Random Primed Gene Walking PCR: A Simple Procedure to Retrieve Nucleotide Fragments Adjacent to Known DNA Sequences

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The development of the polymerase chain reaction (PCR) has simplified the cloning and studying of nucleotide sequences. Modifications of PCR protocols have been used for gene walking. Most of these protocols, such as inverse PCR (5), require multiple steps. Recently, a few variations of a procedure called targeted gene walking PCR (TGW-PCR) have been described to accomplish the same task (1–3). However, in most cases, TGW-PCR amplifies multiple nonspecific PCR products from which gene-specific PCR products must be distinguished and purified before attempting to sequence them (1–3).

We have developed a technique that eliminates the amplification of multiple spurious products and therefore the need for band purifications. Our technique enriches single-stranded (ss) DNA sequences either upstream or downstream from the known sequences followed by a random amplification.

ssDNA was obtained by using high-temperature annealing of a primer to the known DNA sequence. In developing this technique, we used primers that annealed to different fragments of a *Borrelia burgdorferi* *cheA* homolog gene. The PCR used 0.5 µg of borreliar DNA and 100 pmol of specific primer in 100 µL reaction mixture and consisted of 40 cycles (94°C for 1 min; 55°C for 1 min; and 72°C for 1 min). For reaction A, the specific primer was UP3 (5'-CAGCCTCACTGACAAGAT-3'), and for reaction B, the specific primer was UP6 (5'-CCTCAAGATCTGATTCTG-3'). The absence of double-stranded (ds) DNA products after the first set of amplifications was verified by agarose gel electrophoresis (data not shown).

ssDNA (10 µL of the first PCR mixture) was then used in a second PCR

mixture (100 µL) containing the same specific primer and a 10-mer (5'-CAA-GGGCTCA-3') nonspecific primer, both at 100 pmol. The second PCR consisted of 40 cycles (94°C for 1 min; 40°C for 1 min; and 72°C for 1 min). The temperature for the first PCR was determined by trial/failure and that of the second one was determined by the melting point of the specific primer.

By using this technique, 1–3 bands were obtained per reaction, and nearly all the bands appeared to be specific gene sequences as assessed by Southern blot analysis using digoxigenin-labeled internal nucleotide sequences (Genius™ system; Boehringer Mannheim, Indianapolis, IN, USA) as probes (Figure 1). Three PCR products (0.5 and 0.3 kb from reaction A and 0.8 kb from reaction B) were ligated into plasmid pCR™ II (TA plasmid; Invitrogen, San Diego, CA, USA) and were subjected to nucleotide sequencing (4).

The three inserts contained nucleotide sequences corresponding to the *B. burgdorferi* *cheA* homolog gene. Random primed gene walking PCR amplified multiple specific bands with virtually no spurious products. Therefore,

this technique may allow direct sequencing of unknown DNA sequences without the need for DNA cloning.

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Figure 1. Random primed gene walking PCR of *B. burgdorferi* DNA. A) Amplification products obtained by using primer UP3. B) Amplification products obtained by using primer UP6. For both A and B, agarose gel stained with ethidium bromide (lane 1) and Southern blot analysis using internal sequence probes (lane 2).

Improved PCR Amplification of Multiple Specific Alleles (PAMSA) Using Internally Mismatched Primers

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The polymerase chain reaction (PCR) can detect DNA single-base change mutations or polymorphisms using an allele-specific primer designed