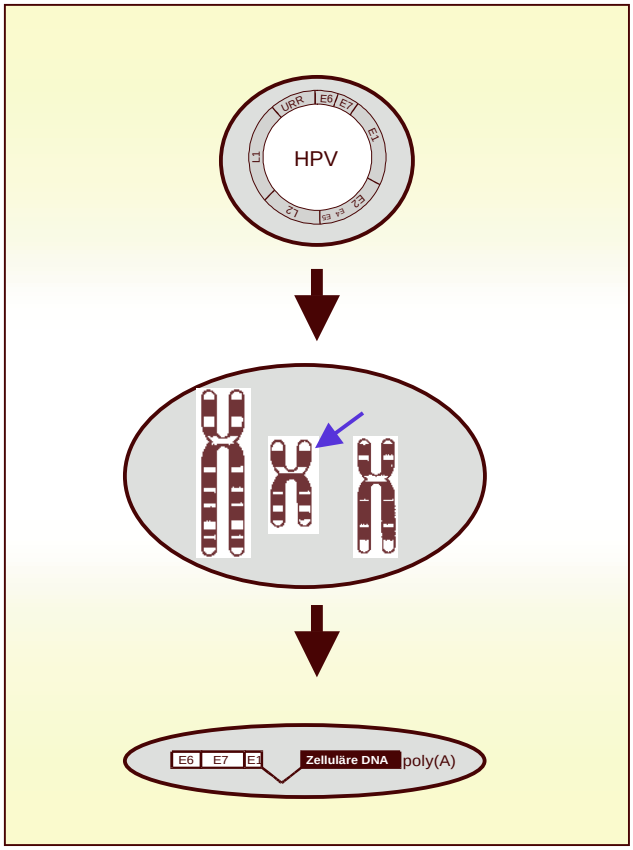
APOT - Assay

Amplification of Papilloma Virus Oncogene Transcripts

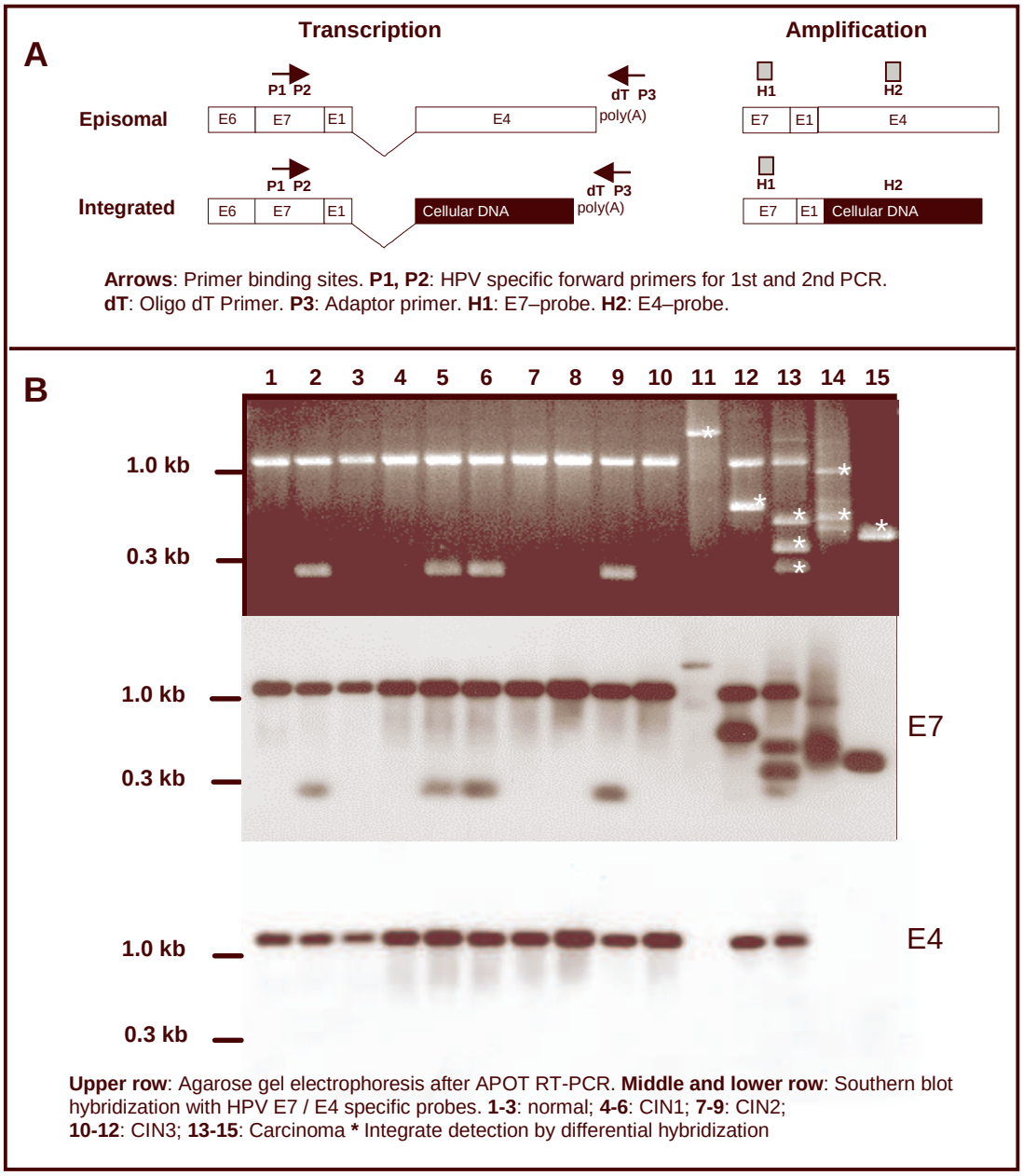


Protocol for HPV16 and 18

Brief summary of the APOT assay

Fig.1A shows the basic principles of the APOT assay for episomal and integrated transcripts. On the left side, transcript schemes are shown, on the right side, the corresponding amplification products are depicted. RT is performed using an adaptor linked oligodT primer. Next, two PCR steps are done using oligo dT / Adaptor primers and HPV E7 specific primers. The amplification products are hybridized to HPV E7 and E4 specific probes to discriminate episomal from integrate-derived transcripts (Fig. 1B). Alternatively, fusion transcripts can be excised from the gel and sequenced to allow detection of integrate derived transcripts.

Figure 1: APOT diagram, amplification and hybridization results



Arrows: Primer binding sites. **P1, P2**: HPV specific forward primers for 1st and 2nd PCR.
dT: Oligo dT Primer. **P3**: Adaptor primer. **H1**: E7–probe. **H2**: E4–probe.

Upper row: Agarose gel electrophoresis after APOT RT-PCR. **Middle and lower row**: Southern blot hybridization with HPV E7 / E4 specific probes. **1-3**: normal; **4-6**: CIN1; **7-9**: CIN2; **10-12**: CIN3; **13-15**: Carcinoma ***** Integrate detection by differential hybridization

Sample material

RNA quality

RNA integrity is very important for a good performance of the APOT assay. When RNA integrity is assured, APOT can be performed from very low amounts of clinical material, such as cervical swabs or small biopsies. It is very important to stabilize RNA immidiately after sample extraction. For optimal results, samples should be frozen in liquid nitrogen. Various RNA preparation methods have been used, including modified phenol/chlorophorm assays, Trizol protocols or column based methods, like RNeasy (Qiagen). All methods showed sufficient results with APOT amplification when good RNA was used as starting material.

RNA stabilization solutions

We have tested RNA stabilization solutions as a substitute for liquid nitrogen. We had good APOT amplification results when samples were immediately transferred to RNAlater (Ambion) and stored up to one week at 25°C and up to one month at 4°C. Long term storage is possible in RNAlater solution at –20°C or -80°C.

The quality of the isolated RNA should be determined by amplification of housekeeping gene mRNAs like GAPDH or beta-actin.

DNAse digest

If problems with DNA contamination occur, a DNAse digest can be performed prior to reverse transcription. However, a general application of DNAse does not seem to be necessary. In our lab, we are analyzing mainly fresh frozen swab and biopsy samples. RNA is extracted using the RNeasy kit. Additionally, using this kit, DNA can be isolated from the RNeasy column initial and first wash flowthroughs. The isolated DNA can be used for HPV typing or genome-based integration detection. DNAse digestion of the isolated RNA is generally not performed.

Detailed Protocol

Reverse Transcription

Total RNA (1ng -1 µg) was reverse transcribed using an oligo(dT)17-primer coupled to a linker sequence **(dT)17-p3.**

	RT reaction	x1
Master Mix 1	**Template**	4 µl
	Water	7 µl
	(dT)17-p3 25 µM	1 µl
Master Mix 2	5 x RT buffer	4 µl
	0,1 DTT	2 µl
	10mM dNTP	1 µl
	MMLV RT SuperScript™	0.1-0.2µl (20-40 U)
Total		**20µl**

		HPV 16	HPV 18
Heat denaturation	Master Mix 1	70^0C 10 min ->quick chill on ice	
Reverse transcription	Master Mix 2	42°C 60 min	
Inactivation		90^0 5 min	
(dT)17-P3-sequence		GACTCGAGTCGACATCGA TTTTTTTTTTTTTTTT	

SuperScript™ II RNase H- Reverse Transcriptase (Invitrogen) is used to synthesize first-strand cDNA and will generally give higher yields of cDNA and more full-length product than other reverse transcriptases.

1st PCR

Pipetting protocol for the first PCR:

1st PCR	**x1**
10 x RT buffer	5 µl
10mM dNTP	1 µl
50mM $MgCl_2$	1,5
P1for16 / P1for18 (25 µM)	0,5 µl
P3 (25 µM)	0,5 µl
Taq-polymerase (5U)	0,3 µl
Water	37,2 µl
Template	4 µl
Total	50 µl

Cycling conditions and primers for the first PCR :

30 Cycles	**HPV 16**	**HPV 18**
Initial denaturation	94°C 3 min	
Denaturation	94°C 40 sec	
Annealing	59°C 30 sec	61°C 30 sec
Extension	72°C 4 min	
Final extension	72°C 7 min	
P1-sequence	CGG ACA GAG CCC ATT ACA AT	TAG AAA GCT CAG CAG ACG ACC
P3-sequence	GAC TCG AGT CGA CAT CG	

2nd PCR

Pipetting protocol for the second PCR:

1st PCR	**x1**
10 x RT buffer	5 µl
10mM dNTP	1 µl
50mM $MgCl_2$	1,5
P2for16 / P2for18 (25 µM)	0,5 µl
(dT)17-P3 (25 µM)	0,5 µl
Taq-polymerase (5U)	0,3 µl
Water	37,2 µl
Template	4 µl
Total	50 µl

Cycling conditions and primers for the second PCR :

30 Cycles	**HPV 16**	**HPV 18**
Initial denaturation	94°C 3 min	
Denaturation	94°C 40 sec	
Annealing	67°C 30 sec	70°C 30 sec
Extension	72°C 4 min	
Final extension	72°C 7 min	
P2-sequence	CCT TTT GTT GCA AGT GTG ACT CTA CG	ACG ACC TTC GAG CAT TCC AGC AG
(dT)17-P3-sequence	GAC TCG AGT CGA CAT CGA TTTTTTTTTTTTTTTT	

Hybridization

The PCR products are electrophoresed in 1.2% agarose gels, blotted on nylon membranes (Hybond N+, Amersham Life Science, Buckinghamshire, England) and hybridized with an E7-specific probe (H1, table) at 55°C. A second parallel filter is hybridised with an E4-specific probe (H2, table) at 55°C to highlight amplimeres that encompass E4 sequences. Labeling and detection of the probes was performed with the ECL oligolabeling and detection kit (Amersham Pharmacia Biotech, Freiburg, Germany) as per the manufacturer's instructions. Alternatively, other ECL detection systems can be used according to your personal preferences. Amplimeres which did not hybridize with the E4-specific probe or which displayed a different size than the major E7-E1`E4 episomal transcript (approximately 1050 bp in length for HPV16 and 1000 bp for HPV18) were suspected to be derived from integrated HPV genomes.

H1-16	TCGTACTTTGGAAGACCTGTTAATG
H1-18	GTTTCTGAACACCCTGTCCTTTGTG
H2-16	GAAGAAACACAGACGACTATCCAG
H2-18	CAGCTACACCTACAGGCAACAACAA

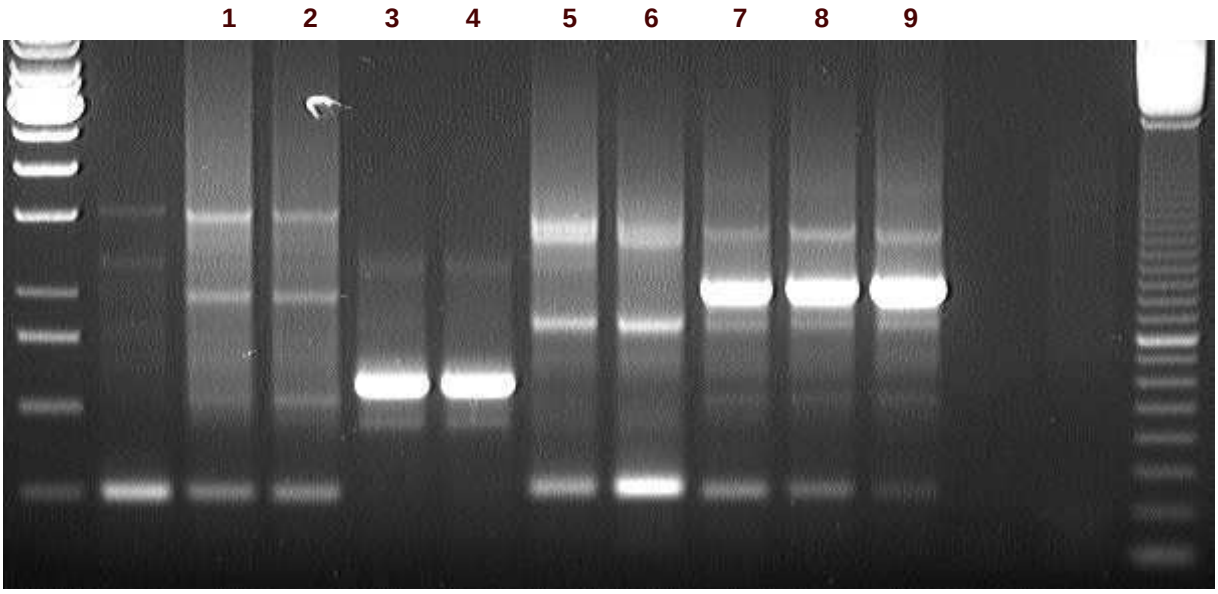
Sequencing

PCR products of interest are excised from the gel and extracted using the Qiagen Gel Extraction Kit (Qiagen). Sequencing reactions are performed using the Big-Dye terminator DNA-sequencing Kit (Perkin Elmer) and analyzed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Sequencing results are compared to public databases using the BLASTN-program provided by the National Cancer Institute, USA.

Examples

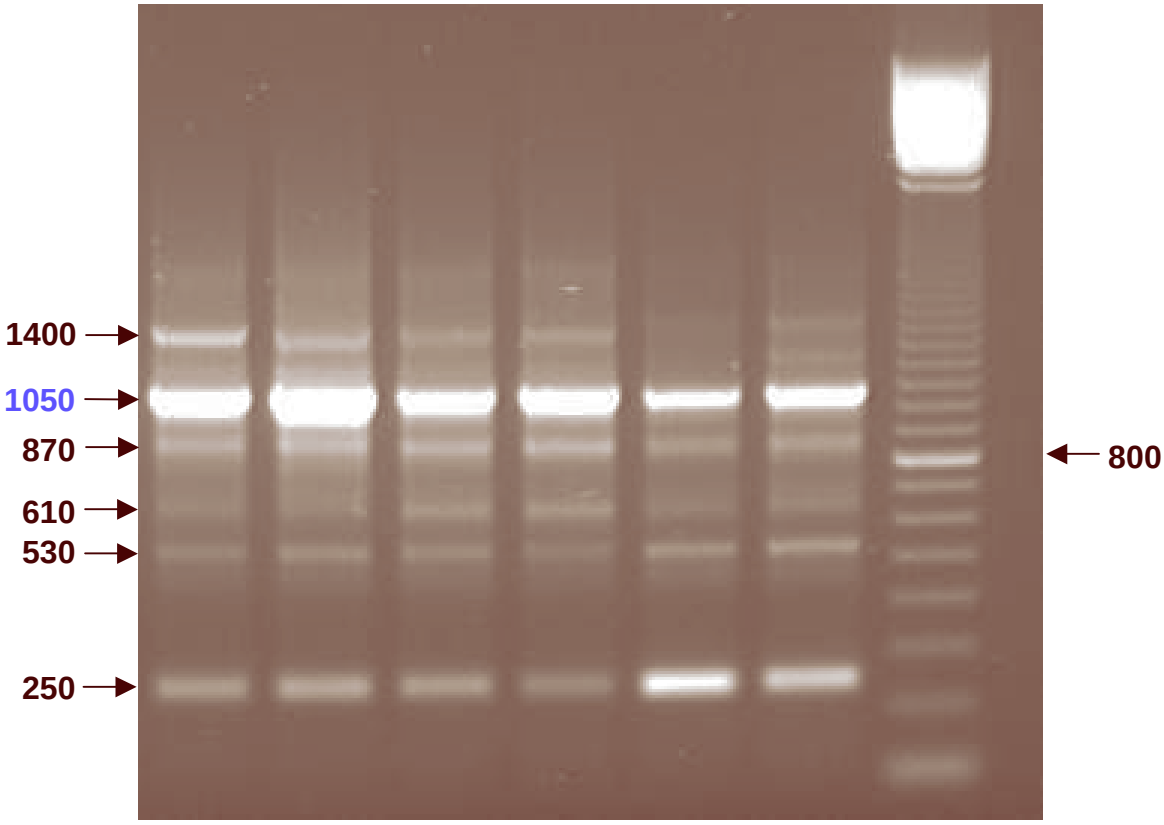
1. Amplification of fusion transcripts from microdissected material



1,2- integrated HPV (two different microdissected tumor regions), **patient N1**
3,4 - integrated HPV (two different microdissected tumor regions), **patient N2**
5, 6 - integrated HPV (two different microdissected tumor regions), **patient N3**
7,8,9- episomal transcripts **patient N4**
(1050bp - specific, 250, 530, 610, 870, 1400bp -weak non-specific amplicons)

The first example shows good reproducible amplification products obtained with microdissected material. Fresh frozen tissue slides were used for laser assisted microdissection. Different areas (200-300 cells) from the same tumor showed identical APOT patterns. Integrate derived fusion transcripts were confirmed by direct sequencing of amplification products.

2. Amplification of episomal transcripts and episome derived artifacts



The gel shows samples that only harbour episomal HPV DNA. When high amounts of episomal transcripts are present, mispriming of the oligo(dT) primer to A-rich regions can lead to amplification of various artifacts that have a different length than the standard episomal transcript (1050 bp). All marked transcripts were cloned and sequenced. Sequence comparism with the HPV16 genome allowed us to identify the most frequent aberrant binding sites. The characteristic artifact patterns shown in the picture above occur only when no integrate derived transcripts can be amplified.

On the following page, the HPV16 sequence from 728 (binding site of the 2nd APOT PCR primer) to 4250 (area of the polyA signal AATAAA that leads to termination of transcription and polyadenylation, regular binding site of the oligo(dT) primer) is provided. A-rich sequences are marked and annotated according to the data from sequenced artifacts. Three artifacts (250, 870 and 1400) derive from non-spliced transcripts or from contaminating episomal DNA, the others from regularly spliced transcripts.

Additional material: sequence map of HPV16 with aberrant binding sites.

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          2nd PCR primer
 721 attgtaacct tttgttgcaa gtgtgactct acgcttcggt tgtgcgtaca aagcacacac
 781 gtagacattc gtactttgga agacctgtta atgggcacac taggaattgt gtgccccatc

                                          s.donor site
 841 tgttctcaga aaccataatc taccatggct gatcctgcag gtaccaatgg ggaagagggt

                                               A-rich 250 bp
 901 acgggatgta atggatggtt ttatgtagag gctgtagtgg aaaaaaaaac aggggatgct
 961 atatcagatg acgagaacga aaatgacagt gatacaggtg aagatttggt agattttata
1021 gtaaatgata atgattattt aacacaggca gaaacagaga cagcacatgc gttgtttact
1081 gcacaggaag caaaacaaca tagagatgca gtacaggttc taaaacgaaa gtatttggta
1141 gtccacttag tgatattagt ggatgtgtag acaataatat tagtcctaga ttaaaagcta
1201 tatgtataga aaaacaaagt agagctgcaa aaaggagatt atttgaaagc gaagacagcg
1261 ggtatggcaa tactgaagtg gaaactcagc agatgttaca ggtagaaggg cgccatgaga
1321 ctgaaacacc atgtagtcag tatagtggtg gaagtggggg tggttgcagt cagtacagta
1381 gtggaagtgg gggagagggt gttagtgaaa gacacactat atgccaaaca ccacttacaa
1441 atattttaaa tgtactaaaa actagtaatg caaaggcagc aatgttagca aaatttaaag
1501 agttatacgg ggtgagtttt tcagaattag taagaccatt taaaagtaat aaatcaacgt

                                                    A-rich 870 bp
1561 gttgcgattg gtgtattgct gcatttggac ttacacccag tatagctgac agtataaaaa
1621 cactattaca acaatattgt ttatatttac acattcaaag tttagcatgt tcatggggaa
1681 tggttgtgtt actattagta agatataaat gtggaaaaaa tagagaaaca attgaaaaat
1741 tgctgtctaa actattatgt gtgtctccaa tgtgtatgat gatagagcct ccaaaattgc
1801 gtagtacagc agcagcatta tattggtata aaacaggtat atcaaatatt agtgaagtgt
1861 atggagacac gccagaatgg atacaaagac aaacagtatt acaacatagt tttaatgatt
1921 gtacatttga attatcacag atggtacaat gggcctacga taatgacata gtagacgata
1981 gtgaaattgc atataaatat gcacaattgg cagacactaa tagtaatgca agtgcctttc
2041 taaaaagtaa ttcacaggca aaaattgtaa aggattgtgc aacaatgtgt agacattata

     A-rich 1400 bp
2101 aacgagcaga aaaaaaacaa atgagtatga gtcaatggat aaaatataga tgtgataggg
2161 tagatgatgg aggtgattgg aagcaaattg ttatgttttt aaggtatcaa ggtgtagagt
2221 ttatgtcatt tttaactgca ttaaaaagat ttttgcaagg catacctaaa aaaaattgca
2281 tattactata tggtgcagct aacacaggta aatcattatt tggtatgagt ttaatgaaat
2341 ttctgcaagg gtctgtaata tgttttgtaa attctaaaag ccatttttgg ttacaaccat
2401 tagcagatgc caaaataggt atgttagatg atgctacagt gccctgttgg aactacatag
2461 atgacaattt aagaaatgca ttggatggaa atttagtttc tatggatgta aagcatagac
2521 cattggtaca actaaaatgc cctccattat taattacatc taacattaat gctggtacag
2581 attctaggtg gccttattta cataatagat tggtggtgtt tacatttcct aatgagtttc
2641 catttgacga aaacggaaat ccagtgtatg agcttaatga taagaactgg aaatcctttt
2701 tctcaaggac gtggtccaga ttaagtttgc acgaggacga ggacaaggaa aacgatggag
2761 actctttgcc aacgtttaaa tgtgtgtcag gacaaaatac taacacatta tgaaaatgat
2821 agtacagacc tacgtgacca tatagactat tggaaacaca tgcgcctaga atgtgctatt
2881 tattacaagg ccagagaaat gggatttaaa catattaacc accaagtggt gccaacactg
2941 gctgtatcaa agaataaagc attacaagca attgaactgc aactaacgtt agaaacaata
3001 tataactcac aatatagtaa tgaaaagtgg acattacaag acgttagcct tgaagtgtat
3061 ttaactgcac caacaggatg tataaaaaaa catggatata cagtggaagt gcagtttgat
3121 ggagacatat gcaatacaat gcattataca aactggacac atatatatat ttgtgaagaa
3181 gcatcagtaa ctgtggtaga gggtcaagtt gactattatg gtttatatta tgttcatgaa
3241 ggaatacgaa catattttgt gcagtttaaa gatgatgcag aaaaatatag taaaaataaa

                                                          s.acc
3301 gtatgggaag ttcatgcggg tggtcaggta atattatgtc ctacatctgt gtttagcagc
3361 aacgaagtat cctctcctga aattattagg cagcacttgg ccaaccaccc cgccgcgacc
3421 cataccaaag ccgtcgcctt gggcaccgaa gaaacacaga cgactatcca gcgaccaaga
3481 tcagagccag acaccggaaa cccctgccac accactaagt tgttgcacag agactcagtg
3541 gacagtgctc caatcctcac tgcatttaac agctcacaca aaggacggat taactgtaat
3601 agtaacacta cacccatagt acatttaaaa ggtgatgcta atactttaaa atgtttaaga
3661 tatagattta aaaagcattg tacattgtat actgcagtgt cgtctacatg gcattggaca

        A-rich 530 bp
3721 ggacataatg taaaacataa aagtgcaatt gttacactta catatgatag tgaatggcaa

                              A-rich 610 bp
3781 cgtgaccaat ttttgtctca agttaaaata ccaaaaacta ttacagtgtc tactggattt
3841 atgtctatat gacaaatctt gatactgcat ccacaacatt actggcgtgc tttttgcttt
3901 gctttgtgtg cttttgtgtg tctgcctatt aatacgtccg ctgcttttgt ctgtgtctac
3961 atacacatca ttaataatat tggtattact attgtggata acagcagcct ctgcgtttag
4021 gtgttttatt gtatatatta tatttgttta tataccatta tttttaatac atacacatgc
4081 acgcttttta attacataat gtatatgtac ataatgtaat tgttacatat aattgttgta
4141 taccataact tactattttt tctttttttat tttcatatat aatttttttt tttgtttgtt

                  poly A episomal 1050 bp
4201 tgtttgtttt ttaataaact gttattactt aacaatgcga cacaaacgtt ctgcaaaacg
```

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Involvement of intact HPV16 E6/E7 gene expression in head and neck cancers with unaltered p53 status and perturbed pRb cell cycle control. (2002)
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Modified APOT protocol used to analyze fusion transcripts from head and neck cancers.

Ziegert C, Wentzensen N, Vinokourova S, Kisseljov F, Einenkel J, Hoeckel M,von Knebel Doeberitz M.
A Comprehensive Analysis of HPV Integration Loci in Anogenital Lesions combining Transcript and Genome Based Amplification Techniques.
Submitted.

Comparison of fusion transcripts and genomic integration sites using APOT.

Conference presentations

Turek L et al.
HPV conference 2001 and 2002

APOT amplification of fusion transcripts from head and neck cancers.

Contact information

We are continuously working to improve the APOT assay and have decided to provide users with our standard procedure protocols. If you encounter any problems or have any further questions, please do not hesitate to contact us.

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