One-Strand PCR: A Comparably Efficient Method for Chromosomal Walking

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Polymerase chain reaction (PCR) is the most powerful method in molecular cloning techniques. However, it is not the ideal method for study of an unknown gene or an unknown region of a chromosome. In chromosomal crawling of genome research, PCR was usually carried out with a restriction enzyme-digested and circularly ligated chromosome with primers that annealed to a known region in opposite directions (5). Beside being time-consuming, lack of data on sizes of chromosomal DNA and restriction enzyme sites greatly reduces successful cloning rates. The expected PCR product sizes are usually arbitrary.

To overcome these difficulties, PCR was initially carried out with one primer that annealed in a known region, followed by a second PCR using the one-strand (OS)-PCR product as a template, with a known and highly degenerate primer set. In OS-PCR, a specific primer is a directional primer, and the product size is roughly restricted with polymerization time (Figure 1). Sometimes, fragmentation of template DNA by a restriction enzyme or mechanical shearing showed good results (data not shown). Varying concentrations of $MgCl_2$, primer and template in the reaction mixtures resulted in quantitative difference and qualitative changes in the PCR products. Optimum conditions of PCR were specific to each reaction, and thus, optimizations for each were a lot more difficult than in OS-PCR (3,4).

The experimental system that we have used is *Thermus caldophilus* GK24, which is an extreme thermophilic bacterium of about 67% G+C in DNA content (1,2). We had studied its xylose utilizing system and t/rRNA genes for the genome mapping (unpublished). Because of the many distinctive natures of the genus *Thermus*, chromosomal crawling was very difficult. Gene fragments were first cloned by PCR and shotgun cloning methods. One primer that annealed to gene fragment was subsequently synthesized and used in OS-PCR for chromosomal crawling. For example, we have tried the cloning of xylose isomerase genes from *T. caldophilus* GK24 and *Thermus flavus* AT62. First, we had cloned

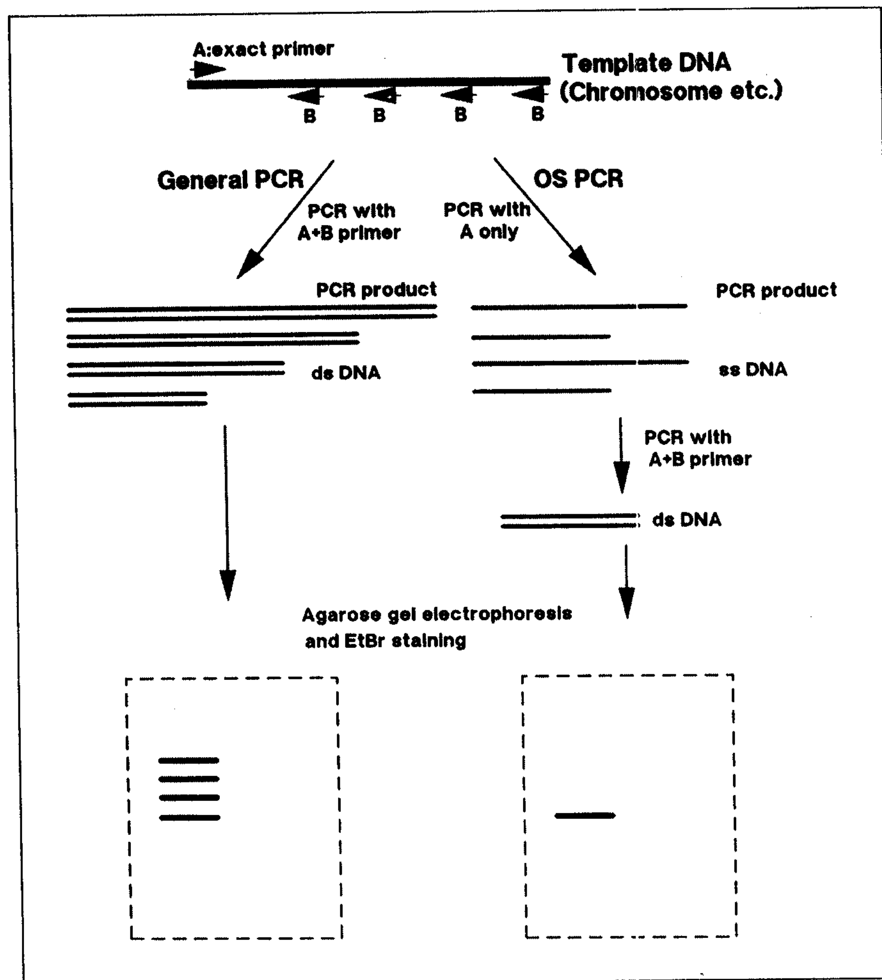

Figure 1. Schematic illustration of the OS-PCR method that was compared to general PCR. The letter A means an exact primer, and B means a degenerate primer that is supposed to be aligned at several sites of the template. During OS-PCR, polymerization time of *Taq* DNA polymerase was roughly calculated as 75 bases per s of the expected product size, since the polymerization rate is 45–100 nucleotides per s, using *Taq* DNA polymerase (5).

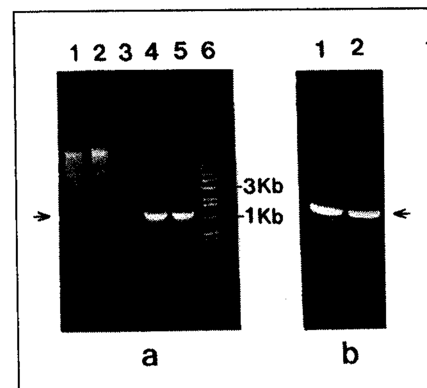

Figure 2. Different efficiencies of general PCR and OS-PCR in ethidium bromide-stained 0.8% agarose gel after electrophoresis of 7 µL of each PCR product. (a) PCR was carried out with the general protocol. Because one of two primers was a highly degenerate random primer, there were no detectable DNA bands at about 1.2-kb region (lanes 1 and 2). The size marker (lane 6) was a 1-kb DNA ladder (Life Technologies, Gaithersburg, MD, USA). After PCR with the non-degenerate one of the two primers, a second normal PCR was carried out with the OS-PCR products (lanes 4 and 5). It shows large amounts of DNA at the expected size of around 1.2 kb (b) after TP-PCR of a 1.2-kb DNA band. On the average, 0.5-µL agarose particles were used as template DNA. The PCR template for lanes 1 and 4 in Panel a and lane 1 in Panel b is the *T. caldophilus* GK24 chromosome and for lanes 2 and 5 in Panel a and lane 2 in Panel b is the *T. flavus* AT62 chromosome. In Panel a, lane 3 is a control sample of PCR without a template.

the internal DNA sequence using primers that were deduced from a conserved amino acid sequence. After determination of the nucleotide sequence, we prepared another primer set for cloning the N-terminal coding region that was deduced from the N-terminal amino acid sequence and the cloned internal DNA sequence. In this case, the primer sequences of PCR constituted one degenerate primer, 5′-ATGTANGANCCNAANCCNGANCANAG-3′, for the sense strand and one exact primer, 5′ -TCACCCCCGCACCCCCAGGAGGTACTCC- 3′, for the antisense strand.

In this experiment, PCR was carried out in a 20-μL reaction volume containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 50 mM Tris-HCl, pH 8.4, 100 nM oligonucleotide primers and 0.5 U *Taq* DNA polymerase, with a DNA thermal cycler (Korea Biotech, Taejon, Korea). The reactions were cycled 30 times at 94°C for 30 s, 60°C for 30 s and 73°C for 50 s as in general methods (5). The reactions of OS-PCR were cycled 30 times at each temperature but 25 s for each cycle with only one exact primer first, and then a general PCR was carried out with 1-μL reaction samples as the template.

Figure 2a shows the results of a general PCR (lane 1 and 2) and OS-PCR (lanes 4 and 5), respectively. OS-PCR with a highly degenerate primer normally shows some bands produced with one primer. Even then, successful results are usually obtained. Therefore, this method is comparably more efficient than the general PCR method. Many cases of OS-PCR result in small amounts of expected DNA products. In this regard, tip-pick-PCR (TP-PCR) was recommended. TP-PCR is a simple method for amplifying a target DNA band from many PCR product bands using a corresponding pipet-picked band in an agarose gel as the template. Figure 2b shows an amplified OS-PCR product with a template obtained from a tip-picked agarose-gel particle.

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Design of Clone-Specific Quantitation Standards for Competitive PCR

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The polymerase chain reaction (PCR) is an exquisitely sensitive technique, which can be used in semiquantitative assays to determine the relative concentrations of target and reference sequences (9). Quantitation in acute lymphoblastic leukemia (ALL) is of the utmost importance for the evaluation of minimal residual disease and prognosis (2,10). To develop quantitative PCR assays, a number of approaches have been applied, most of which use chromosomal translocations or the somatic gene rearrangements occurring at the antigen receptor gene loci. Some systems rely on amplification of a range of dilutions of diagnostic material to produce a calibration curve (1,3,8,10). This method is reliable only if all amplifications proceed with the same efficiency. Furthermore, all data must be obtained before the reactions reach the plateau phase of product formation (6).

The most reliable quantitation is achieved by competitive PCR (cPCR), which uses internal standards with the same primer recognition sites as the target and similar internal DNA sequence (6,14). This type of reference sequence can be produced by modifying a cloned PCR target by site-directed mutagenesis so that it contains a restriction site (13). Known amounts of a modified target are included in a cPCR, and the products are digested to yield fragments of distinct sizes, the intensities of which can be compared following gel electrophoresis. By utilizing cell line T-cell receptor gene rearrangements, a range of competitor molecules sharing the same limited number of proximal and distal (V and J) segments, but with variable intermediate DNA sequences, have been prepared. These competitor molecules have then been used in cPCR to quantify residual disease (5). Another approach uses T4 DNA ligase to bind a PCR primer, which has been made double stranded by hybridization to its complementary oligonucleotide sequence, to a double-stranded DNA fragment (12). It may be possible to use these approaches for immunoglobulin heavy chain (IgH) gene consensus primer PCRs, but most IgH assays now rely on clone-specific PCR. By their nature, clone-specific primers present a problem for the design of cPCR quantification standards since there are too many possible alternative sequences.

We have developed a simple yet effective strategy for the production of double-stranded quantitation standards for clone-specific PCR analysis. This methodology is especially useful for precursor-B ALL evaluation using