DNA–EIA to detect high and low risk HPV genotypes in cervical lesions with E6/E7 primer mediated multiplex PCR

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Accepted for publication 10 October 1997

Abstract

Background—Oncogenicity of human papillomavirus (HPV) DNA in premalignant and malignant uterine cervical diseases is mainly induced by E6/E7 open reading frame (ORF). The presence of an oncogenic HPV DNA may be a diagnostic marker for the detection of cytologically negative smears.

Aims—To evaluate an original polymerase chain reaction enzyme immunoassay (PCR–EIA) for the detection and typing of oncogenic and non-oncogenic HPV types.

Methods—The test was an original multiplex labelled PCR–EIA for the detection and typing of oncogenic and non-oncogenic HPV using three consensus sequence primers within the oncogenic E6/E7 ORF. One primer was dinitrophenyl (DNP) labelled and the DNP labelled amplimers could be further hybridised with specific biotinylated oligoprobes mixed in only two cocktails: oncogenic (16, 18, 31, 33, 35, 52, and 58) and non-oncogenic (6 and 11) HPV types in only two wells; then biotinylated oligoprobes were deposited in streptavidin-coated microplates. The PCR–EIA was validated on HPV plasmids (types 6, 11, 16, 18, 31, 33 35, 52, and 58) and used to evaluate cervical scrapes from 181 patients (median age 32 years) at high risk for cervical cancer.

Results—HPV were detected in the cervical scrapes of 88 of 181 patients (48.6%); nine with non-oncogenic HPV (5.0%) and 79 with oncogenic HPV (43.6%) including 29 coinfections with oncogenic and non-oncogenic HPV. The number of oncogenic HPV infections increased with the presence of high grade lesions: 95.8% of the cervical scrapes from patients with high grade lesions contained oncogenic HPV compared with 32.1% of the specimens from patients without any lesions detectable by colposcopy and/or by cytological examination of the cervical smears. Moreover, 60% of cervical scrapes exhibiting low grade lesions contained oncogenic HPV.

Conclusions—This test is simple, specific, sensitive, safe, fast, reproducible, and easy to use in routine practice. Thus, it is possible to detect simultaneously on a simple cervical scrape, two kinds of HPV—oncogenic and non-oncogenic—in just two microplate wells with non-isotopic oligoprobes.

(*J Clin Pathol* 1998;51:38–43)

Papillomaviruses are quantitatively the most important group of viruses associated with benign and malignant neoplasias in humans. More than 70 human papillomavirus (HPV) genotypes, oncogenic or not, currently are known to infect either mucosa or skin. More than 30 HPV types infect the anogenital mucosa and some oncogenic HPV types (mainly HPV16, 18, and 33) predominate in malignant squamous cell tumours.[1 2 3 4 5] Indeed, 95% of genital carcinomas are now associated with HPV and, on the basis of follow up studies of women with cervical intraepithelial neoplastic (CIN) lesions, it appears that the presence of high risk or oncogenic HPV types has a predictive value for progressive disease.[2 6 7] However, the significance of the presence of low risk HPV types (non-oncogenic HPV6 and 11) is not harmless. The detection of low risk HPV may be an indication that the patient may also be or become a likely candidate for infection by a high risk type.[8] Various nucleic acid hybridisation procedures, with or without gene amplification, have been developed, each of which has specific advantages and drawbacks. Many molecular biology techniques are available and widely varying results have been reported linked to the sensitivity of each method, and to the nature of the biological samples. Polymerase chain reaction (PCR) is the most sensitive method for the detection of HPV sequences in clinical samples, and HPV typing has been performed by sequencing, restriction enzyme analysis, and HPV type specific hybridisation of PCR products.[5 9] However, the presence of many HPV types complicated the design of PCR based assays for HPV detection, because of the dramatic increase of such infections in most countries since the 1970s.[4 10] Consensus PCR tests solved this problem by using primer sequences, frequently in the L1 open reading frame (ORF), which has been highly conserved between HPV types.[11 12] Nevertheless, the use of L1 sequences may miss some HPV16 and other oncogenic HPV types because of the loss of the L1 sequence during viral DNA integration.[13 14] It is well known that oncogenicity of HPV DNA is mainly induced by E6 and E7 ORF. Therefore, we developed an original PCR–enzyme immunoassay (EIA) for the detection and typing of oncogenic and non-oncogenic HPV types using three consensus sequence primers within the oncogenic E6 and

E7 ORF. We used a modified version of the protocol of Fujinaga et al[15] to amplify nine HPV types: 6, 11, 16, 18, 31, 33, 35, 52, and 58, and a multiplex PCR with one dinitrophenyl (DNP) labelled primer.[9] The amplification products or amplimers could then be further hybridised with specific labelled oligoprobes mixed in two cocktails: one oncogenic and the other non-oncogenic biotinylated HPV oligo-probes. Each cocktail was deposited in one well of a streptavidin-coated microplate. This non-isotopic PCR assay was validated on HPV plasmids and applied on clinical specimens.

Materials and methods

CLINICAL SPECIMENS AND CONTROLS

One hundred and eighty one women at high risk for cervical cancer (median age 32 years) were examined in the obstetrics and gynaecology departments of Amiens and Reims. These patients were selected because of their histories of cytological and/or histological cervical/vaginal abnormalities. They were offered HPV testing on cytobrush collected samples as well as routine cytological testing. The smear was obtained with an Ayre's spatula. The cytobrush used to detect HPV was immersed immediately in transport medium phosphate buffered saline pH 7.2. Tubes with cytobrushes were stored at −20°C. Total cellular DNA was extracted classically from cytobrush specimens. Briefly, cells were lysed, proteins were extracted with phenol-chloroform-isoamyl alcohol and precipitated with ethanol, and purified DNA was suspended in sterile water. For an average PCR assay 5–10 µl of genomic DNA template were used. Agarose gel electrophoresis was used to control PCR analysis, and the absence of inhibitors was checked by amplifying a part of exon 10 (250 base pairs) of the cystic fibrosis transmembrane conductance regulator gene whose size is similar to that of the HPV multiplex amplimer.[16]

Positive controls used were HPV plasmids (pHPV types 6, 11, 16, 18, 31, 33, 35, 52, and 58) to check the PCR consensus primers, and the subsequent Southern blot hybridisation. Other positive controls were HPV infected cell lines, CaSki (type 16), and HeLa (type 18).[2] Moreover, we obtained positive HPV types 6 and 11 tissular samples from a condylomata acuminata.

Three negative controls were systematically used: water, human genomic DNA from blood samples, and Phi X 174 phage DNA.

CONSENSUS PRIMERS AND MULTIPLEX PCR

The consensus primers used targeted a portion of the HPV E6/E7 region (228 to 268 base pairs)[15]: two sense primers pU-1M (for oncogenic HPV types 16, 18, 31, 33, 35, 52, and 58) and pU-31B (for non-oncogenic HPV6 and 11), and one antisense primer pU-2R (common to the HPV quoted above). The common primer was labelled with DNP[9] and a multiplex PCR was run with the three consensus primers.

Multiplex PCR amplification was carried out on 5–10 µl of genomic DNA or 1 ng of pHPV in a volume of 100 µl containing 50 mM Tris-HCl (pH 9), 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 µM each of dATP, dTTP, dGTP, and dCTP, 10 pmol of pU-1M and pU-31B primers, 20 pmol of pU-2R-DNP primer, and 1.25 units of taq DNA polymerase. Samples were amplified in a DNA thermal cycler heat block (Thermocis, Cis bio International, Gif-sur-Yvette, France). The first DNA denaturation was done for seven minutes at 94°C, then 30 cycles of PCR were run: denaturation one minute at 94°C, annealing for two minutes at 55°C, extension for two minutes at 72°C, followed by a final extension for 10 minutes at 72°C.

PREPARATION OF MICROPLATES AND BIOTINYLATED OLIGOPROBES

Polystyrene 96-well microplates were purchased ready to use (Streptavidin-coated Combiplates, Breakable Microstrips; Life Science International, Labsystems, Cergy-Pontoise, France). Oligoprobes were chosen according to the HPV sequence database (Los Alamos National Laboratory, New Mexico, USA) and synthesised by Cis bio International. Biotin label was attached to the 5' end during oligoprobes synthesis (table 1).

We developed two biotinylated oligoprobe cocktails, one oncogenic and the other non-oncogenic; 100 µl of each cocktail were deposited in one streptavidin-coated well. Each oligoprobe was diluted to obtain an optical density (OD) of 0.05; various oligoprobe concentrations from 0.05 to 0.2 OD were tested and 0.05 OD gave the optimal results. The oncogenic well contained the seven oncogenic oligoprobes (HPV types 16, 18, 31, 33, 35, 52, and 58—that is, 14.3 µl of each), and the non-oncogenic well (HPV types 6 and 11) contained the two non-oncogenic oligoprobes (50 µl of each). Biotinylated oligoprobes can be deposited in wells a few days before hybridisation and must be stored at 4°C.

HYBRIDISATION SOLUTION AND EIA FOR HYBRIDS

Figure 1 shows the procedure used for multiplex PCR and detection. Briefly, 100 µl of each cocktail of biotinylated oligoprobes were deposited in one well each and incubated for one hour at 37°C. After extensive washing with solution 1 we added to each well 100 µl of hybridisation buffer and 20 µl of PCR amplimer. After 10 minutes of incubation at room temperature, we added 15 µl of neutralisation solution and incubated for one hour at 37°C.

After extensive washing with solution 1, 100 µl of the conjugate solution (anti-DNP monoclonal antibody, horseradish peroxidase) were deposited in each well and incubated for one hour at 37°C. After extensive washing

Table 1 Oligoprobes used for hybridisation in microplates

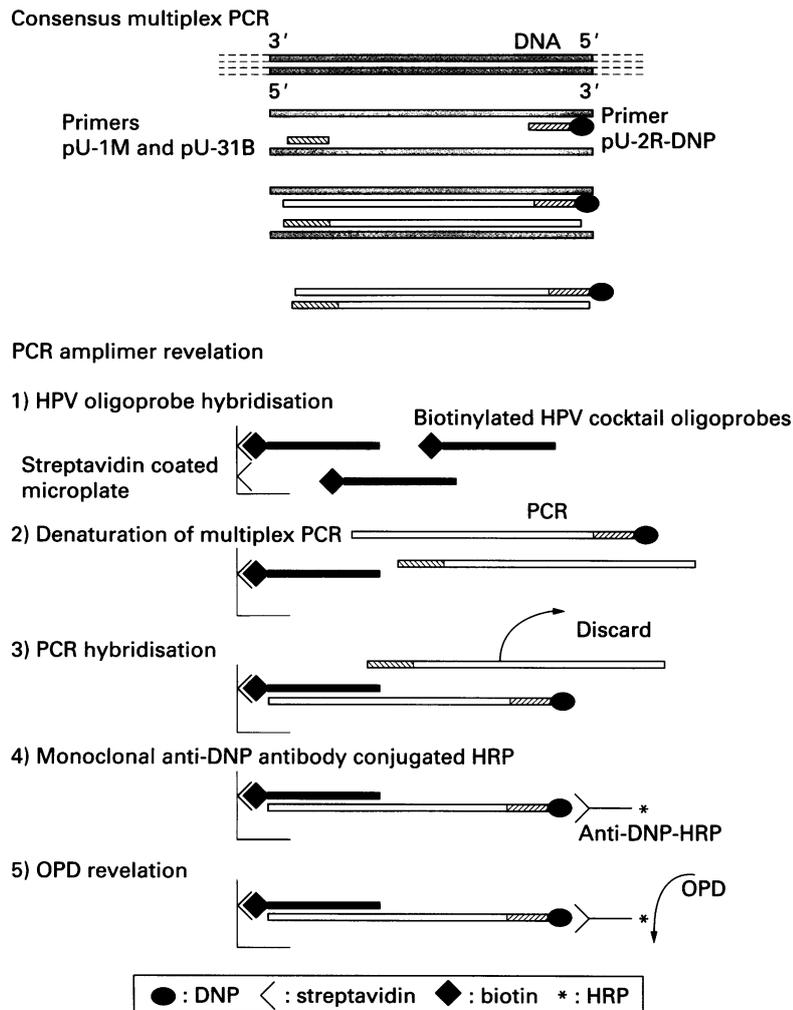| HPV type	Oligoprobes	Base pairs
6B	5' GCT AAA TTG TAC GTG GAA GG 3'	548–567
11	5' TGC ATG GAA GAC TTG TTA CC 3'	545–564
16	5' AAA CCC AGC TGT AAT CAT GC 3'	559–578
18	5' AGA CAC CAA TGA AAA ACG AC 3'	487–506
31	5' AAG ACC TCG TAC TGA AAC CC 3'	544–563
33	5' AGA GAA ACT GCA CTG TGA CG 3'	554–573
35	5' GTA GAG AAA CCG AGG TGT AA 3'	555–574
52	5' TGA CCC AAG TGT AAC GTC AT 3'	549–568
58	5' CTG TGC AGT GTG TTG GAG AC 3'	528–547

Figure 1   PCR–EIA procedure. HRP, horseradish peroxidase; OPD, orthophenylenediamine-2 HCl.

- Neutralisation buffer was 1.6 M acetic acid.

- Washing solution 1 was 0.5 M sodium chloride in fixation phosphate buffer.

- Washing solution 2 was 0.05% Tween 20 in washing solution 1.

- Saturation solution was 1 g of bovine serum albumin diluted in 10 ml of distilled water.

- Conjugate solution was 3 ml of saturation solution, 7.5 ml of washing solution 1, and 1 ml of anti-DNP monoclonal antibody conjugated to horseradish peroxidase.

- Revelation solution used during the last 10 minutes of the last washing step was obtained by dissolving one tablet of OPD in 5 ml of OPD buffer (0.02% H_2O_2) in a dark vial at room temperature.

- Stop solution was made by dissolving a packet of oxalic acid (Cis bio International) in 25 ml of distilled water, and stirring for 10 minutes.

Results

OPTIMISATION OF PCR: SPECIFICITY AND SENSITIVITY

We checked the separation of consensus primers with oncogenic PCR (pU-1M and pU-2R-DNP) and non-oncogenic PCR (pU-31B and pU-2R-DNP). The same consensus PCR was validated with DNP labelled primer and with a multiplex PCR procedure using the three primers. The nine pHPV types were individually amplified with multiplex PCR. Restriction enzyme digestion of the various amplimers was used for the HPV typing on agarose gels (except for type 35) and confirmed pHPV genotypes. In addition, this multiplex PCR, unlike the previously described approach,[15] could also amplify pHPV type 35, as detected by Southern blotting of the consensus amplimers (data not shown). Moreover, with this methodology, it was possible to amplify several pHPV simultaneously (for example, pHPV types 6/11, 6/16, 6/18, 31/33, 11/16/31) at equal amounts (for example, 10 ng of each pHPV in the PCR) or at varying amounts (from 10 ng to 0.1 pg—for example, 10 ng of pHPV6 and 0.1 pg of pHPV16 and inversely in the PCR).

Sensitivity of the detection of amplimers on agarose gels was evaluated by amplifying different amounts of each pHPV, ranging from 100 ng to 1 fg. We also amplified these diluted pHPV in the presence of 1 μg genomic human DNA to mimic true specimens and obtained the same results with or without the latter. The threshold of sensitivity for all these pHPV amplimers detected on agarose gels was 100 fg, with extreme limits of detection being 10 pg for HPV18 and 10 fg for HPV31.

SPECIFICITY OF HYBRIDISATION

Biotinylated oligoprobes were used to detect and type oncogenic and non-oncogenic HPV groups. The specificity of these oligoprobes was controlled by Southern blotting, which generated intense hybridisation bands with

with solution 2, 100 μl of the orthophenylenediamine-2 HCl (OPD) revelation solution were added. The colour was allowed to develop in the dark for 30 minutes and was stopped by the addition of 200 μl of stop solution. The OD was measured at a wavelength of 492 nm on a Dynathec MR 5000 spectrophotometer processor.

A result was considered positive when the OD_{492} value was higher than a control value (C) and C = 0.05 + average NC, where NC is the average of the three negative controls systematically included in each plate. If the value obtained was close to C, the sample was reinvestigated.[17]

REAGENTS

The following reagents were used and some were purchased ready to use from Cis bio International:

- Fixation phosphate buffer was made by diluting one tablet in 200 ml of distilled water that had to be stirred to ensure a dissolution.

- Hybridisation buffer was 0.5 M sodium chloride and 0.2 M sodium hydroxide (final concentrations) dissolved in fixation phosphate buffer.

Table 2 Results obtained with clinical samples

Lesion in cervical scrape	Non-oncogenic HPV	Oncogenic HPV			No HPV
		Oncogenic	Coinfections*	Total	
None (n = 137)	8 (5.9)	23 (16.8)	21 (15.3)	44 (32.1)	85 (62.0)
Low grade (n = 20)	1 (5.0)	8 (40.0)	4 (20.0)	12 (60.0)	7 (35.0)
High grade (n = 24)	0 (0.0)	19 (79.1)	4 (16.7)	23 (95.8)	1 (4.20)
Total (n = 181)	9 (5.0)	50	29	79 (43.6)	93 (51.4)

Values are n (%).
*Coinfections with oncogenic and non-oncogenic HPV.

amplimers obtained from various pHPV with multiplex consensus PCR. In parallel, the same positive hybridisations were obtained in each well of the streptavidin coated microtitre plate with one oligoprobe reacting with pHPV amplimers. On Southern blot, some weak cross reactivities were noted between oligoprobes detecting pHPV types 16, 31, 33, and 35, and pHPV types 52 and 58, as described previously.[2] Weak cross reactivities between HPV could not be abolished by hybridisation at higher temperatures (data not shown) on Southern blot but were overcome on microplates.

The cocktails of oncogenic and non-oncogenic oligoprobes were deposited in only two separate microplate wells. Multiplex amplimers (PCR performed with 1 ng of each plasmid tested) were hybridised in these wells. The non-oncogenic cocktail yielded positive results with the non-oncogenic pHPV only, and the oncogenic cocktail hybridised only with oncogenic pHPV. It must be emphasised that there was a total absence of cross reactivity between the oligoprobes detecting oncogenic pHPV and those specific to non-oncogenic pHPV.

SENSITIVITY OF HYBRIDISATION
The sensitivity of the hybridisation on microplates varied from 1 pg to 1 fg, equal to the picogram level for the less well amplified pHPV types (like pHPV18) (1 pg in the initial PCR) to the femtogram level for the well amplified pHPV types (like pHPV31) (1 fg in the initial PCR).

Positive OD_{492} ranged from 0.119 to 2.800 OD_{492} (or over) and negative values from 0.009 to 0.109 OD_{492}. A close parallel was noted between the initial amount of viral DNA in the PCR and the intensity of the OD_{492}.

REPRODUCIBILITY OF HYBRIDISATION
Reproducibility was evaluated by repeating the PCR and hybridisations of three positive and three negative samples on different days. In negative samples the SD of the mean OD_{492} values ranged from 0.015 to 0.050; in positive samples it was 0.119 to 0.620.

CONTROLS DETECTION OF HPV GROUPS
Positive controls hybridised specifically with oncogenic (CaSki and HeLa cells) and non-oncogenic (condylomata acuminata) DNA cocktails without any cross reactivity. Negative controls never hybridised with the two cocktails.

CLINICAL SAMPLES: DETECTION OF HPV GROUPS
HPV were detected in the cervical scrapes of 88 of 181 patients (48.6%); nine with non-oncogenic HPV (5.0%) and 79 with oncogenic HPV (43.6%) including 29 coinfections with oncogenic and non-oncogenic HPV (table 2). The number of oncogenic HPV infections increased with the presence of high grade lesions: 95.8% of the cervical scrapes from patients with high grade lesions contained oncogenic HPV compared with 32.1% of the specimens from patients without any lesions detectable by colposcopy and/or by cytological examination of the cervical smears. Moreover, 60% of cervical scrapes exhibiting low grade lesions contained oncogenic HPV.

In this series of patients, it was possible to amplify DNA of unknown HPV type from one patient that was detected on an agarose gel but did not hybridise on microplates.

Discussion
Numerous molecular biology techniques to detect HPV types are now available and it is not yet evident which is the best method(s) for routine analysis. In this study, we developed and applied an original system: multiplex PCR with consensus primers for E6/E7 ORF followed by hybridisation with cocktails of biotinylated oligoprobes in microplates. The whole test—from PCR to EIA—can be performed in a single day. At present, PCR is the most sensitive and specific method, and it is easy to use for routine screening.[5 18] Furthermore, hybridisation on microplates increases the sensitivity and specificity of this system. Numerous PCR primers have been described in the literature and can be used to detect one HPV type or a group of HPV types. Consensus primers are thus useful in routine testing or epidemiological studies because they can rapidly screen for many HPV types. These consensus primers generally target L1 or L2 HPV ORF[6 11 12 19 20 21 22] while few target E6 and E7 ORF.[15 23] However, these latter DNA sequences are conserved during viral integration and are responsible for the oncogenicity of HPV. Comparative studies with PCR amplifying L1 ORF and E6 ORF demonstrated a higher rate of oncogenic HPV detection with the E6 primers.[13 14 23] Therefore, to increase the rate of detection of HPV, we amplified the E6 and E7 ORF. Indeed the aim of our HPV detection system is not to replace systems using L1 consensus primers but to complete the HPV detection when those methodologies give negative results. In addition, our PCR protocol generates small amplimers of 250 base pairs that are easier to obtain than the large fragments of 450 base pairs with L1[11] consensus primers, and it has been shown that the efficiency of the primer pair is inversely correlated to the length of the amplimer.[19] We used consensus primers for E6/E7 sequences to detect HPV types 6, 11, 16, 18, 31, 33, 35, 52, and 58. In a recent paper, Lungu et al also used a PCR-EIA system but the procedure to target the E6 gene was different.[23] They were not able to

distinguish non-oncogenic from oncogenic HPV and succeeded in detecting less HPV than expected.

The second step of our method comprised hybridisation and detection of PCR products in microplates coated with streptavidin. This methodology (using PCR with L1 consensus primers) has been described by some authors with microplate hybridisation[10 13 24 25 27] or with dot-blot detection.[6 26 28] However, they detected only one HPV type per well or per blot. To obtain a more informative clinical assay, we preferred to simplify the final HPV detection by dividing them into two groups: oncogenic and non-oncogenic. The corresponding cocktails of biotinylated oligoprobes were deposited in different wells: one contained seven oligoprobes recognising oncogenic HPV types and the second contained two oligoprobes binding to non-oncogenic HPV types. Aliquots of PCR amplimers were deposited in each well. Hybridised products were detected by a monoclonal anti-DNP antibody. This DNP system was previously validated on solid support detection (tubes) with three nested PCR amplified DNA to detect four HPV types 6, 11, 16, and 18.[9] Hybridisation in microplates improves the sensitivity and specificity of the multiplex PCR, especially for PCR products not visible on the agarose gel or when some additional DNA bands exist. Compared to the results obtained with the detection of amplimers on agarose gels, the sensitivity was estimated to be 10-fold higher. The control of specificity showed no overlap between oncogenic and non-oncogenic HPV. In light of HPV phylogeny, it is possible that more HPV types can be detected[1] and we succeeded in detecting the previously undetected HPV type 35. Moreover, similar to Fujinaga *et al*,[15] we were able to amplify the DNA of an unknown HPV present in one patient that was detected on agarose gel but not hybridised on microplates. On that account, a new cocktail of probes can be created easily or new probes may be added to the previous cocktails. An advantage provided by this system is that each microplate has its own internal negative controls. Otherwise, the best sensitivity limit was 1 fg of pHPV, with some variations depending on HPV type (corresponding to 10^{-5} to 10^{-6} copies of HPV genome per cell, on dot-blot hybridisation according to Fujinaga *et al*[15]). Then, HPV18 is detected with a lower sensitivity (picogram level) using our system. However, we emphasise that to give high grade lesions, HPV18 is necessarily integrated in the host genome with the preservation of the E6/E7 sequences, which are selectively amplified with our PCR–EIA method. As viral integration is usually accompanied by the presence of viral DNA in every cell of the lesion, sensitivity of detection is less important.

The efficiency of this approach to HPV detection was tested on cervical scrapes. Forty four of the 137 patients without lesions (32.1%) were infected with oncogenic HPV, 5.9% with non-oncogenic HPV. Oncogenic HPV were found in 12 of 20 patients (60%) with low grade lesions. Twenty three of 24

(95.8%) patients with high grade lesions were infected with oncogenic HPV. These results agree with those in the literature.

In conclusion, this test is simple, specific, sensitive, fast, reproducible, and easy to use in routine practice. Thus, it is possible to detect simultaneously on a simple cervical scrape, two kinds of HPV—oncogenic and non-oncogenic—in just two microplate wells with non-isotopic oligoprobes. The technique can be automated and extensive epidemiological studies with non-isotopic oligoprobes are possible with easy reading by a spectrophotometer. Furthermore, this highly adaptable test can be extended to other DNAs.

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