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Single-Primer Amplification of Flanking Sequences

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A number of PCR-based methods have been described for the amplification of DNA flanking a known sequence. Most of these techniques rely on restriction endonuclease digestion and subsequent ligation of a linker, a single-stranded oligonucleotide, or self-ligation (2). Although these techniques have proven valuable, a major disadvantage is the inefficiency of the enzymatic modification of the substrate DNA. However, several alternative PCR-based methods that require no enzymatic modification before amplification have also been described. Such techniques rely on a single targeted primer specific to the known sequence and nonspecific "walking primers" (1,3,4). Other techniques use one specific primer only and rely on single-primer amplification under certain conditions (5,7). These techniques are highly sensitive but often prone to false positives.

We have developed a variation of this single-primer approach that provides increased specificity. This is a consequence of both specific template capture and purification and the use of a single nested primer in subsequent amplifications. We have used this technique to isolate the 5' regions of genes encoding banana sucrose phosphate synthase (*SPS*), sugarcane actin (*ACT1*), and *Dunaliella tertiolecta* S15 ribosomal protein (*S15*).

Genomic DNA was isolated essentially as described previously (6). A biotinylated primer (B-primer, Table 1) was then used to extend from the sequence of interest. This reaction was cycled using an extension time of 3–5 min, allowing accumulation of long ss-DNA products. Extension cycles and PCR were carried out on a PT-200™ Thermal Cycler (MJ Research, Waltham, MA, USA) using the Expand™ Long Template PCR System (Roche Molecular Biochemicals, Indianapolis, IN, USA). The extension reactions were assembled on ice, each containing 50 ng–1 µg genomic DNA, 200 µM

each dNTP, 5 µL Buffer 2 or 3, 20 pmol B-primer, 0.5 U Expand polymerase, and distilled water to a final volume of 50 µL. Extensions were heated to 92°C for 2 min and then cycled at 92°C for 30 s, 50°C–60°C for 30 s, and 68°C for 3 min for a total of 35 cycles (Table 1). A final extension was carried out at 68°C for 10 min, and the samples were cooled to 4°C. The products from this primer extension were purified using the Dynabeads® Kilobase BINDER™ kit (Dynal A.S., Oslo, Norway) according to the manufacturer's directions. The beads (5 µL) were first washed twice in Binding Buffer (provided by the manufacturer) and resuspended in 20 µL Binding Buffer. A 20-µL aliquot of the extension product was added and incubated for 3 h at 25°C on a roller to keep the beads in suspension. Genomic DNA was removed using 100-µL washes in 0.2 M NaOH (1 × 30 min and 4 × 1 min washes), TE buffer (5 × 1 min washes), and distilled water (5 × 1 min washes), and the beads were then resuspended in 20 µL distilled water. To increase specificity, a nested primer (N-primer, Table 1), designed to bind upstream from the B-primer, was used for amplification. This primer must first misprime and extend on the purified ss-DNA to create a further N-primer annealing site, allowing amplification. PCR was assembled on ice, as it was for the B-primer extension reaction, except that 1 µL captured template instead of genomic DNA and 20 pmol N-primer, instead of the B-primer, was used. A similar range of annealing temperatures equal to or above the primer melting temperature (T_m) (Table 1) were tested, and typically, two rounds of PCR were needed to visualize products on an agarose gel.

Products from the PCR were separated by electrophoresis through a 1% agarose gel. Generally, several bands were present ranging in size from 500 bp to 2 kbp. To determine the specificity of the reaction, the amplified products were transferred to a nylon membrane using a Posiblote® (Stratagene, La Jolla, CA, USA). The membrane was hybridized overnight at 30°C in DIG Easy Hyb (Roche Molecular Biochemicals) using a 3' end DIG-labeled oligonucleotide (Table 1), generated using a 3' end DIG-labeling Kit (Roche Molecular Bioche-

Benchmarks

micals). Unbound probe was removed with two washes in 2× standard saline citrate (SSC)/0.1% SDS for 10 min at 48°C and two 15-min washes in 0.1× SSC/0.1% SDS at 48°C. Hybridized probe was detected using alkaline phosphatase conjugated to antidigoxigenin Fab fragments with CDP-*Star*TM (Roche Molecular Biochemicals), according to the manufacturer's instructions.

Southern analysis on each of the reaction products (Figure 2) indicated that the procedure was specific, with additional nonspecific products only generated in the sugarcane actin reaction. The presence of multiple specific products in each of the reactions and the sizes of the amplified products, which generally did not exceed 2 kbp, were all indications of the random-priming efficiency of the N-primer. This was further demonstrated by the fact that initial low-stringency PCR cycles common to other techniques (3,5) were not necessary to generate specific products. Subsequent analysis of the N-

primer annealing sites indicated that only minimal primer-template homology was required for subsequent extension. For instance, in one of the actin-N priming sites, only 8 of the 18 nucleotides were homologous, which was consistent with previously described random-priming studies (4,5). To demonstrate that the amplified products were upstream of the known sequence in the original genomic DNA, each was

amplified using a specific primer (B or N) and a primer derived from the amplified products. In all cases, a single product of the predicted size was amplified, and subsequent sequence analysis revealed that it was identical to the original product.

Specific B-primer extension was essential to the success of the reaction because nonspecific priming at this point was believed to lead to the majority of

Figure 1. Diagrammatic representation of the technique. In the first step, a B-primer was annealed to genomic DNA and extended. The ssDNA product was purified using Dynabeads, and an N-primer was used to misprime and extend. This created another site for N-primer annealing, allowing PCR amplification. Subsequent hybridization with a labeled oligonucleotide probe allowed identification of positive products, which could then be cloned and sequenced.

Benchmarks

Table 1. Sequences of the Primers and Probes Used

Primer	Sequence (5'→3')	Length (Nucleotides)	Annealing Temperature (°C)
Actin-B	ACCTTGACCATTCCAGTGCC	20	60
Actin-N	AGTGCCATTGTACAGAC	18	60
Actin-P	ATGGCTGACGGCGAGGATATCC	22	—
SPS-B	GGAGGACTTGGCCGCGTCGAT	21	50
SPS-N	CGTCCAGGATCGCCTCCAGA	20	50
SPS-P	GTTGATCCAGTCGTTTCCCGCGT	23	—
S15-B	TCCCTACGCCGATTTAGGAATAGG	24	60
S15-N	AGGCAGCTTGGCTCAAAG	18	60
S15-P	CTTAGTGGAGGGGACTTGCAGTTCC	25	—

Biotinylated oligonucleotides (-B), nested oligonucleotides (-N), and oligonucleotide probes (-P). The primers were designed for sugarcane (*Saccharum officinarum*) actin (*ACT1*), banana (*Musa* spp. cv. Cavendish AAA) sucrose phosphate synthase (*SPS*), and *D. tertiolecta* S15 ribosomal protein gene (*S15*). The annealing temperature at which the primers were used is indicated.

false positives. To limit nonspecific priming, we normally examined a number of annealing temperatures at or above the T_m of the primer. Extension from organisms with small genomes such as banana or algae worked under most conditions, whereas plants with larger genomes, such as sugarcane, re-

quired more extensive optimization of B-primer annealing. The protocol presented in this report should facilitate the amplification of unknown DNA regions flanking known sequences because it is a simple and specific technique that requires no enzymatic modification of the substrate DNA.

Figure 2. Southern analysis of amplified PCR products. The agarose gels show typical products from the sugarcane *ACT1*, banana *SPS* and *D. tertiolecta* *S15* genes, respectively. The reaction products were blotted and probed with the appropriate 3' end DIG-labeled oligonucleotide probe (Table 1).

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