Establishment of an optimized PCR method using sequence-specific primers for screening multiple gene polymorphisms simultaneously

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Abstract. The present study aimed to explore the factors that affect polymerase chain reaction using sequence-specific primers (PCR-SSP) and to establish an optimized PCR-SSP method for detecting multiple gene polymorphisms simultaneously. The amplification system parameters, including the concentrations of Mg^{2+}, dNTPs, pfu Taq, primers and control primers, were optimized using the designed PCR-SSP reactions. The resulting optimized reaction system was used to determine the melting temperature of the genomic DNA and the cycling parameters. The optimized PCR-SSP method was used to analyze the polymorphisms of the following genes: mutations -308A/G and -238G/A in TNF-α, -174G/C in IL-6 and C/T mutation at exon 188 of CYP2D6 *10B. The PCR-SSP amplification system was optimized; in a 20 μl reaction system, the quantities of Mg^{2+}, dNTPs, pfu Taq, primers, control primers and genomic DNA were 3.25 μM, 0.5 mM, 2.5 units, 0.5 μM, 0.2 μM and 0.15 μg, respectively. The cycling system comprised 5 start cycles and took 15 min to melt a genomic DNA sample using a touchdown protocol. The optimized PCR-SSP is suitable for polymorphism analysis of polygenic SNPs in large genomic DNA samples and a number of different genes.

Introduction

The presence of specific human gene single nucleotide polymorphisms (SNP) has been associated with susceptibility to several diseases (1). Evidence has demonstrated that polymorphisms have substantial clinical effects on the prevalence, incidence and prognosis of various major diseases, including asthma in children which is associated with IL-13 (1,2) and TNF-α -308A/G (3), Alzheimer's disease (4,5), human mental diseases (6,7), chronic obstructive pulmonary disease (8,9) and cardiovascular disease (10). These diseases are often associated with the deoxyribonucleic acid (DNA) sequences of the genes, in particular with the SNPs. A very accurate prediction of diseases present may be obtained by genotyping the relevant SNPs. Large-scale samples are required to analyze and reveal the relationships between disease and SNPs.

At present, the following methods are typically used: i) polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), a method which demands highly pure PCR products, large quantities and restriction enzyme sites for the SNP to be detected. Detection using this method would be very costly and time consuming for large samples. ii) Sequencing, a method that has high costs and is unsuitable for the quick analysis of many clinical samples. iii) PCR using sequence-specific primers (PCR-SSP), a method that is based on a critical PCR process with detection of the amplified product by agarose gel electrophoresis. The rapid and simple PCR amplification makes the technique suitable for screening SNPs that are related to disease, treatments and drug choice, particularly by large-sample polygenic genotyping. Thus, it is a more favorable prospect for the clinical testing of gene polymorphisms. However, multiple factors affect the PCR process. During PCR-SSP, the sequence primers are required to be combined and controlled with specific primers in the same reaction mixture in the test tube to ensure that if amplification failure occurs, it results from mismatched primers rather than from a failure of the amplification system. Therefore, the reaction parameters have a significant effect which may limit the applications of the method in the detection of SNPs (11).

For this purpose, the parameters for the cycling and operation systems were optimized to establish a protocol suitable for the convenient, rapid and accurate polygenic genotyping of a large number of samples.

Materials and methods

DNA. Genomic DNA from human peripheral blood donated by researchers was prepared and used by our laboratory.

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Table I. Primer sequences, amplification size for the single locus PCR-SSP and sequencing.

Gene name	Primer sequences (5' to 3')	Product size (bp)
Human β-globin gene (12)	F: GAA GAG CCA AGG ACA GGT AC R: CAA CTT CAT CCA CGT TCA CC	268
TNFα -308A/G (13)	F: TCC TCC CTG CTC CGA TTC CG Ra: CAA TAA GTT TTG AGG GGC ATG A Rb: CAA TAA GTT TTG AGG GGC ATG G	104
CYP2D6 *10B exon 188 C/T (7)	F: ACC AGG CCC CTC CAC CGG Ra: AGG GGG CCT GGT GG Ra: AGG GGG CCT GGT GA	397
COMT 472 coding sequence G/A	F: ACT GTG GCT ACT CAG CTG TG Ra: TGC ACA CCT TGT CCT TCA C Rb: TGC ACA CCT TGT CCT TCA T	138
Mouse β actin (14)	F: CCAGGGTGTGATGGTGGGAATG R: CGCACGATTTCCCTCTCAGCTG	510
Mouse PPARγ (15)	F: GACCACTCGCATTCCTTT R: CCACAGACTCGGCACTCA	265

The animal genomic DNA was from healthy Kunming mice provided by The Animal Experimental Center of Xinxiang Medical University. The DNA extraction kit used was a universal genomic DNA extraction kit SK1341 (Sangon Co. Ltd., Shanghai, China).

Primers. The PCR-SSP method optimized in our laboratory was tested to analyze polymorphisms of the following genes: -308A/G mutations in TNF-α, COMT 472 coding sequence G/A, the C/T mutation at exon 188 of CYP2D6 *10B, mouse PPARγ and mouse β-actin. The primers (synthesized by Sangon Co. Ltd., Shanghai, China) used in the experiment are shown in Table I.

Method optimization. The amplification system parameters, including the concentrations of Mg^{2+}, dNTPs, pfu Taq, primers and control primers, were optimized using designed PCR-SSP reactions. The optimized reaction system was used to determine the melting temperature of the sample DNA and the cycling parameters.

The PCR products were examined on a 2% agarose gel (Shanghai Sangon Biological Engineering Technology & Service Co. Ltd., Shanghai, China). The amplified products were confirmed under UV light following ethidium bromide staining to determine the optimum parameters. The molecular standard used was GeneRuler™ 100 bp DNA Ladder (Sangon Co. Ltd.). Pfu Taq DNA (Promega Corporation, Madison, WI, USA) and PTC-100 PCR machine (Biogene, Richardson, TX, USA) were used.

Results

Reaction system. The amplification was performed in a 20 μl reaction volume and the effect of varying each of the parameters was studied while the others were kept constant. Firstly, the volume of 25 mmol/l Mg^{2+} was varied from 1 to 5 μl. Other parameters were DNA from 50 to 100 ng, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.5 mM of each dNTP, 0.7 μM of each primer (Table I) and 3 units Pfu Taq DNA polymerase. As shown in Fig. 1a, 3 μl was the optimal amount of Mg^{2+}. Secondly, after the optimal Mg^{2+} concentration was determined, the effects of different final dNTP concentrations on the amplification were compared in the range 0.25-1.0 mM, added in increments of 0.25M. Fig. 1b reveals that 0.5 mM dNTPs was optimal. The optimal quantity of pfu Taq DNA polymerase was 2.5 units; it was evaluated in the range 1.0-3.5 units, added in increments of 0.5 units. The optimal common primer concentration was 0.5 μM; it was evaluated in the range 0.3-0.9 μM and added in increments of 0.2 μM (Fig. 1c and d). Finally, the ratio of the internal control primer concentration to the specific primer concentration was optimized after the other optimal concentrations had been determined. As shown in Fig. 1e, the optimum ratio was 2/5.

Cycling and validation. The primers, PCR mixture and electrophoresis conditions were as previously described. The optimal thermocycler conditions for the amplification were evaluated, including 95˚C for 1, 3, 5, 10, 15 and 20 min, 95˚C for 50 sec, 56˚C for 50 sec and 72˚C for 60 sec, followed by 72˚C for 10 min. The denaturation times were evaluated and revealed an optimal duration time of 15 min (Fig. 2a). For certain SNPs that were difficult to amplify by conventional PCR, evaluation was performed by touchdown and modified touchdown PCR (Figs. 2b and c).

The same primers were used in the optimized PCR: 20 μl reaction system contained 2 μl 1X PCR buffer, 3 μl 25 mmol/l $MgCl_2$, positive control primer at a final concentration of 0.2 μmol/l, the common primer and primers a or b at final concentrations of 0.5 μmol/l, dNTP at a final concentration of 0.5 mmol/l and 2.5 units pfu Taq DNA polymerase. The PCR programs used the touchdown cycling method were: 95˚C for 15 min, 95˚C for 30 sec, 70˚C for 1 min and 72˚C for 10 min

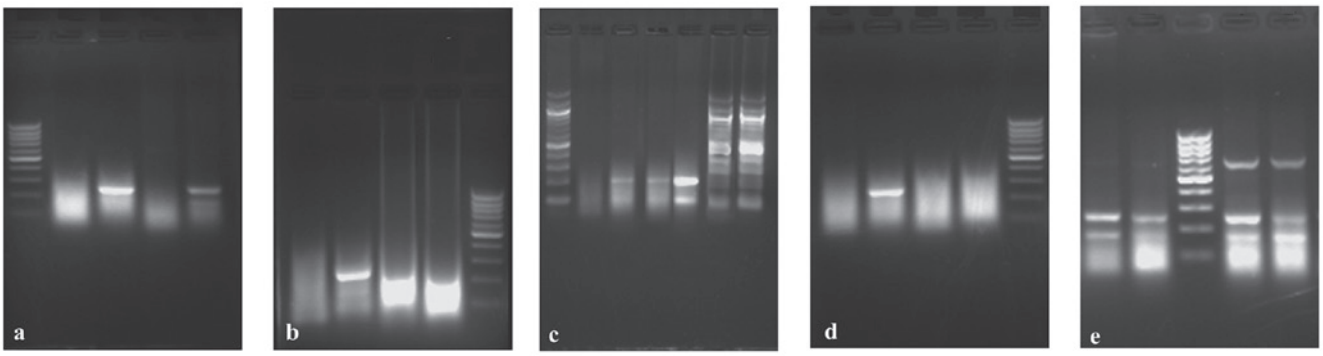

Figure 1. PCR-SSP system parameters optimization. (a) Variation of Mg^{2+} concentration in PCR. Lane 1: GeneRuler™ 100 bp DNA Ladder; lanes 2, 3, 4 and 5: 5, 3, 2 and 1 μl 25 mM Mg^{2+} in buffer, respectively. (b) Effect of different dNTP concentrations on PCR. Lanes 1, 2, 3 and 4: 0.25, 0.5, 0.75 and 1.0 mmol/l dNTPs in the PCR mixture, respectively; lane 5: GeneRuler 100 bp DNA Ladder. (c) Effect of different concentrations of pfu Taq in PCR. Lane 1: GeneRuler 100 bp DNA Ladder; lanes 2, 3, 4, 5, 6 and 7: 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 units of pfu Taq DNA in PCR mixture, respectively. (d) Different concentrations of common primers in the PCR system. Lanes 1, 2, 3 and 4: 0.3, 0.5, 0.7 and 0.9 μmol/l primers, respectively; lane 5: GeneRuler 100 bp DNA Ladder. (e) Different ratios of internal control primers to specific primers in the PCR system. Lane 3: GeneRuler 100 bp DNA Ladder; lanes 1, 2, 4 and 5: the ratio of internal control primers to common primers of 2/5, 4/5, 1 and 1.5. PCR-SSP, polymerase chain reaction using sequence-specific primers; DNA, deoxyribonucleic acid.

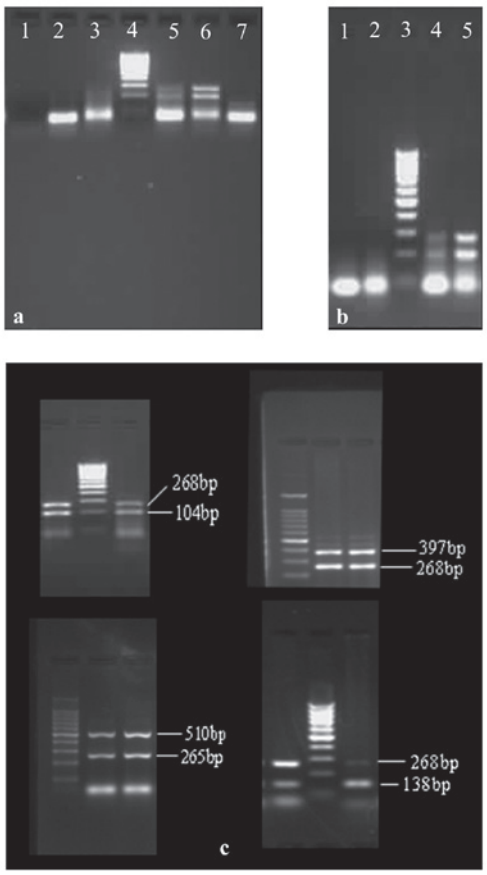

Figure 2. Optimized parameters of the PCR-SSP cycling. (a) Different denaturation times in PCR cycling. Lane 4: GeneRuler™ 100 bp DNA Ladder; lanes 1, 2, 3, 5, 6 and 7: denaturation times of 1, 3, 5, 10, 15 and 20 min, respectively. (b) Different touchdown programs used in PCR. Lanes 1 and 2: conventional PCR; lane 3: GeneRuler 100 bp DNA Ladder; lane 4: a common touchdown program; lane 5: optimized touchdown PCR. (c) Different SNPs tested by the optimized touchdown program. PCR-SSP, polymerase chain reaction using sequence-specific primers.

repeated for 5 cycles; 95˚C for 30 sec, 69.5˚C for 1 min, then decreased by 0.5˚C every cycle, 72˚C for 1.5 min, repeated for 30 cycles; and 72˚C for 10 min.

The modified PCR-SSP reaction system and touchdown cycling were used to evaluate human SNPs and mouse genes. TNF-α -308A/G, C/T at exon 188 of CYP2D6 *10B, COMT 472 G/A, mouse PPARγ and mouse β-actin were analyzed. Fig. 2c clearly shows the amplified bands.

Discussion

To optimize the protocol for the simultaneous polymorphism analysis of SNPs in large samples of multiple genes, the reaction was performed in 20 μl liquid mixture. The Mg^{2+} concentration is more important to the polymerase, hence it was optimized first. For other parameters, conventional concentrations were utilized for smooth optimization.

PCR-SSP is based on the principle that recombinant Taq DNA polymerase is more specific for the oligonucleotide primers that completely match the target gene. If a primer that completely matches one genotype of the allele is designed and the PCR process is strictly controlled, then the matching primer will be amplified (positive results), whereas the mismatched primer will not (negative results). The results of PCR-SSP are interpreted based on whether an amplification band is visible, and the amplified products of two lanes are used to determine the genotype of a sample. Considering that numerous factors affect a PCR procedure, an internal reference must be used for each reaction. The internal reference generally used is the human globin gene, which is universally present in the conserved region of the human genome. In reactions showing positive bands, the amplification band of the internal reference may be present, attenuated or absent depending on the concentration of the specific primer pair and internal reference primer pair (16-18).

Primer concentrations are very significant in PCR-SSP reactions (11) to avoid false negative results. The optimized common primer concentration is 0.5 μM and the ratio of internal control primer to specific primer is 2/5.

If the amplification reaction occurs smoothly, the DNA template must be a single band. Human genes are extremely complicated. Thus, the denaturation time was optimized. The results indicated that 15 min is the optimum duration for

human and mouse genes. To avoid Taq polymerase inactivation, 2.5 units pfu Taq were used, which is slightly higher than the usual concentration.

The proposed optimized method is similar to the touchdown method and standard PCR protocols, with the exception of a special cycling program in which a series of gradually decreasing annealing temperatures was set. Furthermore, a variety of primers were added to the reaction system so that different target gene specific sequences were amplified satisfactorily. The basic principle of this approach was to choose an annealing temperature that was 15˚C higher than the calculated annealing temperature. This temperature was gradually decreased back to the annealing temperature and then finally reduced further to 5˚C lower than the annealing temperature. Such a strategy ensures that the first hybridization between the primer and the template occurs under the most complementary conditions and it may be cycled at different denaturing and annealing temperatures.

In conclusion, a PCR-SSP protocol modified to optimize the diagnosis of diseases associated with multiple gene polymorphisms has been developed. This PCR-SSP protocol is faster and less expensive than the currently used procedures, particularly in the primary screening of large samples and various point mutations.

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