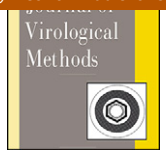
Journal of Virological Methods 187 (2013) 345–351

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet



Simultaneous detection, typing and quantitation of oncogenic human papillomavirus by multiplex consensus real-time PCR

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Article history:
Received 17 January 2011
Received in revised form 11 July 2012
Accepted 30 October 2012
Available online 14 November 2012

Keywords:
HPV
High-risk HPV
Real-time PCR
Cervical cancer
Cervical screening

A B S T R A C T

A consensus multiplex real-time PCR test (PT13-RT) for the oncogenic human papillomavirus (HPV) types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66 is described. The test targets the L1 gene. Analytical sensitivity is between 4 and 400 GU (genomic units) in the presence of 500 ng of human DNA, corresponding to 75,000 human cells. HPV types are grouped into multiplex groups of 3 or 4 resulting in the use of 4 wells per sample and permitting up to 24 samples per run (including controls) in a standard 96-well real-time PCR instrument. False negative results are avoided by (a) measuring sample DNA concentration to control that sufficient cellular material is present and (b) including HPV type 6 as a homologous internal control in order to detect PCR inhibition or competition from other (non-oncogenic) HPV types. Analysis time from refrigerator to report is 8 h, including 2.5 h hands-on time. Relative to the HC2 test, the sensitivity and specificity were respectively 98% and 83%, the lower specificity being attributable to the higher analytical sensitivity of PT13-RT. To assess type determination comparison was made with a reversed line-blot test. Type concordance was high ($\kappa = 0.79$) with discrepancies occurring mostly in multiple-positive samples.

1. Introduction

Human papillomavirus (HPV) infection is the primary cause of cervical cancer (Walboomers et al., 1999). Of the 118 types described by de Villiers et al. (2004) 13 have been confirmed as being oncogenic in genital mucosa (IARC, 2007).

HPV testing has been shown to have a higher sensitivity for high-grade cervical dysplasia than the Papanicolaou test, although the specificity is lower due to the high prevalence of benign, transient HPV infections (Cuzick et al., 2006). Many countries have adopted HPV testing as a secondary screening (triage) test for low-grade and equivocal dysplasia (Wright et al., 2002; Waage, 2005) and its use in primary screening has also been suggested (Dillner et al., 2008). The HPV test used most widely is the Qiagen/Digene hybrid capture (HC2) test. HC2 is commercially available worldwide, straightforward to perform and interpret, and supported by a large body of scientific literature making it the baseline against which other tests are compared. The HC2 test classifies the HPV types detected as high-risk or low-risk, without more detailed genotyping. Where genotyping is required more sophisticated tests based on nucleic acid amplification are required.

There are several advantages to genotyping: it distinguishes benign serial transient infections from type-specific persistent infections which have a high probability of neoplastic progression; it distinguishes HPV types of differing oncogenic potential (Schiffman et al., 2005); and it provides epidemiological information which will be of particular value in monitoring the population effects of vaccination against HPV 16 and HPV 18.

Genotyping tests that cover the full range of oncogenic HPV types are available commercially but all the currently available tests involve post-PCR sample handling which necessitates stringent, labour- and resource-intensive molecular hygiene procedures to prevent carry-over contamination. This limits the applicability of such tests in the routine laboratory.

With the aim of providing a more rapid, less labour-intensive HPV typing test that operates in a closed system, a quantitative real-time PCR method using consensus primers and multiplexed type-specific 5′-hydrolysis probes was developed. This test, allows sensitive and specific detection and typing of the 13 oncogenic HPV types HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66. Using standard 96-well instrumentation, clinical samples may be analysed in batches of up to 22 and the entire analysis, including sample processing and reporting can be completed in one working day.

Abbreviations: HPV, human papillomavirus; GU, genomic units; LNA, locked nucleic acid; MGB, minor groove binder; HC2, hybrid capture test.

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0166-0934
http://dx.doi.org/10.1016/j.jviromet.2012.10.021

2. Materials and methods

2.1. Human DNA

All solutions of HPV control DNA were diluted to the appropriate concentrations in a 100 ng/μl solution of human DNA (Sigma D4642, Sigma–Aldrich, St. Louis, MI) in 1× TE buffer (10 mM Tris, 1 mM EDTA pH 8.0) in order to mimic the composition of a cell-rich clinical sample as closely as possible.

2.2. Plasmids

Plasmids containing entire HPV genomes were provided by John Arrand with the permission of Lutz Gissmann (HPV 6, 11, 16 and 18), Gerard Orth (HPV 33 and HPV 39), Attila Lorincz (HPV 31, 35, 45, 52 and 56), Saul Silverstein (HPV 51) and Toshihaki Matsukara (HPV 58 and 59). Plasmids were transformed into *Escherichia coli* DH5-alpha and isolated by caesium chloride density gradient centrifugation (Sambrook et al., 1989) or Qiagen HiSpeed Midi Kit (Qiagen GmbH, Hilden, Germany). DNA concentrations were determined by ultraviolet spectrophotometry (Sambrook et al., 1989). The HPV 66 plasmid was a synthetic sequence corresponding to nucleotides 6320–6600 in the HPV 66 genome (U31794) cloned into pUC57, synthesised by Genscript Corporation, Scotch Plains, NJ. Ten-fold serial dilutions of plasmid DNA in 100 ng/μl human DNA were prepared in order to provide concentrations appropriate for experimental use.

2.3. Clinical samples

Were cervical cytobrush samples sent to Unilabs Telelab from clinical practises in Southeastern Norway for routine HPV analysis subsequent to findings of low-grade dysplasia, equivocal cytology or inadequate sample according to Norwegian guidelines (Waage, 2005). Samples were collected and transported in CYTYC thin prep transport medium (CYTYC, Crawley, UK) according to the manufacturer's recommendations. 10 ml of sample was centrifuged at 3000 rpm for 10 min, the pellet was resuspended in 100 μl phosphate buffered saline and DNA was extracted using the MagNAPure automatic DNA extraction instrument with the MagNAPure LC DNA extraction kit (Roche Diagnostics, Penzberg, Germany). DNA concentration was measured by determining *A*260 and *A*280 using the Eppendorf Biophotometer (Eppendorf AG, Hamburg, Germany) according to the manufacturer's instructions. Samples yielding DNA concentrations < 5 ng/μl were considered to contain too little material and rejected. Samples ($N = 206$) were tested by hybrid capture test, PT13-RT and reverse line-blot analysis.

2.4. Hybrid Capture test

The Digene Hybrid Capture (HC2) test (Digene, Gaithersburg, MD) was performed using 10 ml of sample according to the manufacturers' instructions.

2.5. Reverse line-blot analysis

Biotin-labelled amplicons were generated using primers PTf and 5′ biotin-labelled PTrGr3 (see Table 2), which were designed to have broad specificity for mucosal HPV types and which were found (data not shown) to amplify all the oncogenic HPV types targeted in the real-time PCR reaction. PCR reactions were run in a 50 μl volume containing 5 μl of sample DNA in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using DyNAmo SYBR green qPCR mix with ROX passive control dye (Finnzymes, Espoo, Finland) and the standard PCR program described in Section 2.8. Sample quality was controlled by a parallel amplification of the human globin gene (Saiki et al., 1985) under the same reaction conditions. Denaturation profiles from 60 to 91 °C (temperature increment 1.75 °C/min) were obtained and melting temperatures were determined by the position of peaks in the first derivative plot using the software provided with the instrument. The success of β-globin PCR was assessed according to the presence of a denaturation peak at 81 °C.

Table 1
Probes for reverse line blot analysis.

Target	Sequence
HPV 16	CTGCAAATTTAGCCAGTTCAAA
HPV 18	GCTTCACCTGGCAGCTGTGT
HPV 31	GGCTCCGGTTCAACAGCTAC
HPV 33	TGCCTCTATTCAAAGCAGTGC
HPV 35	GGTACCACTGGCACATTG
HPV 39	GGCACAGATATACGTGCAAA
HPV 45	GGCACTAGCGCTAATATGC
HPV 51	GTAGTGGTAATGGCCGTG
HPV 52	GGGTCTAACTCTGGCAATA
HPV 56	GGTAGCAATGGTAGAGAAC
HPV 58	GGTCCGGTAATACTGCAG
HPV 59	GGTACTGACATACGTGCC
HPV 68	GGCACTGACATACGTGACA

5′ amino-labelled probes (MWG Biotech, Ebersburg, Germany) were bound to carbodiimide-activated nylon membranes (Pall Biodyne C, Pall Europe Ltd., Portsmouth, UK) as described previously (Schouls et al., 1999). Each probe was applied in two 2 mm parallel stripes of 100 and 200 pmol respectively using a lineblot manifold (Miniblotter MN45, Immunetics, Cambridge MA, USA). The probes, which were selected from regions of high intertypic variability without known intratypic polymorphism, are listed in Table 1.

10 μl aliquots of amplified samples supplemented with tracer dye (0.03% bromophenol blue, 0.03% xylene cyanol, 0.4% Orange G, 15% Ficoll-400, 2 mM Tris–HCL pH 7.5, 50 mM EDTA pH 8.0) were diluted in 150 μl 2× SSPE, 0.1% SDS (2× SSPE is 0.3 M NaCl, 0.02 M NaH_2PO_4, 0.2 M EDTA), denatured at 99 °C for 10 min then placed immediately on ice. 140 μl of sample was applied in the Miniblotter MN45 with the sample channels running perpendicular to the probe stripes. The apparatus was sealed and incubated overnight at 45 °C. After aspiration of samples and washing of the sample channels the apparatus was disassembled and the filter was washed twice in 2× SSPE, 0.5% SDS for 10 min at 50 °C, then incubated in 10 ml 2× SSPE, 0.5% SDS containing 2.5 μl (3.75 mU) streptavidin–peroxidase conjugate (Roche Molecular Biochemicals, Mannheim, Germany) for 30 min at 42 °C with rotary mixing. After washing twice in 2× SSPE, 0.5% SDS for 10 min at 42 °C and two brief rinses in 2× SSPE at 42 °C amplicon detection was performed using the Amersham ECL system (Amersham Biosciences, Piscataway, NJ, USA) using a 10 min film exposure.

2.6. Primer design

The PCR primers (see Table 2) were designed to target conserved regions of the L1 gene corresponding to positions 6348–6373 and 6556–6579 in the HPV 16 genome (K02718). A sequence similarity tree of the concatenated primer target sequences provided the initial basis for the grouping of the target types (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66, plus the internal control, HPV 6) into four primer target sequence similarity groups. A distance tree of these sequences was constructed using CLUSTALW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The initial groups were: HPV 18, 45, 52 and 59; HPV 39, 51, 56 and 66; HPV 16, 33 and 58; and HPV 31, 35 and HPV 6. A single forward primer, PTf was found to be suitable for all groups. Reverse primers were optimised for each group. Variable positions were accommodated by using inosine as a wildcard base, thymine as a

Table 2
Composition of the multiplex PCR mixes.

	Target	Type	Sequence	Label	Concentration
Group 1					
Primers					
PTf	Consensus		TGYAAATATCCWGATTATWTIIAAATG		600 nM
PTrGr1b	Group 1		TGTAGCCAGTATGGYTTATTGAA–3		600 nM
Probes					
PTp18(131)	HPV 18	MGB	ACTGTGCCTCAATCC	FAM	150 nM
PTp52(4)	HPV 52	LNA	TC+TAA+CTC+TGGC+AA+TAC+TGC	ROX	50 nM
PTp59(1)	HPV 59	MGB	TACTGACATACGTGCC	NED	100 nM
Group 2					
Primers					
PTf	Consensus		TGYAAATATCCWGATTATWTIIAAATG		600 nM
PTrGr2	Group 2		TGKAGCCAATAAGGTTTATTAAA		600 nM
Probes					
PTp 39(4)	HPV 39	LNA	CA+CA+GATAT+ACG+TG+CAA+ACC	ROX	50 nM
PTp 51(156)	HPV 51	MGB	AGTGGTAATGGCCGTGAC	FAM	100 nM
PTp 56(2)	HPV 56	MGB	CAATACCTGCAGAGTTATA	VIC	50 nM
PTp 66 (1)	HPV 66	MGB	TTGGAAGGGTGGCAAT	NED	100 nM
Group 3					
Primers					
PTf	Consensus		TGYAAATATCCWGATTATWTIIAAATG		600 nM
PTrGr3	Consensus		TGIAICCAATAIGG(CT)TTATTGAA		600 nM
Probes					
PTp16(6)	HPV 16	MGB	ATTTGCAGTAGACCCAGAG	FAM	150 nM
PTp45(6)	HPV 45	MGB	GTGAAACCCCTGGCAGT	VIC	150 nM
PTp58	HPV 58	MGB	TCCGGTAATACTGCAG	NED	50 nM
Group 4					
Primers					
PTf	Consensus		TGYAAATATCCWGATTATWTIIAAATG		600 nM
PTrGr4	Group 4		TGTAICCAATATGGtTTATTAAA		600 nM
Probes					
PTp 31	HPV 31	MGB	TCCGGTTCAACAGCTAC	VIC	50 nM
PTp 33(9)	HPV 33	LNA	TG+CCTCTATT+CAAAG+CAGTGC	ROX	50 nM
PTp 35(4)	HPV 35	MGB	GTACCACTGGCACATTG	FAM	100 nM
PTp 6(154)	HPV 6*	MGB	TAGTGGAAATCGCACGTCT	NED	50 nM

Oligonucleotide composition of the multiplex mixes. Degeneracies are indicated by IUPAC ambiguity codes. I indicates inosine. LNA residues are indicated by a "+" before the designated nucleotide. FAM, NED, ROX and TaqMan are registered trademarks of Life Technologies Inc. LNA is a registered trademark of Exiqon Life Sciences.

Group	Forward		Reverse	
1	HPV18	TGTAAATATCCTGATTATTTACAAATG	HPV18	TTTAATAAACCATATTGGTTACA
	HPV52	.....G.....A........G.....	HPV52	........R..R..C...T.A..
	HPV59	.....A.....T........A.....	HPV59	........A..A..T...C.G..
	PTf	TGYAAATATCCWGATTATWTIIAAATG	PTrGr1-rc	TTCAATAARCCATACTGGCTACA
			PTrGr1 (3′-5′)	AAGTTATTYGGTATGACCGATGT <-
2	HPV39	TGTAAATATCCTGATTATTTGCAAATG	HPV39	TTTAATAAGCCTTATTGGCTACA
	HPV51	.............T...T.AA.A...	HPV51	........G.........C.C..
	HPV56	.............C...T.AA.A...	HPV56	........A.........T.G..
	HPV66	.............T...Y.AA.R...	HPV66	........A.........T.G..
	PTf	TGYAAATATCCWGATTATWTIIAAATG	PTr2-rc	TTTAATAAACCTTATTGGCTMCA
			PTrGr2 (3′-5′)	AAATTATTTGGAATAACCGAKGT <-
3	HPV16	TGCAAATATCCAGATTATATTAAAATG	HPV16	TTYAATAAACCWTATTGGTTRCA
	HPV45	..T........W......T.GC.....	HPV45	..T.....G..A......T.A..
	HPV58	..C........A......T.AA.....	HPV58	..T.....G..T......C.A..
	PTf	TGYAAATATCCWGATTATWTIIAAATG	PTr3-rc	TTCAATAARCCITATTGGITICA
			PTrGr3 (3′-5′)	AAGTTATTYGGIATAACCIAIGT <-
4	HPV31	TGTAAATATCCAGATTATCTTAAAATG	HPV31	TTTAATAAACCATATTGGATGCA
	HPV33	..T........A......T.AA.....	HPV33	........G.........C.A..
	HPV35	..C........T......C.AA.....	HPV35	........A.........T.G..
	HPV6	..T........A......T.AC.....	HPV6	........G.........C.A..
	PTf	TGYAAATATCCWGATTATWTIIAAATG	PTr4-rc	TTTAATAAACCATATTGGITACA
			PTrGr4 (3′-5′)	AAATTATTTGGTATAACCIATGT <-

Fig. 1. Alignments of primers and their target sequences. A period (".") indicates identity to the reference sequence. IUPAC ambiguity codes indicate variant positions in the target sequence and mixed nucleotides in the primer sequences. I indicates inosine. For the reverse primers the plus strands of the target sequences is shown along with the reverse complement (rc) of the primer sequence; the primer sequence itself is shown in reverse orientation (3′–5′).

universal pyrimidine, guanine as a universal purine (exploiting the stability of the noncanonical G:T basepair), incorporating mixed nucleotides or allowing the mismatch. Later, it was found to be expedient to modify the composition of the groups to group 1: HPV 18, 52 and 59; group 2: HPV 39, 51, 56 and 66; group 3: HPV 16, 45 and 58; and group 4: HPV 31, 33, 35 and 6. This could be achieved without modifying primer sequence as the conserved sequences of the primer targets allow most types to be amplified efficiently by more than one of the primer pairs. Fig. 1 shows the primer sequences along with their target sequences using the final group composition. Primers were obtained from Eurogentech (Herstal, Belgium). It should be noted that the group 3 primer PTrGr3 is a consensus reverse primer for all targeted HPV types; a primer targeting group 3 specifically was tested but was found not to improve performance.

2.7. Probe design

TaqMan MGB probes were designed using Primer Express software (Applied Biosystems, Foster City, CA) using the target amplicon as input sequence and specifying an amplicon length of ≥200 bp. As Primer Express will not select probes unless it can also find a primer pair that satisfies its internal design criteria it was sometimes necessary to facilitate the process by bracketing the input sequence with the 'primer-friendly' sequences GACAATTCTGCCATGCGACAT and CGATTCACGGTTTCCATATCG. Candidate probes were screened by comparison with a CLUSTALW multiple sequence alignment of the amplicon region of the target HPV types with intratypic variant positions marked. Probe sequences were selected if they targeted a sequence that was highly specific for the target type and contained no intratypic polymorphisms. TaqMan MGB probes were labelled with FAM, VIC or NED and obtained from Applied Biosystems. All MGB probes incorporate a dark quencher. Probes suitable for labelling with ROX were either (a) MGB probes redesigned as LNA probes with the help of Sigma Genosys, (Cambridge, UK) and http://lna-tm.com/ or (b) designed as non-MGB TaqMan probes using Primer Express. ROX-labelled probes were fluorophor-labelled at the 5′ end and quenched with the dark quencher BHQ2 at the 3′ end. All probes were designed to have a T_m of 70 ± 2 °C.

2.8. PCR program

For all experiments the PCR program was: 95 °C, 12 min followed by 45 cycles of 95 °C, 15 s; 45 °C, 30 s; 60 °C, 60 s. For experiments using SYBR green, dissociation analysis was appended to the end of the program. All PCR reactions were run on the ABI Prism 7000 or 7300 genetic analyzer (Applied Biosystems, Foster City, CA) with the ROX passive reference function disabled in order to allow the ROX colour channel to be used for probe detection. As the composition of the mastermix may affect the emission spectra of the fluorophors, spectral calibrations were performed with solutions of unquenched ROX-, NED-, FAM- and VIC-labelled oligonucleotides for each of the PCR mastermixes used in order to ensure optimal channel separation.

2.9. Testing of primers

Primer testing was performed by amplifying 10-fold serial dilutions of the target HPV types using Finnzymes DyNAmo SYBR green qPCR kit F400 (Finnzymes, Espoo, Finland). The criterion for primer acceptability was an analytical sensitivity of 4000 genomic units (GU) or better for all target HPV types. Analytical sensitivity was defined as the lowest target concentration that gave an unequivocal type-specific dissociation peak.

2.10. Probe screening and titration

Probes were screened at a concentration of 200 nM by amplifying 10-fold serial dilutions of the target HPV type and the appropriate primer pair in Finnzymes DyNAmo probe PCR kit F450 (Finnzymes, Espoo, Finland) or, in later experiments, PerfeCta qPCR FastMix, UNG (Quanta Biosciences, Gaithersburg, MD). A positive amplification signal from 400 GU of target DNA was the minimum criterion for approval of the probe for further testing.

Probe titration was performed in order to determine the minimum concentration that could be used without loss of analytical sensitivity; probe concentrations tested were 200, 150, 100 and 50 nM. Target DNA concentrations that bracketed the cut-off concentration determined previously for 200 nM probe were used.

2.11. Testing of probes in multiplex

For multiplex testing, the probes were combined at the minimum acceptable concentration determined by probe titration and tested against dilution series of all members of the target group. Criteria for acceptance of a multiplex combination were analytical sensitivity 400 GU or better for all types and no intra-group cross-reactions. Where unacceptable loss of analytical sensitivity or cross-reaction was detected, reactions were repeated with fewer probes in all combinations in order to identify the interfering probe, which was replaced with a new candidate.

Successful groups of probes were tested against 4×10^4 GU of each of the 13 targeted HR HPV types and the internal control, HPV 6 in order to detect cross-reaction. Where cross-reaction was observed, the cross-reacting probe was tested against the cross-reacting HPV type(s) in a simplex reaction. Cross-homology was tested using the LALIGN program available on the Swiss EMBNet node (http://www.ch.embnet.org). Several probes that cross-reacted only in multiplex and lacked significant homology to the cross-reacting type were encountered.

Where probes were rejected due to interference or cross-reaction, new probes were designed and tested as described in Sections 2.10 and 2.11. 1–3 rounds of reformulation were required to arrive at the multiplex formulations described in Table 2. These formulations were used in all subsequent experiments.

2.12. Controls

For each multiplex group a positive control containing a mixture of the targeted HPV types was run in parallel. The concentrations used were 40,000 GU/5 μl (HPV 52, 39 and 66); 4000 GU/5 μl (HPV 16, 18, 31, 33, 35, 45, 56, 58 and 59) or 400 GU/5 μl (HPV 51). The non-oncogenic HPV type HPV 6 was used as an internal control against PCR inhibition. HPV 6 was added at a concentration of 4×10^3 GU per reaction to all multiplex group 4 reactions.

3. Results

3.1. Analytical sensitivity

Analytical sensitivity was determined by amplification of 10× serial dilutions of the target HPV types using the multiplex formulations shown in Table 2. In the presence of 500 ng of human DNA, the detection cut-off was 4 GU (genomic units) for HPV 31 and HPV 51; 40 GU for HPV 16, HPV 18, HPV 39, HPV 56 and HPV 59; and 400 GU for HPV 33, HPV 35, HPV 45, HPV 52, HPV 58 and HPV 66. Lowering the amount of human DNA to 50 ng resulted in a detection cut-off of 4–40 GU for all types.

In order to control that the analytical sensitivities determined using plasmid clones diluted in human DNA obtained from commercial sources are applicable to clinical samples processed

Table 3
Comparison of HC2 and PT13 real-time testing of clinical samples.

		HC2			*N*
		+	+/−	−	
PT13-RT	+	59	7	23	89
	−	1	0	115	116
	Inhibitory	1	0	0	1
	N	61	7	138	206

according to the test protocol, the following tests were performed: (1) substitution of human DNA from commercial sources with DNA from clinical samples as diluent; (2) spiking of clinical samples with serial dilutions of CaSki cells and (3) re-extraction of selected serial dilutions of DNA using the MagNAPure instrument.

Substituting the human DNA diluent from commercial sources (100 ng/μl) with pooled DNA from clinical samples (50 ng/μl) gave unchanged cut-off values for all HPV types. Cq values were up to +2 cycles higher (mean = 0.6 cycles) suggesting that DNA from some clinical samples may have a slight inhibitory effect on the reaction.

When doubling dilutions of CaSki cells were diluted in pooled HPV-negative human cervical samples and extracted according to the standard protocol, HPV16 was detected down to the theoretical limit of 1 CaSki cell per 10 ml aliquot, showing that intracellular HPV DNA is recovered from and detected in cervical samples efficiently.

In order to test recovery of HPV DNA in the MagNAPure DNA extraction process 10 μl of a plasmid dilution of each target HPV type one $\log_{10}$ above cut-off was diluted with 90 μl of PBS and extracted according to the standard protocol. The final volume after extraction was 100 μl. All HPV types were detected also after re-extraction. Cq values were on average +0.7, which corresponds to a 40% reduction in HPV concentration assuming 100% PCR efficiency. This indicates that HPV is recovered with at least 60% efficiency.

3.2. Internal control

In order to provide an internal control a plasmid containing the genome of HPV6 was added to all group 4 reactions. In order to determine suitable concentrations HPV6 was added to the group 4 mastermix at varying concentrations and the mix was used to detect HPV31, HPV33 and HPV35 at the cut-off concentrations determined previously. It was found that HPV 6 could be added to the group 4 multiplex reactions at levels of 400–4000 GU per reaction without compromising the analytical sensitivity for HPV 31, HPV 33 or HPV 35. 4000 GU per reaction was chosen as standard concentration for the internal control.

3.3. Clinical samples

Table 3 compares the results of the PT13-RT test with those of the HC2 test for 206 clinical samples. Of 61 samples that were positive by HC2, 59 were PT13RT positive, one was inhibitory and one was negative, giving a sensitivity of 98% relative to HC2. Of 138 samples negative by HC2, 115 were negative by PT13-RT and 23 were positive, giving a specificity of 83% relative to HC2. Of these latter 21/23 had Cq values >30, indicating low viral loads. By contrast, of the 59 HC2+/PT13-RT+ samples, only four had Cq values >30. This suggests that the discrepancies are due principally to the analytical sensitivity difference between the PT13-RT and HC2 tests. The HPV types detected in the discrepant samples were HPV16, 18, 45 and 51 (four samples each), HPV 31, 52 and 56 (two samples each) and HPV 58 (one sample).

Discrepancy analysis was performed using the Roche Amplicor 4800 HPV test. Discrepancy analysis confirmed the PT13-RT result in 50% (12/24) of discrepant samples, including the single sample that was positive by HC2 and negative by PT13-RT. Five of the confirmed samples as against none of the non-confirmed samples were HPV16 positive. Apart from this there was no apparent difference in type distribution between confirmed and non-confirmed samples.

In order to assess the accuracy of typing, a second typing test using reverse line blot (RLB) was performed. In the reverse line blot test amplification is performed with the consensus group 3 primers but different probes are used. Two hundred and two samples were tested. The overall kappa value (Fleiss et al., 2003) for the 12 types common to both tests (HPV types 16, 18, 31, 33, 35, 39, 45, 52, 56, 58 and 59) was 0.79. One hundred and sixty-two samples gave identical results with both tests: 111 were negative; 44 were positive for a single HPV type and seven were positive for 2 or 3 types. Three samples were positive only by RLB; one of these was partially inhibitory and in the other two the RLB hybridisation signal was weak and equivocal. Twelve samples were positive only by PT13-RT. All had high Cq values (≥30) and seven contained HPV 52 or HPV 56, for which the RLB test has a reduced sensitivity. In 12 samples PT13-RT detected an extra type in a sample where RLB detected only one; in nine of these samples the extra type was HPV 52 or 56 and/or had a Cq value ≥30. Thirteen samples were multiply-positive by both tests but the combination of types agreed only partially. Four had extra types detected only by PT13-RT, five had extra types detected only by RLB and four had extra types in both tests (Table 4).

In cases where discrepancies were found or where correct interpretation seemed in doubt the PCR was repeated in a simplex format with only the probe appropriate to the HPV type in question. In all cases the original interpretation of the PT13-RT test was confirmed.

Table 4
Comparison of typing results between PT13-RT and reverse line blot.

Description	*N*	Remarks
Identical results		
Both negative	111	
Both positive, single type	44	
Both positive, multiple types	7	
Identical results, total	162	
Discrepant results		
RLB negative, PT13-RT positive	12	Cq ≥ 30 (*N* = 12); HPV 52 or 56 (*N* = 7); inhibitory sample (*N* = 1); equivocal result, RLB (*N* = 2)
RLB positive, PT13-RT negative	3	
RLB single positive, PT13-RT multiple positive	12	Cq ≥ 30 and/or HPV 52 or 56 (*N* = 9)
Multiple types, type agreement partial	13	
Discrepant results, total	40	
N	202	

Results for HPV 66 (not included in the RLB test) and HPV 68 (not included in PT13-RT) are disregarded.

3.4. Logistic considerations

The time required for completion of the analysis is 8 h:4.5 h for sample processing, 15 min for PCR setup, 2.5 h PCR run time and 1 h for analysis. Of this, 2.5 h is hands-on time. The batch size is 22 samples, plus controls.

4. Discussion

The PT13-RT test detects and genotypes simultaneously the 13 oncogenic mucosal HPV types recognised in the 2007 IARC monograph (IARC, 2007). The analytical sensitivity is 400 GU (genomic units) or better for all types. Since each test uses 1/20 of a 10 ml sample, this corresponds to an analytical sensitivity per sample of 8000 GU or better. This was determined using reconstructed samples consisting of plasmid-cloned HPV genomic DNA diluted in purified human DNA from commercial sources. Experiments where the reconstructed samples were subjected to the protocol extraction process, where the human DNA diluent from commercial sources was replaced with DNA from clinical samples, or where clinical samples were spiked with the HPV16-containing cell line CaSki, all confirmed the analytical sensitivity estimates determined in reconstruction experiments, indicating that they are likely to be applicable to clinical samples. The background human DNA concentration in clinical samples is usually lower (10–40 ng/μl) than in the reconstruction experiments and, since lowering the concentration of human DNA is found to improve the analytical sensitivity, the analytical sensitivity for real clinical samples is expected to be better. In the rare cases of samples yielding DNA concentrations of over 100 ng/μl, dilution of the sample would be indicated. The analytical sensitivities reported here are comparable to those reported by Moberg et al. (2003) for a multiplex real time PCR test for HPV which uses type-specific rather than consensus primers.

In clinical samples, PT13-RT had a 98% sensitivity and 83% specificity for HPV detection relative to the HC2 test. Since the HC2 test is not 100% specific for oncogenic HPV types (Poljak et al., 2002; Castle et al., 2002) and has a lower analytical sensitivity than PT13-RT this result is not unexpected; similar patterns of specificity and sensitivity have been seen when comparing PCR tests with the Hybrid Capture test (Molijn et al., 2005). Samples with Cq values >30 (corresponding to low viral loads) comprised 91% of the PT13-RT positive/HC2 negative samples but only 7% of PT13-RT positive/HC2 positive samples, which is consistent with differences in analytical sensitivity causing the discrepancies. Third-test discrepancy analysis confirmed the positive PT13-RT result in 11/23 samples. Discrepancy analysis for the single sample that was positive by HC2 and negative by PT13-RT also confirmed the PT13-RT result.

The accuracy of typing with the PT13-RT test was assessed by comparison with a reverse line-blot (RLB) hybridisation test. Of the 59 samples that were positive for a single HPV type by RLB, the same type was detected by PT13-RT in 56 samples, with an additional type being detected in 12 of these samples. For the 20 RLB multiple-positive samples there was complete type agreement in seven samples and partial agreement in 13. In no case was there complete disagreement of type. The results were consistent with a high degree of typing concordance ($\kappa = 0.79$), but with PT13-RT having a higher sensitivity, particularly for HPV 52 and HPV 56. Previous experiments (data not shown) indicate that the RLB test has a reduced analytical sensitivity for these types.

The PT13-RT test is designed to be highly error-resistant, the salient features being use of a closed-system real-time PCR format, inclusion of an internal control and measurement of sample DNA concentration. These features minimise interference from carry-over contamination, inhibition and sample insufficiency respectively. In real-time PCR amplification, detection and typing occur simultaneously in a closed tube. There is thus no need for handling amplified material and the risk of false positive results caused by carry-over contamination is avoided. The internal control, HPV6 is amplified in the group 4 reaction and will be negative if amplification is inhibited by impurities in the sample or if there is competition from large amounts of an amplifiable non-oncogenic HPV type. However, samples that are strongly positive for an oncogenic HPV type can, and do, out-compete the internal control. A positive result thus takes precedence over the internal control result, which is relevant only if no oncogenic HPV types are detected. Finally, measuring DNA concentration by UV spectrophotometry provides a simple way to check that the sample contains sufficient cellular material and has yielded sufficient DNA for a reliable result to be obtained. Inter-genotype competition is the main residual source of error and may affect the combination of genotypes detected in samples containing more than one genotype, particularly where the viral loads are very unequal. Most typing discrepancies observed in this study were in multiple-positive samples.

The PT13-RT test is intrinsically semi-quantitative since results are read off as Cq values which are proportional to the initial concentration of target DNA. It would also be possible to run the reaction in a fully quantitative mode by including dilution series of the target type(s) in order to construct standard curves. The quantitative nature of the test has several advantages. In epidemiological studies it would be possible to establish a constant cut-off value for all types, thus eliminating the biased prevalence estimates that can result from type-specific differences in analytical sensitivity. In clinical use the viral load may be determined; clinical value of viral load measurements remains a matter of ongoing research, viral load is reported to be correlated with disease severity for HPV 16 but not for HPV 18 (Woodman et al., 2007). The PT13-RT test would allow viral load cut-off values to be established on a type-specific basis as type-specific clinical data become available. It has been suggested (Schiffman et al., 2000) that the HC2 test owes its higher specificity for cervical dysplasia relative to PCR tests to its relatively low analytical sensitivity. By adjusting cut-off values for the PT13-RT test it would be possible to configure the test to mimic the analytical sensitivity of the HC2 test. Finally, use of a quantitative test may be of use in following the progress of infections and the effects of treatment.

In the present format of the test all stages of the analysis including reporting results can be completed in 8 h, 2.5 h of which is hands-on time; the batch size is 22 samples. By overlapping runs, three runs per day could be achieved, which would utilise the capacity of a single MagNAPure extractor and ABI7300 PCR machine optimally. Incremental improvements in analysis time could be achieved by optimising step times of the PCR program, by using rapid cycling instruments and/or by employing faster DNA extraction methods. Larger batches of samples could be analysed using 384-well format real-time PCR instruments and 96-well DNA extractors, both of which are now available on the market.

This test will be applicable in diagnostic, screening and research applications where HPV genotyping and/or viral load estimates are required and in epidemiological studies where unbiased type-specific prevalence estimates are needed.

Conflicts of interest

The present test is the subject of Norwegian patent 330943, and dependent patent applications worldwide held by the company Allum-Jenkins AS, of which A.J. and A.G.A. are sole shareholders. L.S. is a co-inventor.

Author contributions

Primer and probe design was by A.J. Experimental work was planned by A.J., L.S. A.G.A. and R.K.Aa. and executed by L.S., A.G.A. and R.K.Aa. All authors contributed to the analysis of the data and the drafting of the manuscript.

Acknowledgements

The authors wish to thank John Arrand, Lutz Gissmann, Gerard Orth, Attila Lorincz, Saul Silverstein and Toshihaki Matsukara for providing plasmids, Andreas Matussek for his support and interest and the Unilabs Organisation for financial support. We also wish to thank Helena Enroth, Christine Jonassen and Mona Lindsethmo Hansen for analysing discrepant samples. The project was supported in part by tax refunds via the Norwegian *Skattefunn* system. This work builds on preliminary work done by Jónleyg Danielsen under the supervision of A.J. and L.S. in the course of her M.Sc. project.

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