

Allele specific amplification by tetra-primer PCR

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Genotyping a known point mutation or polymorphism can be achieved by restriction endonuclease digestion or allele specific oligonucleotide (ASO) hybridization. Recently, allele specific amplification (ASA) by the amplification refractory mutation system (ARMS) has been introduced, avoiding the use of restriction enzymes and radioisotopes (1). This method requires two separate polymerase chain reactions (PCR) to amplify each allele using different primers. We propose a protocol to accomplish ASA in a single PCR, using two annealing temperatures (2) and four primers. In contrast to ARMS, which requires a mismatched 3'-residue in the mismatch in tetra-primer PCR is in the middle of the internal primers.

A pair of flanking primers (1 & 2) and two internal primers (3 & 4), in conjunction with two temperature programs, are required in this method (Figure 1). The Tms (4(G + C) + 2(A + T)) of the flanking primers should be at least 10°C higher than those of the internal primers (3), which can usually be achieved by designing longer flanking primers and shorter internal primers. Primer 3 is completely complementary to the sense strand of allele 1 and primer 4 to the antisense strand of allele 2. Primer 3 is therefore refractory to PCR on allele 2, because its annealing is unstable. This also applies to primer 4 and allele 1.

The first block of cycles are performed with a higher annealing temperature and subsequent cycles at a lower annealing temperature. In the first program, a DNA fragment is produced by utilising only the flanking primers because the temperature is too high for the intemal primers to anneal. During the second program, this DNA fragment acts as a concentrated template which facilitates the low Tm internal primers to anneal specifically. Two DNA fragments are generated in a heterozygote, one from primers 1 and 3, another from primers 2 and 4; but only one DNA fragment is amplified in an allele 1 homozygote (from primers 1 and 3) or an allele 2 homozygote (from primers 2 and 4). Because the internal primers are not centered in the fragment generated by the flanking primers, the two DNA fragments created in the second program are of unequal length, allowing them to be distinguished by agarose gel electrophoresis.

We applied this method to genotype a HaeIII polymorphism of the β fibrinogen gene on a number of individuals (Figure 2a). The bands of the PCR products were unambiguous and genotypes were supported by the results of ASO hybridization (Figure 2b). We have also employed this method to distinguish a single base pair mutation from a dilution of 1 mutant in 40 normal DNA molecules by using an 8 fold greater amount of the mutant internal primer (data not shown). Tetra-primer PCR method should be easily applicable to genotyping diallelic polymorphisms where the mutation is known and may have wider applications.

ACKNOWLEDGEMENTS

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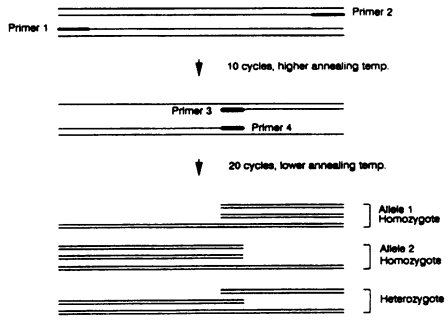

Figure 1. Schematic representation of tetra-primer PCR.

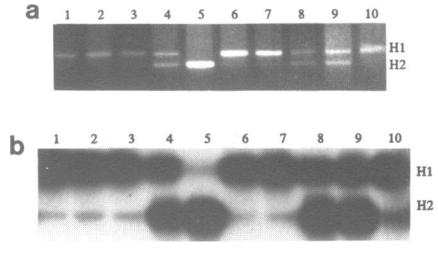

Figure 2. (**a**). Results from tetra-primer PCR. PCR conditions were as follows: each PCR reaction with the total volume of 50 μl contained 1 pmol primer 1 (5'-ctcctcattgtcgttgacaccttgggac-3', Tm = 86°C), 1 pmol primer 2 (5'-gaatttgggaatgcaatctctgctacct-3', Tm = 80°C), 35 pmol primer 3 (5'-aaaggggccattaaaat-3', Tm = 46°C), 35 pmol primer 4 (5'-attttaatagccccttt-3', Tm = 46°C), 1 U Taq polymerase, 100–300 ng genomic DNA, 50 mM KCl, 10 mM Tris–HCl, 2.0 mM $MgCl_2$, 0.2 mM each dNTP, 0.01% (w/v) gelatin, and 0.05% W1. The solution was overlaid with 50 μl liquid paraffin and underwent 10 cycles of 93°C (1'), 63°C (1') and 72°C (1.5'), and followed by 20 cycles of 93°C (1'), 46°C (1') and 72°C (1'). PCR products were subsequently electrophoresed on a 1% agarose gel. Genotypes of individuals 1, 2, 3, 6, 7, 10 are H1H1, individuals 4, 8, 9 are H1H2 and individual 5 is H2H2. (**b**). Autoradiograph of results from allele specific oligonucleotide hybridization. Genotypes, determined by comparing the relative intensity of bands derived from each allele, are identical with those from tetra-primer PCR.