Improved Detection and Characterization of Mutations by Primer Addition in Nonradioisotopic SSCP and Direct PCR Sequencing

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Single-strand conformation polymorphism (SSCP) has become the most widely used method to screen new mutations or already-established polymorphisms. Several protocols have been described to overcome the two main problems related to this technique, which are separation of both strands and detection. For the former, there are methods using a strong denaturant such as methylmercury hydroxide to enhance strand separation. Others try to enrich one of the strands by asymmetric polymerase chain reaction (PCR), biotinylated PCR primers or RNA SSCP (3). To improve detection, radioactive isotopes (4), gel silver staining (1) or fluorescence-labeled primers (5) have been used.

We describe a simpler method that, without using radioisotopes or previous treatments such as re-amplification or transcription, improves both single-strand separation and simple ethidium bromide (EtdBr) detection. It is based on the observation that the combination of heat denaturation and primer addition before loading onto the gel greatly prevents re-annealing of DNA during electrophoresis, giving a higher yield of single strands. This allows its EtdBr detection and eliminates artifactual bands that result from partial denaturation of PCR fragments. This protocol allows the use of the PCR product directly without previous treatments. Moreover, we managed to directly sequence aberrant bands after recovering them from the gel. We have used this protocol to improve strand separation and/or mutation detection in exons of the p16 and p53 tumor-suppressor genes with size ranges from 100 to 267 bp. Here, the improvement of the strand separation in exons 6, 8 and 9 of p53 and the detection of the already-known polymorphism in codon 213 of this gene are

Table 1. SSCP Conditions of Exons 6, 8 and 9 of p53

Exons	% Acrylamide	TBE in Gel	Temperature	Size	Time
6	15	1×	19°C	185 bp	5 h
8	8	0.5×	22°C	200 bp	2.5 h
9	15	1×	18°C	102 bp	5 h

presented.

p53 exons 6, 8 and 9 were amplified using the Human p53 Amplimer Panel (Exons 2–11) (CLONTECH Laboratories, Palo Alto, CA, USA) following the manufacturer's instructions. PCR products (10 μ L) were diluted 1:3 with water, and after addition of 3 μ L of loading buffer (98% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol, pH 5.3), 10 pmol of forward (exon 8), reverse (exon 6) or both (exon 9) primers were added. This mixture was heated to 100°C for 4 min and quickly plunged into ice. Then, 35 μ L were loaded onto 16-cm $\times$ 16-cm polyacrylamide gels (39:1 acrylamide/bisacrylamide) (Protein® II xi Cell; Bio-Rad, Hercules, CA, USA). Optimal conditions for separation of each exon were accurately calculated (2) as specified in Table 1. All gels were run at 300–350 V in standard 1×

TBE (0.089 M Tris-HCl, 0.089 M boric acid, 10 mM EDTA, pH 8.2), and the electrophoresis lasted 2–5 h. Gels were stained with EtdBr for 15 min and destained in distilled water for 5 min. Strands with different mobilities were recovered from the gel with a scalpel and transferred to 1.5-mL tubes. A mixture containing 50 μ L of 0.3 M sodium acetate (pH 6.8) and 1 mM EDTA was added and incubated at 37°C overnight. The supernatant was pipetted off and filtered using 0.45- μ m filter tubes (Milipore, Bedford, MA, USA). Two microliters of this solution (or 1:1000/1:5000 dilutions) were re-amplified by PCR, purified with columns (Boehringer Mannheim GmbH, Mannheim, Germany) and subjected to the *fmol*® DNA Sequencing System (Promega, Madison, WI, USA).

As Figure 1 shows, addition of reverse primer in exon 6 and forward primer in exon 8 notably increases the visualization of single-stranded DNA. Otherwise, in exon 9, only addition of both primers allows discrimination of both bands. It is remarkable that in these three cases, the amount of double-stranded DNA has decreased considerably in those exons where primer

Figure 1. SSCP analysis of exons 6, 8 and 9 of the p53 gene. Lanes 1–5: p53 exon 6 SSCP. Control DNA without adding primer before loading onto the gel (lane 1), after addition of 10 pmol of forward primer (lane 2), after addition of 10 pmol of reverse primer (lane 3), after addition of 10 pmol of both primers (lane 4) and DNA of patient with acute myeloid leukemia with a known A→T substitution after addition of 10 pmol of reverse primer (lane 5). Lanes 6–9: p53 exon 8 SSCP. Control DNA without primer (lane 6), with forward primer (lane 7), with reverse primer (lane 8) and with both primers (lane 9). Lanes 10–13: p53 exon 9 SSCP. Control DNA without primer (lane 10), with forward primer (lane 11), with reverse primer (lane 12) and with both primers (lane 13). Arrow shows migration of double-stranded DNA.

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was added. Moreover, in exon 6, primer addition suppresses formation of an intermediate artifactual band. Extraction and sequencing of the new upper band in exon 6 (Figure 1) showed an A to T substitution at the last base of codon 213 (data not shown).

The PCR-SSCP modification described here highlights that: (i) the addition of primer allows strand separation of unresolved fragments by standard conditions; (ii) once the optimal conditions of separation of each exon are calculated, this method gives reproducible patterns of SSCP; (iii) primer addition in some exons avoids re-annealing of DNA during electrophoresis, decreasing the amount of double-stranded product, increasing single-strand concentration and improving its EtdBr visualization because of its better intercalation in the partial bicatenary structure formed by primer annealing; and (iv) characterization of

abnormal strands can be determined by directly cutting the band from the gel and re-amplifying without further purification. This gives a clearer sequence of aberrant bands without contamination of the standard product.

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