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Nonisotopic discontinuous phase single strand conformation polymorphism (DP-SSCP): genetic profiling of D-loop of human mitochondrial (mt) DNA

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Several methods exist for the rapid detection of small DNA sequence differences such as point mutations. Single strand conformation polymorphism (SSCP) analysis (1), based on the differential sequence-dependent electrophoretic mobilities of single-stranded (ss) DNA in non-denaturing polyacrylamide gels, is widely used on account of its speed, sensitivity and ease of use. Recently, nonisotopic SSCP analysis using alkali denaturation and ethidium bromide staining was described (2), and applied to the quantification of DNA by competitive PCR (3). However, with this protocol a minority of DNA templates do not yield sufficient ssDNA, due to either inefficient denaturation or excessive reannealing. We have developed a method that overcomes this problem. therefore allowing the use of ethidium bromide SSCP analysis for a wider range of template DNA.

The principle of this method is the use of discontinuous phase gel electrophoresis, whereby DNA is completely denatured by initial passage through a 'stacking' gel containing formamide and allowed to assume secondary conformations in a non-denaturing 'resolving' gel. The lower resolving gel comprised 6% (w/v) polyacrylamide:bis−acrylamide (49:1 ratio) and 5% (w/v) glycerol in 0.5× TBE buffer, with 0.05% (w/v) ammonium persulphate and 0.2% (v/v) tetramethyl−ethylene−diamine (TEMED). The 0.4 mm thick gel was poured using a vertical format apparatus (Protean II. Biorad) as described previously (2), such that there was a 1 cm gap between the top of the resolving gel solution and the intended bottom of the wells. Using a Pasteur pipette, isopropanol was gently added as a layer to the top to allow polymerization to proceed, uninhibited by the presence of air. After 30 min, the isopropanol was removed by tilting the gel and absorption with tissue paper.

The upper stacking gel comprised 8% (w/v) polyacrylamide:bis−acrylamide (19:1 ratio) and 75% (v/v) formamide in 0.2× TBE buffer, with 0.25% (w/v) ammonium persulphate and 1% (v/v) TEMED. With the well-forming comb in place, the solution was allowed to polymerize for 6−16 h at 20°C or for 2 h at 42°C (since formamide retards polymerization). The comb was very carefully removed from the delicate upper gel. 1 μl of 10× loading buffer (0.5% bromophenol blue (w/v), 0.5% xylene cyanole (w/v) in formamide) was added to 10 μl of PCR DNA sample and heat denatured at 95°C for 5 min before snap cooling in ice and loading. Electrophoresis was performed in 0.5× TBE buffer at 4−20°C. The gel was stained in ethidium bromide solution (0.5 μg/ml) for 5 min, before visualisation by UV transillumination.

We demonstrated the increased sensitivity of this method by applying it to the genetic profiling of individuals using the highly polymorphic D-loop region of human mitochondrial DNA (4). Using the previously described SSCP protocol (2), the maternal inheritance of this locus was demonstrated but ssDNA bands were very weak-staining (Figure 1a). However, with discontinuous phase SSCP (DP-SSCP), ssDNA bands stained with much greater intensity as well as better resolution, using conventional UV transillumination and Polaroid photography (Figure 1b). The single phase protocol for routine use and the discontinuous phase protocol for DNA that are difficult to denature, facilitate the use of SSCP analysis for the rapid and nonisotopic detection of small/point mutations and polymorphisms.

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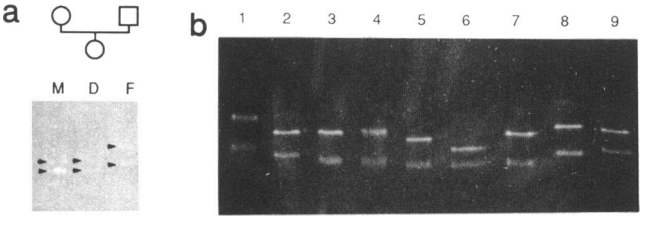

Figure 1. Genetic profiling of human individuals by SSCP analysis of 446 bp PCR fragment from the hypervariable D-loop region of mitochondrial DNA (4). (**a**) Conventional ethidium bromide SSCP analysis of family members (M = mother, F = father, D = daughter) shows maternal inheritance of very weakly-staining ssDNA bands (arrows). (**b**) Discontinuous phase SSCP of same fragment from unrelated individuals (lanes 1−9) results in intensely staining bands demonstrating highly polymorphic mobilities.

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