performed by a silica-based method (Boom et al., 1990) using the NucliSens Extractor (BioMérieux, Boxtel, Netherlands) and the protocol for automated isolation. RNA and DNA were isolated and co-purified after lysis of 1 ml cervical smear sample in lysis buffer, from a total of 9 ml (BioMérieux, Boxtel, Netherlands). The extracted DNA/RNA was eluted in 40–50 µl elution buffer (1 mM Tris-HCl, pH 8.5) and stored at –70 °C prior to analysis.

2.6. PCR

Isolated DNA (stored at –70 °C for a maximum of 6 months) was subjected to PCR using the consensus GP5+/6+ primers (Husman et al., 1995). Gp5+/6+ PCR was carried out in a 50 µl reaction volume containing 75 mM Tris-HCl (pH 8.8 at 25 °C), 20 mM (NH₄)₂SO₄, 0.01% Tween 20, 200 µM each of dNTP, 1.5 mM MgCl₂, 1 U recombinant *Taq* DNA Polymerase (MBI Fermentas), and 50 pmol of each primer. Three microlitres of DNA sample were used. A denaturation step of 2 min at 94 °C was followed by 40 cycles of amplification in a PCR processor (Primus 96, HPL block, MWG, Ebersberg, Germany). Each cycle included a denaturation step at 94 °C for 1 min, a primer-annealing step at 40 °C for 2 min and a chain elongation step at 72 °C for 1.5 min. The final elongation step was prolonged by 4 min to ensure a complete extension of the amplified DNA.

HPV type-specific PCR for HPV type 16, 31, 33, and 35 was performed according to Karlsten et al. (1996), and HPV types 6/11, 18, 39, 45, 51, 52, and 58 according to the PCR protocol described below (NorChip AS, Klokkearstua, Norway). The PCR primers are directed against different regions of the genome compared to the NASBA primers.

The HPV 6/11, 18, and 51 PCR reagent mixtures were similar to the Gp5+/6+ PCR reagent-mixture, except for one change in the protocol: 2.0 mM MgCl₂ was used instead of 1.5 mM. The

HPV 39, 45, 52, and 58 PCR was carried out without $(\text{NH}_4)_2\text{SO}_4$ and Tween 20, but included 10 mM Tris–HCl (pH 8.3 at 25 °C), 50 mM KCl, and 0.001% gelatin. HPV type specific PCR utilized 25 pmol of each primer. A 2 min denaturation step at 94 °C was followed by 35 cycles of amplification. Each cycle included a denaturation step at 94 °C for 0.5 min, a primer-annealing step at 57 °C for 0.5 min and a chain elongation step at 72 °C for 1 min. The final elongation step was prolonged by 10 min to ensure a complete extension of the amplified DNA. The primer annealing temperatures for HPV 6/11 and 51 were 51 and 55 °C, respectively. A β -globin primer set was used to assess specimen adequacy (Operating procedure, University Hospital Vrije Universiteit, Amsterdam, The Netherlands). DNase free water was used as negative control.

Visualization of the PCR products was performed on a DNA 500 chip according to the manufacturer's protocol (Agilent Technologies, CA, USA). The DNA chip utilizes a micro scale gel electrophoresis with an LOD of 0.5–50 ng/ μl . The results were interpreted using the Bioanalyzer 2100 software.

2.7. Real-time singleplex NASBA

Singleplex NASBA was performed as described by Kieivits et al. (1991) with some modifications. The NASBA reaction (Fig. 1) is isothermal at 41 °C and utilizes the activity of AMV reverse transcriptase (RT), RNase H and T7 RNA polymerase, together with two primers to amplify single-stranded RNA (Compton, 1991). The products can be detected in real-time by the use of molecular beacons (Tyagi and Kramer, 1996). Molecular beacons are single-stranded oligonucleotide probes having a stem-loop structure that fluoresce only upon hybridization to their target. The loop contains a sequence that is complementary to the target and a stem region that is unrelated to the target but has a double-stranded structure. One arm of the stem

is labelled with a fluorescent dye (fluorophore) and the other arm of the stem is labelled with a non-fluorescent quencher. In the non-hybridized state the fluorescent signal is “captured” by the quencher (Stryer, 1978), but upon hybridization the fluorophore and the quencher are separated and a fluorescent signal is transmitted. A premix of reagents (nucleotides, dithiothreitol and MgCl_2) was made in 80 μl diluent (Tris–HCl, 45% DMSO) followed by addition of 16 μl KCl stock solution (BioMérieux, Boxtel, The Netherlands), 5 μl of each primer (10 μM), 2.5 μl of probe (20 μM), and 11.5 μl NASBA quality water. Five microlitres of RNA sample (of a 5 \times dilution) was added to 10 μl NASBA pre-mixture and incubated for 4 min at 65 °C to destabilize secondary structures of RNA. The mixture was then cooled down to 41 °C for 4 min. The amplification reaction was started by addition of 5 μl enzyme mixture (AMV-RT, RNase H, T7 RNA polymerase and BSA). Total reaction volume was 20 μl .

2.8. PreTect™ HPV-Proofer; real-time multiplex NASBA

Real-time multiplex NASBA is based on the same principles as singleplex NASBA. The assay PreTect™ HPV-Proofer was optimized by testing different combinations and concentrations of primers and probes together, ending up with two primer and probe pairs in each reaction vessel that gave similar relative fluorescent units (RFU). Three premixes were made by the reconstitution of reagent sphere(s) in the reagent sphere diluent, followed by addition of either U1A/HPV 16, HPV 33/45, or HPV 18/31 primer/molecular beacon mixes and KCl stock solution. The primer and probe mixtures have been optimized for multiplex reaction. Ten microlitres of this premix were distributed to each well in a reaction plate, followed by addition of 5 μl RNA sample (of a 5 \times dilution) and incubation for 4 min at 65 °C and 4 min at 41 °C. The reaction was started by addition

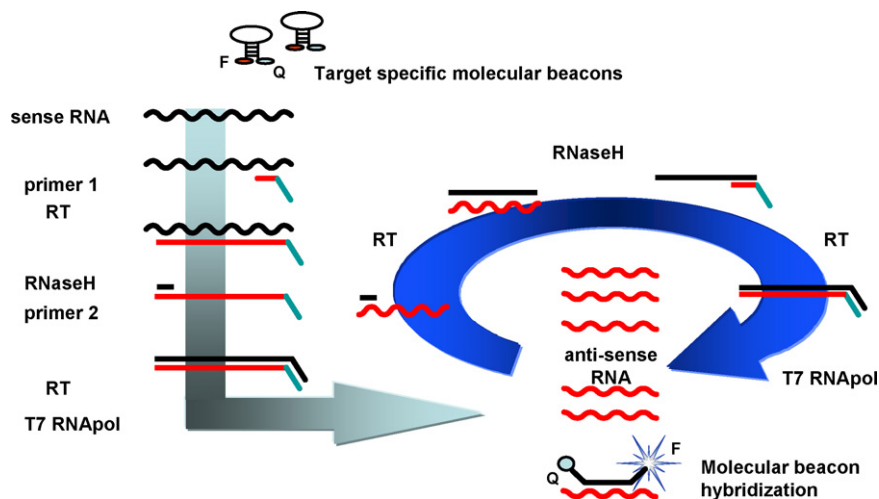


Fig. 1. Schematic presentation of NASBA including molecular beacon detection. The light arrow represents the initiation phase and the dark arrow (curved) represents the cyclic phase. The activities of reverse transcriptase (RT), RNase H, T7 DNA dependent RNA polymerase (T7 RNA pol) and the primer and molecular beacon binding activities are indicated. In the absence of a complementary sequence, the stem of the molecular beacon hairpin structure is closed and the quencher (Q) prevents the detection of the fluorescent signal emitted by the fluorophore (F). By hybridization of the loop sequence of the beacon with a complementary sequence, the stem region is forced open and the fluorescent signal becomes detectable. This figure is a modification of a figure published by Deiman et al. (2002) and reproduced with permission granted from copyright holder (Humana Press Inc.).

of enzymes and measured in real-time using the Lambda FL 600 fluorescence reader (Bio-Tek, Winooski, USA) at 41 °C for 2 h and 30 min. The PreTect™ Analysis Software (NorChip AS, Klokkestua, Norway) was used for routine analysis of the experimental data. The excitation λ (nm) filters for FAM and Texas Red were 485/20 and 590/20, respectively, and the emission λ (nm) filters were 530/25 and 645/40, respectively.

Artificial and standardized oligos, corresponding to the viral sequence, were used as positive controls for HPV 16, 18, 31, 33, and 45. RNase free water was used as negative control. U1A served as mRNA quality control.

2.9. Visualization of NASBA product by electrochemiluminescence (ECL)

ECL is a well documented and sensitive method for the visualization of NASBA products and therefore used as comparison for the molecular beacon assay. ECL detection was performed according to the NucliSens® Detection Kit protocol (BioMérieux, Boxtel, The Netherlands). The NASBA reaction products were diluted 20× in detection diluent and 5 μ l of this mixture was added to 20 μ l hybridization mixture and hybridized at 41 °C for 30 min. The hybridization buffer (5× SSC) contained 2×10^{12} copies of an ECL [Tris(2,2-bipyridine)ruthenium(II) complex]-labelled detection probe and 20 μ g of streptavidin-coated magnetic beads pre-coated with 2×10^{12} copies of a biotinylated capture probe. The ECL detection was performed using the NASBA QR system (BioMérieux, Boxtel, The Netherlands). A positive result by the ECL assay was defined as 10,000 ECL units or more.

2.10. DNA sequencing

A selection of HPV DNA and mRNA positive samples was sequenced for confirmation of genetic specificity. The sequencing was performed at the University of Oslo (Norway) with the MegaBACE Sequence Analyzer from Amersham Biosciences (Little Chalfont, England) according to their protocol. The sequences were compared to other known sequences in a database search (BLAST from National Center for Biotechnology Information [NCBI]: <http://www.ncbi.nlm.nih.gov/BLAST/>).

2.11. Thermal denaturation profiles

The melting point or temperature (T_m) is defined as the midpoint of the temperature range at which the double stranded DNA molecular beacon probe is denatured. For molecular beacons, this means that 50% of the molecules have adopted a hairpin structure. The melting points for the different molecular beacon probes in the PreTect™ HPV-Proofer kit was determined by real-time detection of fluorescence (iCycler, Bio-Rad, Hercules, CA) using a standard NASBA reaction mixture. First, the reaction mixture was heated to 65 °C for 1 min followed by cooling down to 20 °C for 5 min followed by an increase of 0.5 °C per minute and a temperature range of 20–80 °C. The melting profile of the molecular beacons in the presence of template was

also determined using target sequences to which the molecular beacons could bind directly (anti-sense probe). No amplification can take place for these anti-sense probes. The template was present in a concentration that was ten times higher ($\sim 2.0 \mu$ M) than the molecular beacon ($\sim 0.2 \mu$ M). In total 16 parallel runs were tested for each molecular beacon and the mean melting point was calculated. The fluorescence versus temperature was calculated as the mean between the start and the stop of the exponential amplification phase.

2.12. mfold analysis

For predicting the secondary structure of single-stranded DNA, we used mfold version 3.1 software-based on minimal free energy (<http://www.bioinfo.rpi.edu/applications/mfold/>) (Zuker, 2003), using default settings, 100 mM Na⁺, 3 mM Mg²⁺ and a temperature of 55 °C.

3. Results

3.1. Assay setup

Our NASBA primers and molecular beacon probes were positioned in the transcript region coding for the oncogenic E6 and E7 proteins from the five most common carcinogenic HPV types 16, 18, 31, 33 and 45. Two primer/probe-sets were combined in each reaction vessel. HPV 18 is run together with HPV 31, HPV 33 together with HPV 45, and HPV 16 together with the U1A sample integrity control.

3.2. Thermo stability

The mean melting points for the different molecular beacons used in the PreTect™ HPV-Proofer kit are listed in Table 1. The melting points for the molecular beacon probes were in the range of 48–55 °C, well above the temperature of the NASBA reaction, which is 41 °C. At 41 °C, the molecular beacons still have a hairpin structure (as illustrated in Fig. 2 where 41 °C is in the beginning of the exponential phase of the sigmoid curve of the melting temperature). At lower temperatures a low fluorescent signal was observed indicating that the arms of the molecular beacons were closed. As the temperature increased,

Table 1
Melting temperatures (T_m) for the molecular beacon probes in the PreTect™ HPV-Proofer kit

Molecular beacon	T_m (°C)
HPV 16	49
HPV 18	51
HPV 31	49
HPV 33	50
HPV 45	55
U1A	51

At 41 °C, the molecular beacons for the different HPV types still have a hairpin structure (as illustrated in Fig. 1 where 41 °C is in the beginning of the exponential phase of the sigmoid curve of the melting temperature).

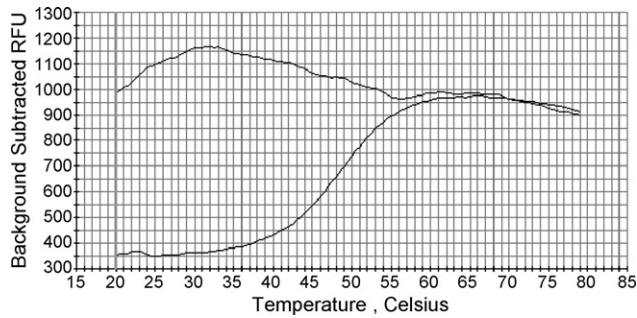


Fig. 2. Molecular beacon melting profiles of PreTect™ HPV-Proofer, exemplified by HPV 16. Melting points for the molecular beacons were determined by detection of fluorescence by the iCycler (Bio-Rad). The sigmoid curve represents the molecular beacon melting profile without template while the other curve represents the melting profile of the molecular beacon with template present.

the fluorescent signal increased due to the opening of the arms, resulting in separation of quencher and fluorophore. In the presence of a template, a fluorescent signal was observed even at low temperatures due to hybridization of molecular beacons and template. An illustration of a molecular beacon melting curve, with and without a complementary template, is shown in Fig. 2. The components of the NASBA reaction mix had a sufficiently stabilizing effect on the molecular beacon probes so as to obtain a melting curve consistent with the stem-loop conformation of the probes.

3.3. Reproducibility

The reproducibility of the PreTect™ HPV-Proofer assay was tested by using four to seven samples of each type (i.e. positive for E6/E7 mRNA). Each sample was run 6–27 times (Table 2). A relative increase of the fluorescent signal of more than 1.7 (defined as positive) was observed in 96.4–100% of the runs. Oligos used as positive controls in the PreTect™ HPV-Proofer kit were tested with 30 parallels and a positive result was observed in all cases (data not shown).

3.4. Genetic specificity

The PreTect™ HPV-Proofer assay was tested for its genetic specificity (cross reactivity) by performing the assay on 357

Table 2
Reproducibility of PreTect™ HPV-Proofer

HPV type	Number of samples	Number of runs per sample	Relative increase in fluorescent signal	Reproducibility of positive results (%)
HPV 16	6 ^a	13–26	>1.7	100
HPV 18	7	7–26	>1.7	99.1
HPV 31	4	16–26	>1.7	96.4
HPV 33	7	11–27	>1.7	100
HPV 45	7	9–27	>1.7	100

A positive result is defined as an increase of the fluorescent signal of more than 1.7.

^a In addition, one sample was positive in 12 out of 26 runs. This is most likely explained by a low number of transcripts.

Table 3
Cross reactivity with other HPV types

PreTect ^a primers	HPV type (demonstrated by PCR)									Total number
	16	18	31	33	45	6/11	35	51	X	
16	29	0	0	0	0	0	0	0	0	0
18	0	22	0	0	0	0	0	0	0	1
31	0	0	13	0	0	0	0	0	0	0
33	0	0	0	9	0	0	0	0	0	1
45	0	0	0	0	14	0	0	0	0	1
Tested	29	22	13	9	14	28	8	13	221	357

All 357 samples were positive by consensus PCR and type specific PCR for the respective HPV types. HPV X was positive by consensus Gp5+/6+ PCR, although negative for HPV 6/11, 16, 18, 31, 33, 35, 45, and 51.

^a PreTect™ HPV-Proofer.

cytological samples in which a single HPV infection had been established by PCR (Table 3). These samples were typed by PCR for HPV types 6/11, 16, 18, 31, 33, 35, 45, and 51 (samples that were positive for Gp5+/6+, yet negative for the specified HPV type are termed HPV X). In addition, five samples, which were positive by PCR for HPV 39 ($n=2$), 52 ($n=1$), and 58 ($n=2$) were tested (data not shown). A single HPV infection is defined as positive for only one of the HPV types tested by PCR and does not exclude infection with other HPV types not included in the PCR. No cross reactivity was observed with the HPV types that were tested. However, three samples defined as HPV X were positive for E6/E7 mRNA from HPV 18, 33, or 45 by PreTect™ HPV-Proofer, yet negative by type-specific PCR. In addition, a selection of 30 samples, which were both HPV DNA and E6/E7 mRNA positive for HPV types 16 ($n=7$), 18 ($n=7$), 31 ($n=5$), 33 ($n=6$), and 45 ($n=5$), were sequenced using the Gp5+/6+ PCR primers and compared to other known sequences in a BLAST database search. NASBA, PCR and DNA sequencing gave an identical identification of the samples.

3.5. Limit of detection (LOD)

The LOD for the PreTect™ HPV-Proofer assay was determined by testing oligos in serial dilutions in four parallel runs without other nucleic acids present (Table 4). Some differences between the different HPV types were observed. The HPV 16 primers and probes were the most sensitive ones, detecting oligos at 0.1 nM. While the HPV 18 and the HPV 45 primers and probes were the least sensitive, detecting oligos at 10 nM.

Table 4
Limit of detection (LOD) for PreTect™ HPV-Proofer on oligos

Concentration (nM)	U1A	HPV 16	HPV 18	HPV 31	HPV 33	HPV 45
10	+	+	+	+	+	+
1	+	+	–	+ ^a	+	–
0.1	+	+	–	–	+ ^a	–
0.01	+	–	–	–	–	–
0.001	–	–	–	–	–	–

A positive test result is defined as a relative increase in the signal of more than 1.7 in four parallel runs.

^a Three out of four runs gave a positive test result.

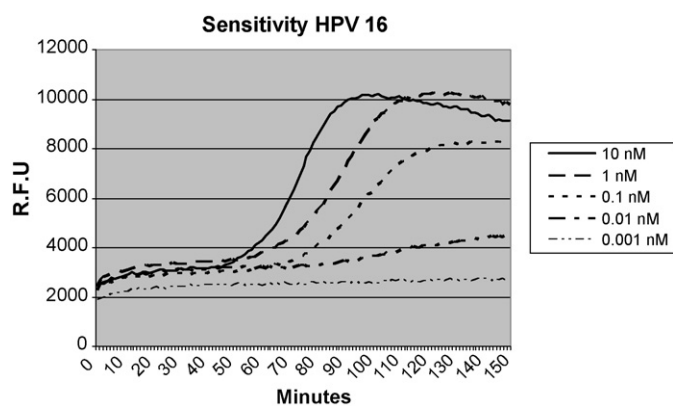


Fig. 3. Limit of detection (LOD) of PreTectTM HPV-Proofer using oligos as template.

An illustration of the real-time NASBA reaction in response to different concentrations of template is given in Fig. 3. To further ascertain the LOD of PreTectTM HPV-Proofer (real-time multiplex NASBA), cell lines containing HPV 16 (SiHa, CaSki) and HPV 18 (HeLa) were tested with real-time singleplex NASBA, and electrochemiluminescence (ECL) assays in four 10-fold serial dilutions containing from 10^5 cells to less than one cell. For comparison, cell lines were also tested by consensus and type specific PCR. The LOD was defined as the concentration (number of cells) producing a 100% hit-rate in four separate trials. The LOD for HPV 31, HPV 33 and HPV 45 has not been established as cell lines containing these HPV types could not be obtained at the time.

The consensus and type specific PCR assays had a LOD ranging from 1 to 100 cells, depending on the cell line (Table 5). The LOD of singleplex and multiplex NASBA ECL assays, detecting 1–10 cells, was almost identical, and corresponded to the LOD of the real-time NASBA molecular beacon assay. However, the LOD of the singleplex NASBA molecular beacon assay was about 10-fold lower than the corresponding multiplex reaction for HPV 16. This was not observed for HPV 18. The difference was minimal in that the multiplex reaction detected approximately 75% of the samples at the LOD of the singleplex reaction. The LOD was also tested in a background of HPV negative MA-11 cells (data not shown). This did not notably influence the performance, neither of the singleplex nor the multiplex HPV 18/HPV 31 reactions. However, the LOD of the HPV 16 multiplex reaction was reduced. The LOD threshold for the HPV

16/U1A multiplex reaction was in a ratio of one SiHa or one CaSki cell, in a background of 100 HPV negative cells.

3.6. Cut-off

The cut-off level for a NASBA application with real-time detection is defined as the level of fluorescence that discriminates between a positive and a negative test result. In order to reflect whether a given specimen is positive or negative for a particular target RNA, it is critical that the cut-off level is determined accurately. Two principal parameters are involved in determining the cut-off level; baseline fluorescence and the variation observed in the baseline fluorescence signals. Depending on the molecular beacon and the fluctuations in the reaction medium, the level of the background signal as well as the baseline fluorescence may vary between reactions. Instead of determining baseline fluorescence values by a significant number of confirmed negative specimens, it was decided to calculate mathematically the baseline level by using the 1st and 2nd derivative for each sample. By doing this, the negative control does not need to contain the same sample matrix (for example cervical cell samples) as the biological specimens that will be investigated with the NASBA assay. A RFU ratio above 1.7 is regarded as a positive result and a RFU ratio below 1.4 is generally regarded as a negative test result. A RFU ratio between 1.4 and 1.69 is regarded as indeterminate. A repeated indeterminate result should be considered positive. For cases where a sigmoid curve is observed but the RFU ratio is below 1.4, it is recommended to re-extract the sample with a higher input of template as the sample may contain a low level of template. The determination of the cut-off level is based on comparisons to ECL detection results and evaluation of the difference between the highest and the lowest values for the baseline fluorescence in a random sampling of a significant number of negative and positive samples (data not shown).

A PreTectTM HPV-Proofer reaction which is positive for HPV 16, as well as for the U1A mRNA control is shown in Fig. 4a; a reaction which is positive only for U1A is shown in Fig. 4b. RNA that was stored at -70°C for more than 12 months was well conserved and amplifiable by PreTectTM HPV-Proofer.

4. Discussion

This report describes the design, design verification and analytical performance of PreTectTM HPV-Proofer, a real-time

Table 5
Limit of detection (LOD) for PreTectTM HPV-Proofer on cell lines

Primer	Single NASBA ^a /ECL assay ^b			Multiplex NASBA ^c /ECL assay ^b			PCR		
	SiHa	CaSki	HeLa	SiHa	CaSki	HeLa	SiHa	CaSki	HeLa
GP5+/6+	–	–	–	–	–	–	100	1	100
U1A	10/10	10/10	1/1	10/10	10/1	10/1	–	–	–
HPV 16	1/1	1/1	–	10/1	10/1	–	100	1	–
HPV 18	–	–	<1/<1	–	–	<1/<1	–	–	10

^a Real-time singleplex NASBA assay.

^b The ECL detection was performed with the product obtained from the NASBA molecular beacon assay.

^c Real-time multiplex NASBA assay, PreTectTM HPV-Proofer.

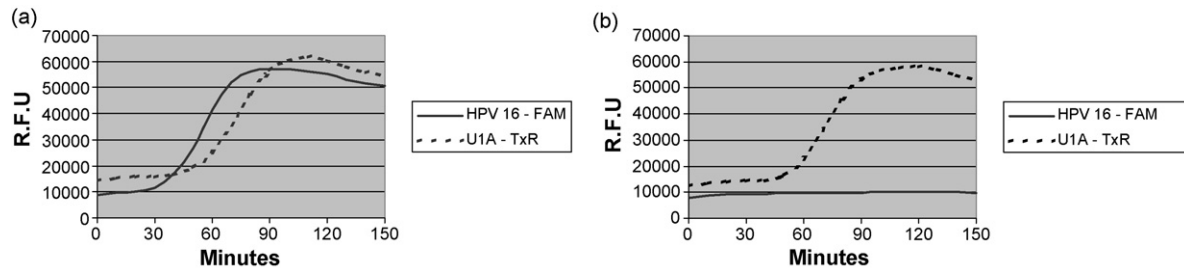


Fig. 4. Real-time multiplex HPV 16/U1A NASBA. (a) A SCC sample positive for HPV 16 E6/E7 mRNA and the U1A mRNA performance control. (b) A SCC sample negative for HPV 16 E6/E7 mRNA but positive for the U1A mRNA performance control. U1A, sample mRNA quality control. R.F.U., relative fluorescence units. This is a visualization of the one-tube multiplex NASBA run and it is not identical to how it is presented in the software from NorChip AS.

multiplex NASBA assay for rapid detection of E6/E7 mRNA from each of the carcinogenic HPV types 16, 18, 31, 33 and 45. Real-time multiplex NASBA with molecular beacon detection is carried out in sealed vessels, thereby minimizing the risk of carry-over contaminations.

To date, the focus of HPV detection has been on DNA. However, the majority of HPV infections are transient or non-transforming, creating a high false positive rate and a low clinical specificity for HPV DNA testing methods. In contrast, as the E6/E7 oncoproteins are the main cause of a malignant transformation of the cell, E6/E7 mRNA detection may be better suited for diagnostics regarding the development of severe dysplasias. Upon integration of HPV into the human genome, which is regarded as an important factor for SCC development, E6/E7 mRNA expression is deregulated and will increase to a level causing transformation (Jeon et al., 1995).

No cross-reactivity was observed between PreTect™ HPV-Proofer and E6/E7 mRNA from phylogenetically related HPV types that were tested. However, three samples, which were positive by consensus PCR, but negative by type-specific PCR, were E6/E7 mRNA positive with PreTect™ HPV-Proofer. This could be due to differences in sensitivities of the methods. In general, the specificity of a molecular beacon has been demonstrated to be better than a standard linear probe and it can be designed to distinguish targets that differ with a single nucleotide base-pair (Bonnet et al., 1999). For example, no false-positive results were obtained for the West Nile (WN) and St. Louis encephalitis (SLE) viruses, for any of the serologically related flaviviruses tested (Lanciotti and Kerst, 2001).

The LOD for the Gp5+/6+ PCR assay was found to be in the range of 1–100 cells for HPV 16 (SiHa, CaSki) and for HPV 18 (HeLa). This is in good agreement with other methods, like the enzyme immunoassay variant of the Gp5+/6+ PCR and Southern blot analysis (Jacobs et al., 1996). The LOD of the real-time NASBA assay and the NASBA ECL assays performed with E6/E7 mRNA from HPV 16 (SiHa, CaSki) and HPV 18 (HeLa) was better than the LOD of PCR by a factor of 10. This most likely reflects the higher number of detectable mRNA copies compared to the DNA copy number (Jeon et al., 1995) or a possible difference in sensitivity of the assays. The copy number of the viral genome in SiHa cells is 1–2, while CaSki cells have as much as 600 copies of the genome. However, it has been reported that SiHa cells have 10–20 times higher E7 mRNA expression

levels than CaSki per HPV DNA copy (Kozuka et al., 2000) and a similar LOD of SiHa and CaSki cells therefore indicates that the PreTect™ HPV-Proofer reaction amplifies mRNA and not DNA. The LOD for PreTect™ HPV-Proofer was also determined using oligos as templates. However, the amplification of the target is dependent on the length as well as on the secondary structure of the product and this is therefore not fully comparable to the clinical situation. The oligos are shorter than the viral mRNA sequence and therefore do not reflect the overall efficacy of amplification, but rather indicate efficacy of primer and probe annealing.

Regarding single- and multiplex NASBA, only minor differences were observed. A benefit of running the reactions multiplex, meaning two or more real-time amplifications in each tube, is the smaller amount of consumables and sample material used. A multiplex reaction also makes preparations easier and allows for a higher throughput of samples. The use of molecular beacons in real-time PCR, with multiplex detection of up to four targets, has been reported (Marras et al., 1999; Vet et al., 1999).

The reproducibility of a positive result by the PreTect™ HPV-Proofer assay was in the range of 96–100%, with the exception of a few samples. For example, one sample was positive for E6/E7 mRNA from HPV 16 in 12 out of 26 runs. This is most likely explained by a low number of transcripts.

RNA integrity is monitored by amplification of the human U1A transcript. U1A is a low abundance housekeeping gene and a negative test result will reveal sample degradation or low number of cells in the original sample. Accordingly, in the three cell lines HeLa, CaSki and SiHa, the LOD of U1A was found to be equal to or lower than the LOD of HPV. A detectable level of U1A mRNA shows that the RNA is preserved and a negative HPV E6/E7 mRNA result is most probably not due to reduced quality of the sample. We have shown in a previous study that U1A and HPV E6/E7 mRNA are stable for at least 12 months when stored at -70°C (Molden et al., 2005). This shows that mRNA is stable over time when stored under the correct conditions and is suitable for routine diagnostic assays.

In conclusion, the PreTect™ HPV-Proofer assay, identifying individually E6/E7 mRNA expression from five carcinogenic HPV types, is a reproducible assay that may serve as a valuable tool in monitoring HPV infections producing proteins with a transforming potential.

Acknowledgements

This work was supported by grant number 145316/213 from The Research Council of Norway. NorChip AS provided the consumables. We also thank everybody at NorChip AS for their valuable help, especially Siri Hovland, Marit Øder Øye, Inger Marie Falang and Petter Grønn for technical assistance. We would also like to thank Gunhild Mælandsmo at the Norwegian Radium Hospital for providing an HPV negative cell line.

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