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Multiplex co-amplification of 24 retinoblastoma gene exons after pre-amplification by long-distance PCR

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Polymerase chain reaction (PCR) amplification has become the method of choice for preparing the DNA template in mutation analysis from complex mixtures of DNA or RNA molecules. This strategy is optimal for small genes or genes with mutational hotspots. However, PCR-based mutation detection is neither labour nor cost effective when large or multiple genes, with many target fragments, are involved. In addition, such an approach is limited by sample quantity. A typical example is the identification of all possible mutations, scattered along the length of disease genes. The problem is compounded by the necessity of processing large numbers of samples.

It has been known for some time that multiple target sequences in complex (higher animal) genomes can be amplified simultaneously, i.e., by multiplex PCR. In multiplex PCR, different DNA fragments are co-amplified under identical conditions, in the same reaction. When the aim is simply to amplify many fragments simultaneously, it is possible to overcome limiting primer kinetics and fragment competition to design optimal conditions for a multiplex system. However, when other constraints are also pertinent, the design of a set of conditions that allows multiplexing of a large number of gene fragments is not trivial (1). The first extensive multiplex reactions of nine fragments for the dystrophin gene were described by Chamberlain *et al.* (2) and Beggs *et al.* (3). These are exceptions: most multiplex systems do not involve more than about five amplicons. The obvious reason for this is that with each primer set added, the permissive reaction conditions allowing each fragment to reach its annealing temperature while evading spurious amplification products become increasingly less flexible. Ultimately, this lack of flexibility is due to the complexity of the genomic sequence environment which allows ample opportunity for non-specific priming (1).

When primers must be selected according to specific criteria, such as in mutation analysis, special constraints greatly lower the flexibility in experimental design of the multiplex system. A good example is denaturing gradient gel electrophoresis (DGGE) in which primers must be designed to encompass fragments with optimal melting behaviour. In such experiments, one of the primers is usually coupled to a GC-rich 'clamp' sequence (4). In an optimal situation, this will generate a two-domain structure with the GC-clamp as the higher melting domain, a configuration which will allow the detection of all possible mutations in the target sequence (5). It will often turn out that primers positioned in such a way as to generate optimal domain structures fit poorly in a multiplex design and vice versa.

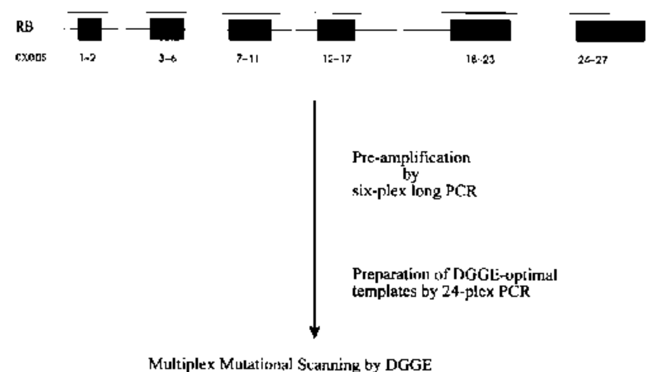

Figure 1. Multiplex PCR design for 24 exons of the RB1 gene.

Recently, it has become possible to PCR-amplify very long fragments directly from genomic DNA (6). We reasoned that this would be the most suitable way of reducing the sequence complexity for a multiplex PCR design. According to this principle, we designed an extensive multiplex PCR reaction for the retinoblastoma tumor suppressor gene, RB1, involving 24 exons (schematically depicted in Fig. 1). Primers for the long-PCR were positioned to obtain all target regions in the smallest possible number of fragments that could still be amplified through long-PCR, i.e. up to 20 kb. Multiplex long-PCR reactions were carried out in a GeneE thermocycler (Techne, Cambridge, UK) in a 50 µl volume with 100 ng genomic DNA as template and 0.25 µM of each primer, using the LA PCR kit (TaKaRa). The conditions were as follows. First, one cycle of 94°C, 1 min, followed by 32 cycles of 98°C, 20s/68°C, 12 min with 15 s incremental increases, and finally one cycle of 72°C, 5 min. The purpose of this first step was only to obtain overrepresentation of the RB1 genomic sequences relative to all other genomic DNA. The products of the long-PCR served as template for short-PCR (see below).

Primer pairs for subsequent short-PCR (with a GC-clamp attached to one primer of each pair; ref. 4) were selected for generating fragments with optimal melting characteristics in denaturing gradient gel electrophoresis. By using the computer program

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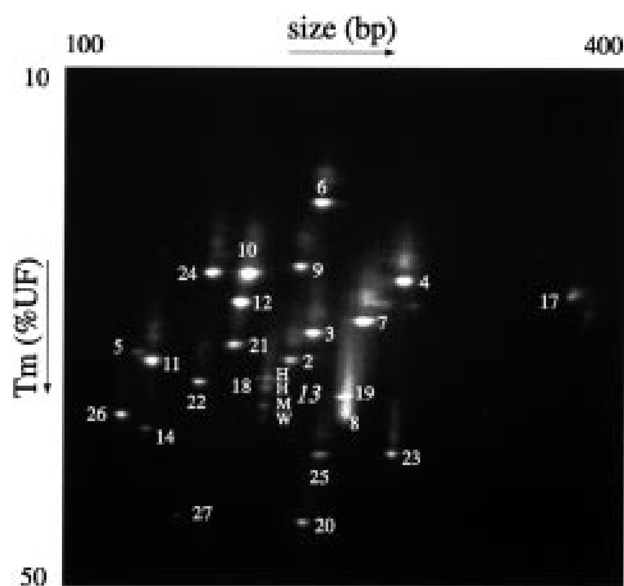

Figure 2. Multiplex PCR products obtained after one 6-plex long-PCR followed by one 24-plex short-PCR of 24 exons of the RB1 gene and separation by two-dimensional denaturing gradient gel electrophoresis. A mutation in exon 13 is indicated by four spots rather than one. The spot representing exon 18 overlaps with one of the exon 13 heteroduplexes. W = wild-type homoduplex variant; M = mutant homoduplex variant; H = heteroduplex variant.

MELT87 (7), each fragment was designed to consist of two melting domains: the GC-clamp (the higher melting domain) and the lower-melting target sequence, which was between 157 and 380 bp long. Two-domain structures were also confirmed empirically by using perpendicular denaturing gradient gel electrophoresis (results not shown). Short PCR reactions were carried out in a 50 µl volume and consisted of 4 µl long-PCR product, 0.125–0.5 µM of each primer, 0.25 mM dNTPs, 8 mM $MgCl_2$, 5 U *Taq* polymerase (Gibco BRL) and 1% DMSO. The PCR conditions were as follows. Five cycles of 94°C, 45 s/52°C, 40 s/68°C, 2 min, followed by 5 cycles of 94°C, 45 s/47°C, 40 s/68°C, 2.5 min, then 32 cycles of 94°C, 50 s/55°C, 10 s/40°C, 40 s/67°C, 2.5 min (with 3 s incremental increases per cycle). After the short PCR, fragments were heteroduplexed by one complete round of denaturation/renaturation: 67°C, 12 min/98°C, 12 min/52°C, 30 min/44°C, 30 min. After PCR and heteroduplexing, 1/10 vol of loading buffer was added and the mixtures were resolved by two-dimensional electrophoresis (8,9) in 9% polyacrylamide (PAA; 37.5:1 = acrylamide:bisacrylamide) gels (size separation) and 10% PAA gels containing 10–50% urea/formamide (UF) gradients (100% UF = 7 M urea/40% deionized formamide), using the DGGE system from C.B.S. Scientific Co. (Solana Beach, CA).

Figure 2 shows the multiplex reaction products obtained after two-dimensional denaturing gradient gel electrophoresis. In this high-resolution system, all 24 fragments are visible as spots with xy-coordinates indicating both size and melting temperature of the target fragments. In this particular genomic DNA sample, which was derived from a patient with heritable retinoblastoma [kindly provided by Dr Thaddeus P. Dryja (Massachusetts Eye and Ear Infirmary, Boston, MA)], a 1 bp deletion mutation was detected in exon 13. This is indicated by the appearance of four spot variants rather than one for the wild-type exon (the two homoduplex variants and the two heteroduplex variants).

From these results, it can be concluded that PCR multiplexing is no longer the limiting factor for rapid mutation scanning of complex genes, like RB1. In combination with the high resolving power of two-dimensional electrophoresis, this form of extensive multiplexing lends itself well to the rapid mutational scanning of genes with many exons. Indeed, we have shown that such gels easily allow complex mixtures of fragments to be resolved in a reproducible manner on the basis of both size and base sequence (8). This also allows unequivocal identification of each fragment and mutations therein. It was demonstrated that all PCR-amplified exons of the CFTR gene could be resolved by 2-D DGGE under one set of experimental conditions allowing 17 out of 17 identified mutations to be detected (9).

In the current design for the RB1 gene, a few fragments that should be included in routine mutational scanning of this gene were left out, i.e., the promoter region, exon 1 and the very small exons 15 and 16. The promoter region and exon 1 are very GC-rich and no attempt was made to include them in the PCR multiplex reaction or the 2-D separation; they are analyzed separately under different conditions. Exons 15 and 16 were later added to the multiplex as one additional fragment, bringing the total to 25. Indeed, without knowing the upper limit of PCR multiplexing, it seems that more primer pairs can be added to the reaction, if more attention is given to optimizing conditions.

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