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Genome Res. 1993 2: 250-252

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Sensitive Two-stage PCR of *p53* Genomic DNA Exons 5–9

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To assess the status of the tumor suppressor gene *p53* from samples with low levels (sub-nanogram amounts) of genomic DNA (e.g., from exfoliated cells, skin, small biopsies, mucosa), a technique based on two successive rounds of PCR was developed. In the first round, a 1.84-kb fragment spanning exons 5–9 was generated using a "touchdown" protocol. After purification by spun-column chromatography, this fragment was used as a template for the amplification of the individual exons 5–9 with inner primer sets specific for the adjacent intronic regions. Using this nested primer approach, several micrograms of each individual exon were obtained starting from as little as 62.5 pg of total genomic DNA. This material proved ideal for future analyses by single-strand conformational polymorphism (SSCP) analysis and DNA sequencing.

Recent studies indicate that mutations in the *p53* gene may be the most common genetic target observed in human malignancies.^(1,2) Mutations in the *p53* gene have been implicated in many forms of human neoplastic disease.^(1,3,4) The analysis of mutant and wild-type *p53* alleles in a wide variety of tumors and cell lines has now established *p53* to be a tumor suppressor gene. The arsenal of molecular techniques for the analysis of mutations has recently been enhanced by the application of the PCR.⁽⁵⁾ As part of an experimental system for the analysis of DNA sequence to investigate the origins of cancer, we developed a sensitive two-stage PCR for individual exons 5, 6, 7, 8, and 9 of human *p53*. Although a sensitive two-stage technique for amplification and sequencing of exons 5–9 has been described recently by Kovach et al.,⁽⁶⁾ this method does not yield individual exons suitable for analysis by SSCP. The method described here provides individual exons (around 250 bp) ideal for analysis by SSCP. We were expecting low amounts of DNA (below 1 ng total, e.g., for exfoliated bladder epithelium cells obtained from urine samples) in some of our samples to be analyzed for mutations in *p53*. To reduce mispriming and to increase the efficiency of the PCR, we developed a protocol using "touchdown" PCR amplification⁽⁷⁾ of a 1.84-kb fragment containing exons 5–9 as a first step, followed by amplification of the individual exons in a second PCR. In the touchdown program, the annealing temperature starts 4°C above the calculated value for the specific primer pair and is sequentially lowered to the annealing temperature during the PCR (see Materials and Methods). This paper describes the PCR protocol for the amplification of *p53*

exons 5–9 starting from sub-nanogram amounts of genomic DNA.

MATERIALS AND METHODS

Source of DNA Templates and DNA Extraction Procedure

Template DNAs used in this study were derived from cell lines, bladder cancer biopsies, and exfoliated cells from urine samples. Cell lines used in this study included: retinoblastoma cell line Y79 (ATCC HTB 18, RFLP in exon 4, codon 72 of the *p53* gene),⁽⁸⁾ lymphoblastoid cell line GM1 (wild type for *p53* exons 5–9, unpublished results), and COLO 320 DM (ATCC CCL 220, point mutation in exon 7, codon 248 of *p53*).⁽⁸⁾ DNA was extracted by proteinase K digestion and phenol extraction as described.⁽⁹⁾ Extracted and purified DNA was quantitated by OD₂₆₀ measurements.

Performance of the PCR

A Perkin-Elmer 9600 series PCR machine was used in this study. The following solutions were used: (1) PCR buffer, 10 mM Tris/HCl (pH 8.5), 20 mM KCl, 3 mM MgCl₂, final concentration. (2) dNTPs (Pharmacia, 100 mM stock), 0.5 mM final concentration each. (3) Oligonucleotide primers (see Table 1 for primer sequences; all primers were synthesized by Dalton Chemicals, North York, Ontario, Canada) were used at 5 pmoles each per PCR assay. (4) *Taq* polymerase (Perkin-Elmer Cetus AmpliTaq) was used at 0.5 μl (2.5 units) were used per PCR assay. (5) Templates, genomic DNA template was extracted as described above; 2 ng of template per assay was used routinely. Using this nested primer approach, 62.5

TABLE 1 Primer Sets Used for the Amplification of p53 Exons 5–9 from Genomic DNA

PCR of exon	Nucl.# ^a	Size	T_m ^b	Sequence (5' → 3') ^c			
1. Round of PCR:							
5–9	12,985	1841	56.4	F:CGTGT	TCCAG	TTGCT	TTATC
	14,806			R:AAGAA	GAAAA	CGGCA	TTTTG
2. Round of PCR:							
5	13,024	252	55.9	TTCA	ACTCT	GTCTC	CTTCC
	13,256			AACCA	GCCCT	GTCGT	CTCTC
6	13,271	224	58.7	TGGTT	GCCCA	GGGTC	CCCAG
	13,475			CGGAG	GGCCA	CTGAC	AACCA
7	13,941	237	58.9	CTTGC	CACAG	GTCTC	CCCAA
	14,158			AGGGG	TCAGC	GGCAA	GCAGA
8	14,346	288	57.3	AAAGG	ACAAG	GGTGG	TTGGG
	14,614			CTGCA	CCCTT	GGTCT	CCTCC
9	14,610	216	52.8	CGGTG	GAGGA	GACCA	AGGGT
	14,806			AAGAA	GAAAA	CGGCA	TTTTG

^aRefers to the position of the first nucleotide used in the primer; the numbers are taken from Hump53G 20303 bp ds-DNA, EMBL Accession X54156.

^bThe T_m values given here were determined by the Oligo software.

^c(F) Forward primer; (R) reverse primer.

pg of genomic DNA has been amplified successfully. Filter-sterilized nanopure water (>17 Megohm resistance) was used throughout this study.

Conditions for the Amplification of 1.84-kb Fragment Spanning Exons 5–9

The assay included: 10× buffer, 5 μl; 10× dNTPs, 5 μl; forward and reverse primer at a concentration of 5 pmoles each in 4 μl of water; *Taq* polymerase, 0.5 μl (2.5 units); template DNA, 62.5 pg to 8 ng; and water to a final volume of 32.5 μl.

Amplification program: Denaturation at 95°C for 5 min.

“Touchdown” protocol: 94°C for 30 sec/60°C for 1 min/72°C for 30 sec: 2 cycles, 94°C for 30 sec/59°C for 1 min/72°C for 30 sec: 4 cycles, 94°C for 30 sec/58°C for 1 min/72°C for 30 sec: 8 cycles, 94°C for 30 sec/57°C for 1 min/72°C for 30 sec: 10 cycles, 94°C for 20 sec/56.5°C for 40 sec/72°C for 40 sec: 10 cycles. Soak cycle: 4°C hold.

After PCR, purification of the 1.84-kb fragment from the products of the first round of PCR was performed using a Pharmacia spun column Sephacryl S-400 according to manufacturer's specifica-

tions. After the purification step, 5 μl of the purified 1.84-kb fragment was used as a template for the amplification of individual exons.

Conditions for the Amplification of Individual Exons 5, 6, 7, 8, and 9

The assay included: 10× buffer, 5 μl; 10× dNTPs, 5 μl; forward and reverse primer at a concentration of 5 pmoles each in 4 μl of water; template, 5 μl; *Taq* polymerase, 0.5 μl (2.5 units); and water to a final volume of 50 μl.

PCR program: Denaturation at 95°C for 5 min. Amplification: 94°C for 20 sec/53°C for 40 sec/72°C for 20 sec: 22 cycles. Soak cycle: 4°C hold.

RESULTS AND DISCUSSION

Serial two-fold dilutions (eight) of DNA templates from cell lines COLO 320 DM, Y79, and GM1, ranging from 8 ng to 62.5 pg were used in the first round of PCR. PCR assays were analyzed by running 5 μl on a 1% agarose gel at 70 Volts for 40 min. A 1-kb ladder (GIBCO BRL) was used as a standard in the agarose gel electrophoresis procedures. A typical result with DNA from cell line COLO 320 DM is shown in Figure 1. After ethidium bromide staining, a band was visible at 1.84 kb for template concentrations above 0.5 ng. For lower template concentrations, usually no band or only a very weak band was visible. The products of the eight PCR reactions shown in Figure 1 were separated from primer and low-molecular-weight components by gel filtration using a Sephacryl S-400 spun column (Pharmacia). We found that this purification step increased the purity of the individual exons obtained in the second PCR. As a standard procedure, two PCR assays were combined (60 μl) for this purification step. Five microliters of purified 1.84-kb fragment was used as template for the amplification of individual exons. PCR assays after the second round of amplification (template amplified from 1 ng of genomic DNA) analyzed by running 5 μl on a 1% agarose gel at 70 Volts for 30 min are shown in Figure 2. Individual exons 5–9 were obtained for all eight serial dilutions of template, down to 62.5 pg of template. In subsequent studies, DNA templates from 25 tumor tissues and 39 exfoliated cells samples were amplified using the above protocol. The template concentra-



FIGURE 1 Amplification of a 1.84-kb DNA fragment spanning exons 5–9 of human of genomic p53. Serial dilutions of template DNA extracted from cell line COLO 320 DM were amplified as described in the text. Five microliters of the PCR assays were loaded onto a 1% agarose gel, stained with ethidium bromide, and photographed under UV light. (Lane 1) 8 ng of template; (lane 2) 4 ng of template; (lane 3) 2 ng of template; (lane 4) 1 ng of template; (lane 5) 0.5 ng of template; (lane 6) 0.25 ng of template; (lane 7) 0.125 ng of template; (lane 8) 62.5 pg of template; (lane 9) size standards (1-kb ladder, GIBCO BRL).

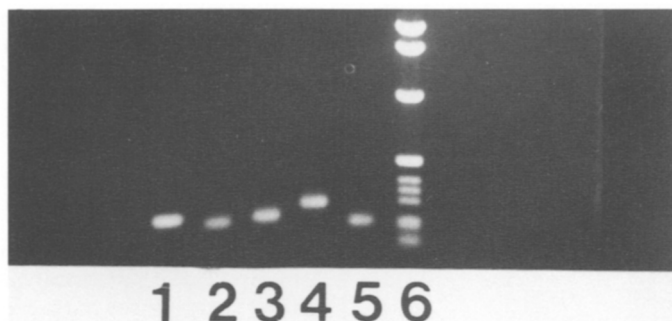


FIGURE 2 Amplification of individual exons 5, 6, 7, 8, and 9 of human *p53* using a purified 1.84-kb fragment obtained from 1 ng of genomic DNA (cell line COLO 320 DM) as template. Five microliters of template was amplified as described in the text. Five microliters of the PCR assays were loaded onto a 1% agarose gel, stained with ethidium bromide, and photographed under UV light. (Lane 1) Exon 5, 252 bp; (lane 2) exon 6, 224 bp; (lane 3) exon 7, 237 bp; (lane 4) exon 8, 288 bp; (lane 5) exon 9, 221 bp; (lane 6) size standards (1-kb ladder, GIBCO BRL).

tions ranged from 0.05 ng/ μ l to several 100 ng/ μ l. All 5 exons could be amplified successfully using this procedure. The DNA sequence of exons 5–9 was analyzed after PCR. The published DNA sequences of exons 5–9 and the point mutation in exon 7 of the COLO 320 DM cell line⁽⁸⁾ could be confirmed (data not shown). Amplified exons were kept at -20°C for future analysis (e.g., SSCPA, DNA sequencing).

ACKNOWLEDGMENTS

We thank Xili Miao and Michael Wetklo for their valuable assistance. This work was funded by the National Institute of Occupational Safety and Health (NIOSH) under contract #211-91-0007. The work is part of a joint NIOSH/National Cancer Institute Study of biological markers of occupational bladder cancer.

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Received July 23, 1992; accepted in revised form September 24, 1992.