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An improved PCR method for walking in uncloned genomic DNA

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Several PCR-based methods are available for walking from a known region to an unknown region in cloned or uncloned genomic DNA. The methods are of three types: inverse PCR (1), randomly primed PCR (2) and adaptor ligation PCR (3–6). However, these methods have not been generally applied to walking in uncloned genomic DNA because they are either complicated or inefficient. Recent improvements to these methods have been applied to uncloned genomic DNA (7,8), however, walks have been limited to distances of <1 kb.

We have investigated the application of 'long and accurate PCR' (9,10) to walking in uncloned genomic DNA. We initially examined the use of 'unpredictably primed PCR' (3), a new method based on randomly primed PCR and 'vectorette PCR' (5), which is based upon adaptor ligation. As a model system we attempted to walk upstream from exon 1 of the human tissue-type plasminogen activator (TPA) gene which has been previously characterized (11).

In our hands both 'unpredictably primed PCR' and 'vectorette PCR' generated multiple PCR products even after nested PCR was performed. Although upon analysis some of the PCR products were found to be derived from successful walks, the presence of multiple PCR products would complicate further characterization without Southern blot hybridization or extensive cloning.

We have improved upon the adaptor ligation method by combining 'vectorette PCR' with a newly developed method termed 'suppression PCR' (12). A special adaptor is ligated to the ends of DNA fragments generated by digestion of human genomic DNA with *Eco*RV, *Sca*I, *Dra*I, *Pvu*II and *Ssp*I separately. Following adaptor ligation, a small amount of the DNA is used as a template for PCR using adaptor primers and gene specific primers. The enzymes used were selected because they have six-base recognition sites and generate blunt ends.

The sequences of the walking adaptor and primers are shown in Figure 1. One end of the adaptor is blunt so that it will ligate to both ends of any DNA fragment generated by restriction enzymes that yield blunt ends. The adaptor also contains two rare restriction enzyme sites for *Not*I (staggered ends) and *Srf*I/*Sma*I (blunt ends) to allow cloning into commonly used vectors such as pBluescript (Stratagene).

The 'vectorette' feature of the adaptor is the presence of an amine group on the 3′-end of the lower strand. This blocks

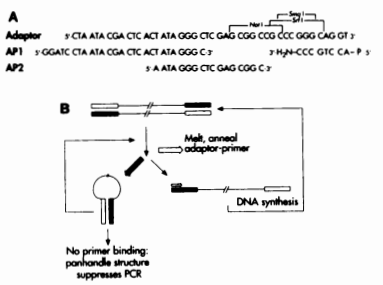

Figure 1. (**A**) Sequences of the walking adaptor and adaptor primers and gene-specific primers. (**B**) Illustration of the suppression PCR effect. Structure of PCR products formed by non-specific amplification. The sequences on the ends of the products contain inverted terminal repeats and will form 'panhandle' structures which suppress PCR.

polymerase catalyzed extension of the lower adaptor strand, preventing the generation of the primer binding site unless a defined, distal, gene-specific primer extends a DNA strand opposite the upper strand of the adapter. The 'suppression PCR' technology uses an adaptor primer which is shorter in length than the adaptor and is capable of hybridizing to the outer primer binding site. If any PCR products are generated which contain double-stranded adaptor sequences at both ends (due to non-specific DNA synthesis), the ends of the individual DNA strands will form 'panhandle' structures following every denaturation step, due to the presence of inverted terminal repeats (Fig. 1B.). These structures are more stable than the primer–template hybrid and therefore will suppress exponential amplification. However, when a distal gene-specific primer extends a DNA strand through the adaptor, the extension product will contain the adaptor sequence only on one end and thus cannot form the 'panhandle' structure. PCR amplification can then proceed normally.

To prepare the adaptor-ligated DNA, 2.5 µg of human genomic DNA (Clontech) is digested in 100 µl reaction volumes with 80 U of restriction enzyme overnight at 37°C using buffers recommended by the supplier (Life Technologies). The DNA was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) vol/vol, once with chloroform, and then precipitated by addition of 1/10th vol 3 M NaOAc, 20 µg glycogen (molecular biology grade, Boehringer Mannheim) and 2 vol of 95% EtOH.

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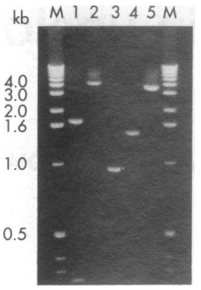

Figure 2. Amplification products generated from walking upstream from exon 1 of the human tissue-type plasminogen activator gene. PCR products in lanes 1–5 were obtained from the *Eco*RV, *Sca*I, *Dra*I, *Pvu*II and *Ssp*I adaptor-ligated DNA libraries, respectively. Lanes M contain 1 kb DNA size markers (Life Technologies). The sequence of the TPA specific primers were: Primary PCR; 5′-AGA AAC CCG ACC TAC CAC GGC TTG CTC CTT-3′. Secondary PCR: 5′-CCC TTT CCT CGC AGA AAT TTT CTC TCC AGC-3′.

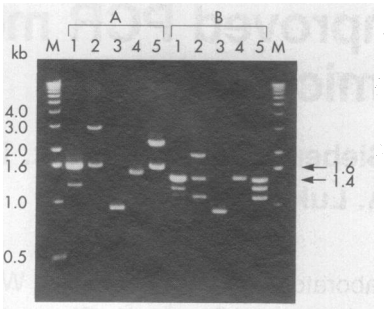

Figure 3. Restriction enzyme digestion of the PCR products shown in Figure 2. Lanes 1–5 correspond to the products obtained from the *Eco*RV, *Sca*I, *Dra*I, *Pvu*II and *Ssp*I adaptor-ligated DNA libraries, respectively. (**A**) Digestion with *Bam*HI. (**B**) Digestion with *Pvu*II. Lanes M contain 1 kb DNA size markers.

After vortex mixing, the tubes were immediately centrifuged at 15 000 r.p.m. in a microcentrifuge for 10 min. The pellets were washed with 80% EtOH and immediately centrifuged as above for 5 min, air dried and dissolved in 20 µl of 10 mM Tris–HCl, pH 7.5, 0.1 mM EDTA. Ten µl of DNA was then ligated to an excess of adaptor overnight at 16°C under the following conditions: 50 mM Tris–HCl, pH 7.6, 10 mM MgCl, 0.5 mM ATP, 10 mM dithiothretitol, 5 µM adaptor and 10 U T4 DNA ligase (Life Technologies) in a total volume of 20 µl. The ligation reaction was terminated by incubation of the tubes at 70°C for 5 min, then diluted 10-fold by addition of 180 µl of 10 mM Tris–HCl, pH 7.5, 1 mM EDTA and stored at –20°C.

PCR amplifications were performed using a long distance thermostable DNA polymerase mixture of Tth (5 U/µl Toyobo) and Vent (2 U/µl New England Biolabs) at a ratio of 20:1 vol/vol (10). We also utilized a commercially available enzyme mixture, rTth XL (2 U/µl Perkin Elmer) with similar results. Before use, 2 µl (11 µg) of Tth Start™ Antibody (Clontech) was mixed with 10 µl of the Tth/Vent enzyme mixture to facilitate a hot start PCR.

Primary PCR reactions were conducted in 50 µl volumes containing 1 µl of ligated and diluted DNA, 40 mM Tris–HCl, pH 9.3, 85 mM KOAc, 1.1 mM MgOAc, 0.4 µM adaptor primer AP1 and gene-specific primer, and 0.9 µl of Tth/Vent polymerase/antibody mixture. The cycle parameters were as follows: denaturation at 94°C for 30 s and annealing/extension at 68°C for 6 min, except for an initial denaturation step of 1 min and a final annealing/extension time of 15 min, 35 cycles of PCR were used. A secondary PCR reaction was conducted with 1 µl of a 100-fold dilution of the primary PCR using adaptor primer AP2 and the nested gene-specific primer. The same reaction composition and cycle parameters, except that 0.6 µl of the Tth/Vent polymerase/antibody mixture was used and 20 thermocycles performed. PCR products were examined on 1% agarose/EtBr gels. For restriction enzyme digestion of PCR products, 10 µl of PCR products were mixed with 1.2 µl of 10 × enzyme buffer and 10 U restriction enzyme (Life Technologies) and incubated for 4 h at 37°C.

The result of an experiment in which we walked upstream from exon 1 of the tissue-type plasminogen activator gene is shown in Figure 2. In all cases, single major PCR products were generated which had the following sizes: 1.8, 4.5, 0.9, 1.5 and 3.9 kb. The sizes of the three smaller PCR products were predicted from the known sequence of the TPA gene (11). Not enough upstream sequence was available in GeneBank (release 81) to predict the sizes of the two larger PCR products.

We then mapped the PCR products by digestion with *Bam*HI and *Pvu*II. The restriction enzyme pattern obtained is shown in Figure 3. The release of common DNA fragments at 1.6 kb with *Bam*HI and 1.4 kb with *Pvu*II from the larger of the two PCR products was predicted from the known sequence. These data thus verified the gene-specificity of the walk for all five adaptor-ligated DNA libraries. The results also show that provisional restriction maps of the unknown sequence can be obtained without cloning.

We have found that the high specificity of the method described is primarily due to the suppression effect of the adaptor/adaptor primer combination, although in some cases additionally having an amine group on the lower strand of the adaptor (vectorette blocking) yielded better results.

We have also utilized the same adaptor-ligated DNA to walk upstream from 5′-end coding regions of the human transferrin receptor gene with similar clean results. In this case the walks obtained ranged in length from 0.2 to 6 kb.

The improved DNA walking method described here will be valuable for rapidly finding promoters and regulatory elements from sequences obtained from cloned cDNAs, to determine the exon–intron boundaries of genes, and to walk upstream or downstream from sequence tagged sites generated from human genome studies.

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