Excess PCR primers may dramatically affect SSCP efficiency

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SSCP analysis is one of the most commonly used techniques for screening of small genetic alterations and a very convenient alternative to direct sequencing. In this technique, PCR products are denatured and electrophoresed on non-denaturing polyacrylamide gels. Any change in the sequence theoretically causes a shift in the mobility of the analysed conformers. However, it is known that certain changes remain undetected by SSCP analysis. We also failed (not shown) to discriminate between two 144 bp cathepsin D sequences which only differ by a C/T base substitution at position 224 (2), using the original or modified (4) SSCP protocols on PCR products obtained from homozygous (T/T), heterozygous (C/T) and homozygous (C/C) breast cancer cell lines (MCF7, MDA-MB-231 and T47D respectively). We solved part of the problem (Figure 1a) by increasing the polyacrylamide concentration of the SSCP gels and by slowing down the speed of the electrophoretic run. The best results were obtained when gels were run at room temperature in 0.5X TBE, 5% glycerol and 15% acrylamide for at least 6 h (the first 2 h at 50 volts and the end of run at 100 to 150 volts), and then silver stained (Kit Biorad, Paris, France). However, in these conditions where T/T genotypes were easily identified (lane 1), C/T heterozygotes (lane 2) and C/C homozygotes (lane 3) were still hardly or not at all separated. To identify migration of each of the two strands, we performed 'unbalanced' PCRs in which one of the two primer concentrations was lowered. Whereas lowering the downstream primer led to no amplification for unknown reasons, in SSCPs performed on unbalanced PCR products obtained using a downstream/upstream primer ratio of 12 (Figure 1a, lanes 4-6), all three genotype patterns were surprisingly different and bands migrated a little further than regular PCR products. SSCP analyses were discriminative when using unbalanced PCR. As the only difference between the two PCR protocols involved the primer concentrations, we hypothesized that excess primers in samples at the end of amplifications interfered with the DNA strands, changing their conformation and subsequently their SSCP profiles. To address this question and test the effects of each PCR reactive on the SSCP, we first performed a quick purification step of the 144 bp fragment: the specific DNA band was excised from the PCR control gel (5% polyacrylamide) and eluted in 50 µl H2O at 65°C for 1 h. The eluted products were then loaded onto an SSCP gel. SSCP patterns from unpurified (Figure 1a) and purified (Fig. 1b) products were again very different. However, in the latter protocol (Fig. 1b), products from regular (lanes 1 - 3) and unbalanced (lanes 4 - 6) PCRs exhibited comparable and distinctive patterns. We then compared (Figure 2) SSCP patterns obtained using unpurified PCR products (lane 1) to those obtained when the 144 bp fragment was eluted in water alone (lane 2), or in water supplemented with all PCR reagents singly: Taq polymerase (lane 3), nucleotides (lane 4), buffer (lane 5) or primers (lane 6). We found that, in each cell line, i.e. MCF7 (Figure 2a), T47D (Figure 2b) or MDA-MB-231 (not shown), primers were the only molecules interfering with the 144 bp sequence. We finally determined (Figure 2, lanes 8 - 12) the lowest primer concentration able to affect the SSCP profiles as being around 6 nM. The effects were so tightly dose-dependent that we obtained intermediate patterns as shown in Figure 2a (lane 10) and Figure 2b (lane 11). They were also strain-specific (lanes 13 and 14). Primer dose-dependent SSCP patterns were further observed when regular PCRs were performed in the presence of equal but decreasing (150 to 30 nM) amounts of primers without subsequent purification. In these conditions (not shown), only intermediate SSCP profiles were detected due to a loss of amplification yield when the primer concentration in the PCR mixtures was lower than 30 nM.

We also improved SSCP detection of point mutations 2 kb upstream in the same cathepsin D gene by performing unbalanced PCRs or purifying the products of regular amplifications as described above (not shown). This work highlights that: (i) In some systems, primers may interfere with the amplified sequence and inhibit the discriminative potential of SSCP. The interaction is likely due to amplified DNA-primer re-pairing during the run and probably explains previous inefficient 'double-stranded' (classical) SSCP and the superiority of 'single-stranded' SSCP which first require either two rounds of PCRs, the second one using only one primer (5), or in vitro transcription of the PCR products into RNA (6, 7); (ii) This cause of inefficient SSCP, which might be very frequent, is easily avoided by lowering primer concentrations prior to genotyping the amplified DNAs. We thus propose one of the two rapid and inexpensive protocols described in this paper: Unbalanced PCR or regular PCR followed by amplicon purification.

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Figure 1. PCR amplifications were carried out using the Bioprobe PCR kit system (Montreuil-sous-bois, France). Regular PCRs contained 500 nM of each primer (lanes 1–3) and unbalanced PCRs 42 nM upstream and 500 nM downstream primers (lanes 4–6). Primer sequences and PCR cycle were as described previously (2). SSCP samples were loaded as follows: Lanes 1 and 4: MCF7 (T/T); Lanes 2 and 5: MDA-MB-231 (C/T); Lanes 3 and 6: T47D (C/C).

Figure 2. SSCP were performed on unpurified (lanes 1) or purified (lanes 2–7) regular PCRs. Samples were purified in water supplemented with no reactive (2); 1.25 U of Taq polymerase (3); 50 µM nucleotides (4); 1 xPCR buffer (5); 500 nM of each primer (6); all PCR reactives (7); in the lower panel, primer dose responsiveness and specificity were tested as follows: Prior to SSCP, the PCR products were either not purified (lanes 8), or purified in water containing decreasing amounts of both primers: 150 nM (9); 30 nM (10); 6 nM (11); 0 nM (12); or 500 nM of only one primer: upstream primer (13); downstream primer (14).