Chapter 7

Designing PCR Primers for the Amplification-Refractory Mutation System

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Abstract

The recent development in genetic research indicates that there exists intraspecific genetic variability in many organism groups. These variations, which result in a variety of genotypes and phenotypes within a population, are called polymorphism. Mutations in different ways can alter the organism's phenotype and affect its fitness, for example, by altering disease susceptibility or resistance. Therefore, the detection of point mutations in different genes of a population is of particular importance. The amplification-refractory mutation system technique is a PCR-based method to detect single nucleotide polymorphisms in the genome. The high repeatability, low cost, high accessibility, and no need for sophisticated technology are the main advantages of the ARMS-PCR technique, compared with other available methods such as PCR-RFLP. This chapter describes the design and analysis method of primers for the ARMS-PCR technique.

Key words Primer design, Molecular biology, ARMS-PCR

1 Introduction

Individuals within a community may show up to 99.9% similarity in their DNA sequences; however, even such fine genetic variations (e.g., single nucleotide polymorphism, SNP) could have a major impact on population structure and function [1]. SNPs represent differences resulting from the substitution of single nucleotides through point mutation. SNPs are the most common type of genomic variability and occur almost on average 1 per 1000 bp in the genome [1]. The study of SNPs is important because some SNPs reflect the health and fitness of individuals [2]. The existing methods to study SNPs have some limitations. For example, Sanger sequencing cannot rapidly screen large numbers of mutations, next-generation sequencing (NGS) is still expensive, and PCR-RFLP is time consuming and nonspecific [1, 3, 4]. The ARMS-PCR (Amplification Refractory Mutation System-PCR)

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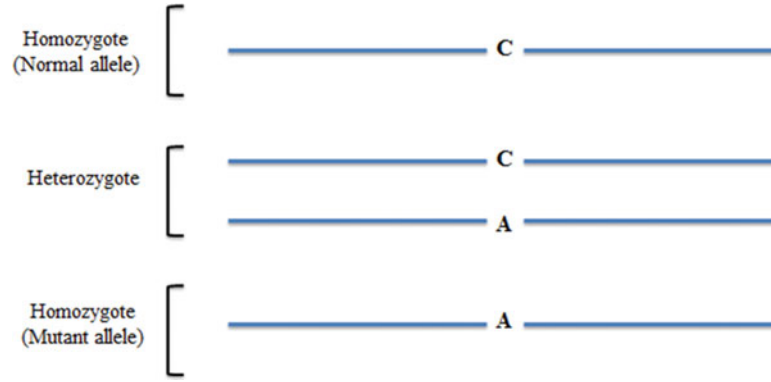

Fig. 1 The normal and mutant allele of a hypothetical gene

technique is an easy and rapid method for detecting point mutations, polymorphisms, and a heterozygous or homozygous gene locus (Newton et al. 1989) [2, 5]. Newton et al. introduced a PCR assay method for detecting SNPs, in which the allele-specific primers have the additional mismatched nucleotides in the 3′ region of the primer for the nonallelic [3]. Unlike other techniques used to detect alleles, the ARMS-PCR method does not require the use of restriction enzymes or determination of the sequence of PCR products [2]. In this technique, primers specific to target sequences are needed. These primers strictly amplify the target region present in samples, and other regions remain non-amplified. Thus, the presence or absence of PCR products indicates the presence or absence of the target allele. However, in the ARMS-PCR method, SNP is detected by the size of PCR amplicon using gel electrophoresis. As such, this method is simple, rapid, and affordable.

Figure 1 shows the normal and mutant alleles of a hypothetical gene. In this gene, the normal allele contains nucleotide G, and the mutant allele contains nucleotide A. In such conditions, the two alleles can be distinguished by the ARMS PCR method that requires two pairs of primers to be designed. A pair of primers specified here as outer forward and outer reverse must be designed such that the considered region will be located between them. In other words, the two primers are common to both alleles (Fig. 2) [6, 7].

In designing primers, care must be taken that the studied mutation is not located within the primers. It is also critical to consider that the location of outer forward and outer reverse primers must be considered, such that if the distance between the studied mutation to the outer forward primer is α, and its distance to the outer reverse primer is β, α divided by β or β divided by α is greater than 1.5 (Fig. 3).

The target region can be homozygote for the normal allele, homozygote for the mutant allele, or heterozygote. The size of the

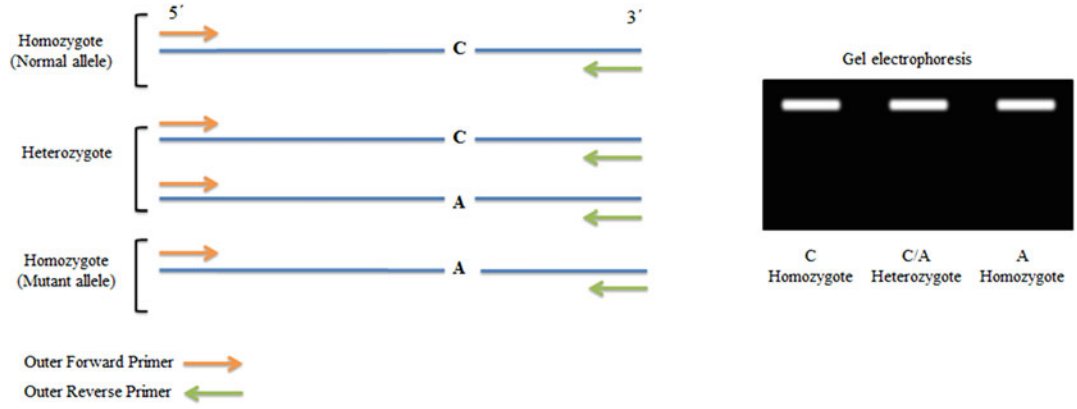

Fig. 2 Position of outer forward and outer reverse primers in normal and mutant alleles and the amplification of the same fragment in both allele types and gel electrophoresis results

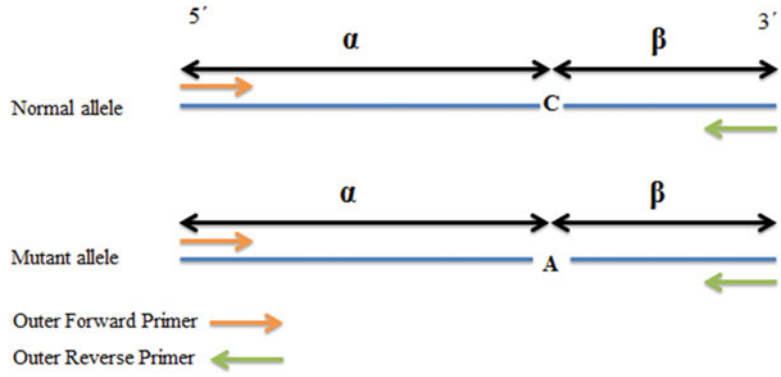

Fig. 3 The PCR product generated by outer forward and outer reverse primers in homozygote for the normal allele, homozygote for the mutant allele, and heterozygote states

PCR product generated by outer forward and outer reverse primers is the same in all three above cases. Therefore, this fragment is used as a control and should be present in all samples [6, 7].

In addition to the above primers, two other primers named inner forward and inner reverse are also required. These primers need to be designed such that the inner reverse primer is specific to the normal allele, and the inner forward primer is specific to the mutant allele [6, 7].

The inner reverse primer, which is specific to the normal allele (here allele G) at its 3′ end, has a complementary base with normal nucleotide (here nucleotide C), indicating that it cannot bind to the mutant allele. On the other hand, the inner forward primer that is specific to the mutant allele (here allele A) at its 3′ end has a mutant nucleotide (here T) and, thus, cannot bind to the normal allele (Fig. 4) [6, 7].

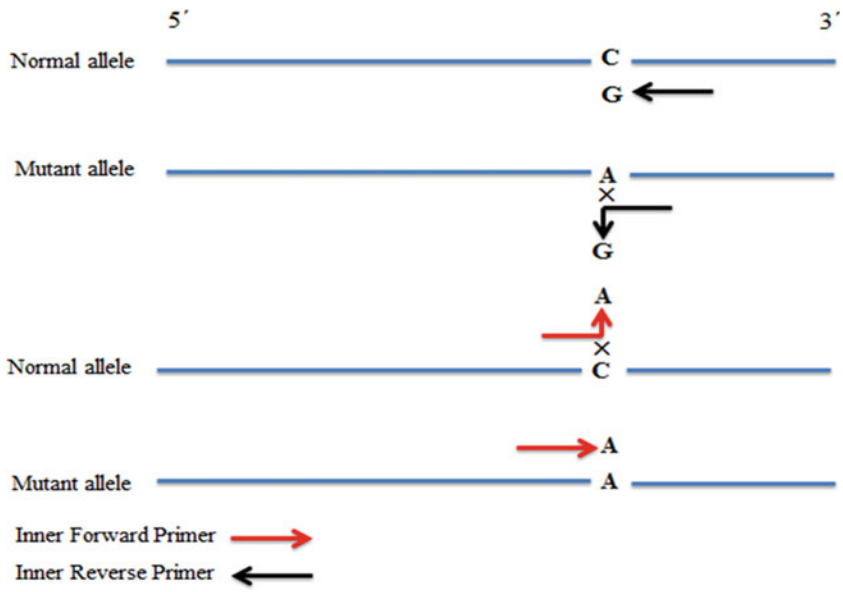

Fig. 4 Position of inner forward and inner reverse primers in normal and mutant alleles and the amplification of the same fragment in both allele types

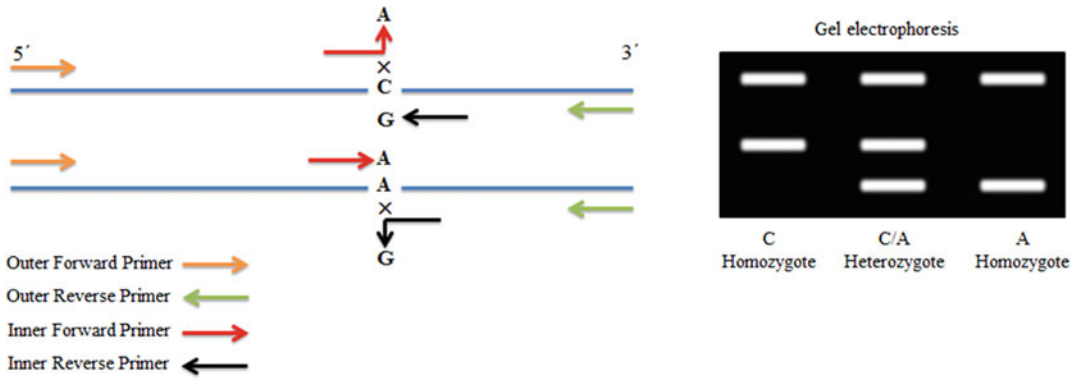

Fig. 5 Position of inner and outer primers in normal and mutant alleles and gel electrophoresis results

If multiplex PCR is performed with all of the above primers, one of the results seen in Fig. 5 will be observed.

2 Methods

To design a primer, it is necessary to first determine the sequence of the gene and SNP in question. To do so, the sequence of the gene in question can be obtained from databases such as https://www.ncbi.nlm.nih.gov/ or https://asia.ensembl.org/index.html. If needed, the SNP in question can be determined by searching for

the SNP name in the box via the following link https://www.ncbi.nlm.nih.gov/snp/?term=.

Primer design can be performed using the website http://primer1.soton.ac.uk/primer1.html, which is accessible free of charge. The sequence in question must be selected on this website as described in the introduction section and entered into the box related to the source sequence (up to 1000 bases). Then, in the next box, the position of SNP from the start of the sequence must be specified. It is recommended to insert the nucleotide related to the normal allele in the allele 1 box, and the nucleotide related to the mutant allele in the allele 2 box. The rest of the options can be modified depending on the test conditions. However, it is recommended not to select a number less than 100 for minimum (inner) product size, which may outperform the optimum primer size. In addition, we recommend the maximum primer melting temperature (Tm) to be 22 nucleotides and less than 70 °C. It should be noted that the values of maximum complementarity and maximum 3′ complementarity are defined as 8 and 3 by default, respectively. Both these primer features strongly correspond to the probability of formation of secondary structures in the primer, i.e., their lower values facilitate setting up of PCR process. After making the desired changes, the Pick Primers option should be selected (Fig. 6).

After selecting this option, the results related to the designed primers will be displayed. Each segment consists of four primers of forward inner primer, reverse inner primer, forward outer primer, and reverse outer primer. In addition, the nucleotide sequence, positioning, melting temperature of primers as well as the length of PCR product fragments for each state are specified.

To increase specificity and reduce error in the PCR process, usually, the third nucleotide from the end 3 in inner primers is designed by the software as a mismatch. This will enable that only the target region is amplified; thus, care must be taken that this "false" error is not corrected by the user.

2.1 Examination of Primers Using Integrated DNA Technology (IDT) Website

The primers designed by the formation of self-dimer, hetero-dimer, and hairpin need to be examined. Integrated DNA Technology website, which is free of charge, can be used for this purpose. After entering the site at https://eu.idtdna.com/pages/tools/oligoanalyzer, each of the primers should be entered separately in the sequence box, and the analysis option must be selected. It should be noted that the more negative Gibbs free energy (ΔG) would indicate a more spontaneous reaction. Therefore, the ΔG resulted from the analysis of self-dimer, hetero-dimer, and the hairpin is recommended to approach zero or positive values (Fig. 7).

Fig. 6 An overview of the website http:/primer1.soton.ac.uk/primer1.html and the changeable options in this site

2.2 Examination of Specificity of the Designed Primers

Once the primers have been designed, their sensitivity and specificity must be analyzed by the Primer Blast method to ensure that the designed primers only amplify the fragment in question. Both these features could be assessed using either ProbeMatch (https://rdp.cme.msu.edu/probematch) or the NCBI website (https://www.ncbi.nlm.nih.gov/). It is necessary to note some points during Primer Blast. The first point is that the specificity value of the outer forward and outer reverse; outer forward and inner reverse; and inner forward and outer reverse primers should be studied separately. Secondly, in the results of Primer Blast, nonspecific products with different lengths may be displayed. In such cases, if there is a mismatch at the end 3′, the probability of the

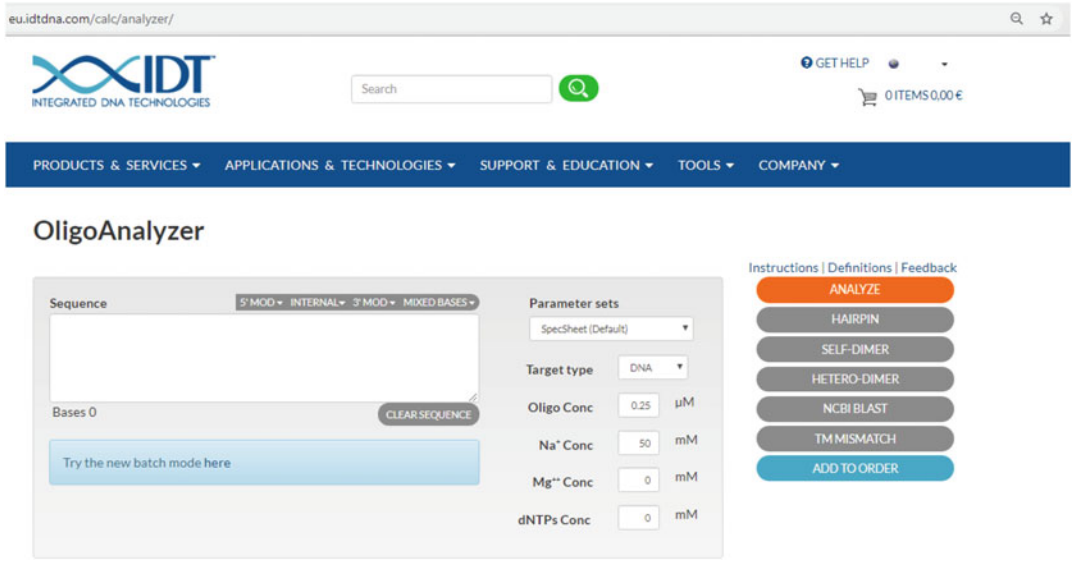

Fig. 7 Analysis of primers using Oligo analyzer link in Integrated DNA Technology (IDT) website

amplification of these fragments will be lower. The increased number of these mismatches at the end 3 will reduce the probability of the amplification of such fragments. On the other hand, since the third nucleotide from the end 3 in inner primers is designed as a mismatch by the software, such fragments are not actually amplified and do not cause any disruption in the results. In terms of wet-lab specificity, one may rely on the ratio between amplified targeted and non-targeted regions in the final products to further assess the performance of the tested primer sets.

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