Benchmarks

Benchmarks are brief communications that describe helpful hints, shortcuts, techniques or substantive modifications of existing methods.

Fluorescent Allele-Specific PCR (FAS-PCR) Improves the Reliability of Single Nucleotide Polymorphism Screening

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The detection of single nucleotide polymorphisms (SNPs) is becoming increasingly important in both the evaluation of candidate gene polymorphisms and fine mapping studies (4,7). Many techniques exist for the detection of SNPs within random sequences, such as single-strand conformation polymorphism (SSCP) (5,6), heteroduplex analysis and direct sequencing, but few techniques specifically identify the different alleles efficiently enough to use in a population screen. Neither SSCP (5,6) nor heteroduplex analyses detect all polymorphisms. Restriction fragment-length polymorphisms (RFLPs) can only be used with genetic variations that either create or abolish a restriction enzyme site. Direct sequencing of polymerase chain reaction (PCR) products can become time-consuming and expensive if analyzing a large population. DNA chip technology (3,7) is evolving rapidly, but is currently too expensive and is not available to the majority of investigators. Other allele-specific techniques, such as allele-specific oligonucleotide hybridization and oligonucleotide ligation assay combined with PCR (1), are very specific but require a large amount of time to perform an analysis with multiple polymorphisms. The most specific and potentially most efficient technique is allele-specific PCR (AS-PCR) (8).

AS-PCR depends on the observation that if the 3′ nucleotide of a primer does not match exactly, then the amplification will not be successful. Allele specificity is determined by selecting alternative primers that are identical except for the 3′ base and performing two amplification reactions. In this situation, DNA from heterozygotes will produce product in both reaction tubes, while DNA from homozygotes will produce product in only a single tube. It is possible to incorrectly score DNA from a heterozygote as a homozygote, if one of the PCRs fails. A control for successful amplification can be added, but, for technical reasons, the optimal conditions for this control might be different from the product of interest. In an effort to evaluate the Q576R polymorphism in the Interleukin-4 receptor (IL-4R) gene (2), we have modified the standard method for AS-PCR in a manner that controls for the absence of amplification and adapts the methodology to Models 310/373/377 DNA Analysis Systems (PE Biosystems, Foster City, CA, USA).

With this technique, the allele-specific primers are modified so that the two distinct products, produced in a single PCR, can be identified on a standard sequencer lane. A second advantage of this new method is that it decreases the amount of Taq DNA polymerase required to detect the two alleles.

Allele-specific primers were designed for the IL-4R Q576R polymorphism from the IL-4R mRNA sequence (GenBank® Accession No. X52425) and previously published sequences (2). Two forward primers were designed with different fluorescent labels. These primers differed in the number of nucleotides on the 5′ end and in the allele-specific nucleotide on the 3′ end. The differences between the two forward primers are underlined. The primer specific for the Q576 allele was tet-5′-GGCCCCACCAAGTGGCTATC-3′ and the primer specific for the R576 allele was fam-5′-CCCCCAACCAGTGCTATCA-3′. The same reverse primer, 5′-CCAGTCCAAAGGTAAC-3′, was used for both.

Figure 1. Densitometric tracings of blue and green lanes from four samples using FAS-PCR. Individuals 1 and 4 are homozygotes with the Q576 and R576 alleles, respectively, and individuals 2 and 3 are heterozygotes of the Q576R polymorphism. The scale at the right displays the intensity signals for each tracing.
AGGGG-3′, was used to detect each of the allele-specific products. The R576-specific product (blue) was arbitrarily chosen as allele one and the Q576-specific product (green) as allele two. The 2-bp difference was chosen to avoid 1-bp shadow bands often seen in PCR-amplified DNA fragments. The Q576 allele was 266 bp and the R576 allele was 264 bp. PCR was performed in a GeneAmp® PCR System 9600 Thermal Cycler (Perkin-Elmer, Norwalk, CT, USA) using a 10-μL vol consisting of 60 ng DNA, 0.5 μM R576-specific primer, 0.125 μM Q576-specific primer, 0.5 μM reverse primer, 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 0.2 mM each dNTP, 1.5 mM MgCl₂ and 0.5 U Taq DNA Polymerase (Life Technologies, Gaithersburg, MD, USA). The Q576-specific primer was used at one-fourth the concentration of the R576 primer to compensate for preferential signal production of the tet-labeled Q576 allele. Cycling parameters included an initial denaturation step of 94°C for 4 min, followed by 30 cycles of 94°C for 30 s and 72°C for 1 min and a final extension step of 72°C for 6 min. The resulting PCR products were diluted 15-fold in water and loaded onto 5.25% Long Ranger™ gel files were analyzed with the GENESCAN Version 3.0 software, Rockland, ME, USA) sequencing gels using a Model 377 DNA Sequencer with 36-cm plates and 32-well, square-tooth combs. We have also used 96-well combs with comparable results. The sizes of the PCR products were determined by the presence of GS500-Tamra internal lane standards in each lane. The gel files were analyzed with the GENESCAN™ Version 2.1 software, and the alleles were scored with the Genotyper® Version 2.0 program (both from PE Biosystems). We have also used GENESCAN Version 3.0 software with comparable results. Four individuals with each genotype were sequenced retrospectively with the ABI PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems) using the same gel conditions described above. The sequences of all four individuals were consistent with the results from the allele-specific method.

Heterozygotes and homozygotes for the IL-4R Q576R polymorphism were easily distinguished using fluorescent allele-specific PCR (FAS-PCR) (Figure 1). The Genotyper software displays a densitometric tracing for each of four colors in each lane. The blue and green tracings, which detect the two possible alleles, are shown for four individuals (Figure 1). Individual 1 is homozygous for the Q576 allele, as is shown by the presence of a green peak of the expected size, which is produced by the tet-labeled primer, with only background in blue. Individuals 2 and 3 are heterozygotes, as evidenced by peaks in both the blue and green lanes. In addition, the blue peak is 2-bp smaller in size than the green peak, which is consistent with the fmm-labeled primer being 2-bp shorter than the tet-labeled primer. The 2-bp difference allows the alternative alleles to be distinguished from the less intense 1-bp shadow bands. Individual 4 is homozygous for the R576 allele, as evidenced by the presence of a blue peak, with only background in green.

SNPs are becoming increasingly important in the analysis of complex diseases and the development of high-resolution genetic maps. Allele-specific methods are optimal for detecting particular sequence changes, but finding the proper controls for each reaction can be difficult. To overcome these disadvantages, we have modified AS-PCR so that each allele acts as an internal control for the other. The alternative alleles can be distinguished in two ways: (i) the alternative primers have different fluorescent tags and (ii) the primer ending in A or T is extended by 2 bp on the 5′ end. This short extension raises the melting temperature of the primer and also provides a second unambiguous method of distinguishing the alternative alleles. If the primers are the same length, it is possible that the combination of preferential amplification of one allele and bleed-through from fluorescent color into the next could cause misinterpretation of a homozygote as a heterozygote. Preferential amplification might still occur, but because the ratio of the two alleles can be read quantitatively using the Genotyper software, the ratio of the fluorescence primers can be adjusted to compensate for the amplification bias. This method can be used to analyze numerous SNPs in large populations and might also aid in the development of high-resolution SNP maps used in fine mapping studies.

REFERENCES


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