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Gene isolation programs often begin with a gene fragment which is then used to isolate longer, more informative clones. Traditional methods of recovering 'full-length' cDNAs by synthesizing cDNA, cloning into appropriate vectors, transforming/transfecting bacteria and screening the resultant library with radioactive probes, generally require two to three weeks and are rather material and labor-intensive. No information on the length of the cDNA insert is available until a purified clone is isolated, thus several clones must be processed to ensure that a useful clone is obtained. PCR-based techniques such as 5′-RACE and 3′-RACE have been developed for obtaining missing sequence from partial cDNA clones (6). These methods permit a composite sequence to be developed but, often, no single cDNA contains all the coding information necessary for protein synthesis. This is a problem if transgenic work or protein characterization is planned. Recent PCR-based methods such as SMART™ PCR (Clontech, Palo Alto, CA, USA) permit capture of a high proportion of 'full-length' cDNAs by exploiting the features of RT and by incorporating a primer sequence into the synthesized cDNA, which later is used to amplify the cDNA products. The cDNA synthesized by this method is usually cloned into vectors, transformed/transfected, plated and screened in conventional manner.

Use of streptavidin-coated beads together with biotinylated DNA fragments to enrich for nucleic acids such as microsatellite-containing regions of genomic DNA (5) and cDNAs (3,8) is well documented. Expanding on this use of biotinylated DNA fragments as probes that hybridize to specific target nucleic acids, we have developed a simple method for selecting 'full-length' cDNAs from amplifiable cDNA in three working days. Unlike conventional library screening, no vectors, plating or radiolabelling is required. The method presented involved hybridization of SMART™ cDNA with a biotinylated probe and capture of hybrids via

streptavidin beads. This is an iterative process that enriches for cDNAs of interest with each round of hybridization.

In our experiments, the starting materials were cDNA fragments isolated by differential display of RNA from developing loblolly pine embryos (2,9). These differential display fragments were biotinylated through PCR with dUTP-biotin to produce a probe. Probe and SMART™ cDNA were mixed, denatured and allowed to hybridize. Streptavidin-coated beads (Dynal® Inc., Lake Success, NY, USA) were added which capture biotin probe-cDNA hybrids. After unbound cDNAs were washed away, cDNAs hybridized to the probes were eluted and amplified by PCR using primers specific to SMART™ flanking regions to produce double stranded cDNA molecules. The result was a population of cDNAs that had been enriched for a specific cDNA. Products of PCR were separated by electrophoresis, excised, purified, and cloned into a plasmid vector and transformed into bacteria. Alternatively, if upon electrophoresis no band was distinguishable above the smear, the products were used for another round of enrichment. Colonies were screened for insert size by PCR using vector primers flanking the cloning site (bacteria are used directly in the PCR reaction with no plasmid isolation). The presence of the desired insert was established by southern hybridization (using the original probe and an appropriate detection system). If sequence information was available for the probe, PCR with one vector-specific and one EST-specific primer distinguished desirable cDNAs from background.

In this way, 'full-length' cDNAs from abundant messages may be captured through a single round of enrichment in three working days without knowledge of 5′ end sequence or the use of radioactivity. Clones of less abundant messages take about one week. By using more than one probe per hybridization, enrichment for several cDNAs can be performed in the same

hybridization. In addition, screening colonies for insert size will identify the longest clones so that selection and sequencing of truncated positive inserts can be avoided.

In our work, loblolly pine differential display cDNA clones used for probe synthesis were generated as described by Xu et al. (9). Biotinylated probes were synthesized by using PCR to amplify 1 μL (10 ng/μL) of cloned differential display cDNA (i.e., from plasmid source) with 0.5 μL of both forward and reverse modified M13 primers (100 μM each) (forward: 5′-GACGTTGTAAAACGACGGCCAG-3′; reverse: 5′-CAGGAAACAGCTATGACCATG-3′), 5.0 μL of 10X Advantage cDNA Polymerase Buffer (Clontech), 1.0 μL each of dATP, dCTP, and dGTP (10 mM each), 16.7 μL of dTTP (0.5 mM), 3.3 μL of dUTP-Biotin (0.5 mM) (Clontech), and 1.0 μL of 50X Advantage cDNA Polymerase Mix (Clontech). The PCR was performed in a GeneAmp® 9600 (PE, Applied Biosystems, Foster City, CA, USA) thermocycler with the following conditions: 95°C, 2 min; 30X(95°C, 15 s; 65°C, 30 s; 74°C, 1.5 min); 74°C, 5 min; hold at 4°C. After electrophoresis in 1% agarose, probes were gel purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and stored at -20°C in Tris-HCl (pH 8.5).

For cDNA synthesis, the mRNA Direct Kit (Dynal®) was used to isolate poly-A RNA from loblolly pine zygotic embryo tissue judged to be at stage 3 (7). This RNA was used as a template for SMART™ cDNA synthesis according to manufacturer's instructions (Clontech protocol #3041-1).

Dynabeads® M-280 Streptavidin (Dynal®) were used to capture probe-cDNA hybrids. All calculations were based on a standard amount of beads (10 μg) per enrichment because this amount of beads is easily visible in the tube following

immobilization with the magnet, but did not appear to create excessive nonspecific binding area. According to Dynal®, 1 mg of beads will bind ~10 pmol of a 1-kb DNA molecule. Since the hybrids were expected to be about 1-kb, 0.1 pmol of probe was used per enrichment. Finally, an amount of cDNA used was determined by estimating an abundance of 1/100,000 (0.001%) to 1/1,000,000 (0.0001%) of the target cDNA in the population of cDNA that had an average molecular length of 2-kb. From these estimates, the amount of cDNA used was chosen so the probe would be in slight molar excess of the target cDNA (10 to 100 times greater).

Hybridizations of probe and cDNA were done in 1.5-mL microcentrifuge tubes. The cDNA, biotinylated probe, and 1/10 volume TE buffer were mixed together and denatured at 95°C for 5 minutes and then place immediately on ice. After 5 minutes on ice, DNA was precipitated at -20°C by adding 1/10 volume 3.0 M Na acetate (pH 5.2) and 2.5 volumes ethanol. The DNA was washed (70% ethanol), dried under vacuum, suspended in 50 μL of 65°C 1X SSPE, and incubated at 65°C with rotation for at least 2 hours to permit hybridization to occur.

Dynabeads® M-280 Streptavidin were prepared for binding by suspending completely and dispensing 20 μL of beads (10 mg/mL) into a 1.5-mL tube. Beads were immobilized with a magnetic stand and the storage buffer was removed. Beads were washed 2 times in 1.0% Tween 20 followed by 3 times in wash/binding buffer (11X SSPE, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM Na Pyrophosphate), and then resuspended in 1 mL of wash/binding buffer. Just prior to addition of beads, the hybridization reactions were removed from the 65°C oven and placed immediately on

ice. Fifty microliters of prepared beads (10 μg) were added to the hybridization mixture, and the binding reaction was performed for 1 to 2 hours at 25°C with gentle agitation.

After the binding step, beads were immobilized by placing the tube in a magnetic stand for 5 to 10 minutes. Supernatant was carefully removed and discarded. Beads were washed two times, 2 min each wash, in 100 microliters of 0.1X SSPE at room temperature and then 4 more times, 10 min each wash, in 100 microliters of 0.1X SSPE at 60 to 65°C. The elevated temperature reduces nonspecific binding of DNA to beads. Most cDNA-probe hybrids were stable at 65°C. However, some probes required a lower washing temperature (60°C) to preserve hybridization. After the final high temperature wash, captured cDNAs were eluted from beads by adding 50 μL of TE (pH 8.0) and incubating for 10 min at 80°C. Beads were quickly immobilized with a magnetic stand and the supernatant was removed and saved.

The following reagents were used to amplify the enriched eluate: 5.0 μL, eluate; 36 μL, dH_2O; 5.0 μL, 10X Advantage cDNA Polymerase Buffer (Clontech); 1.0 μL, dNTPs (10 mM each); 2.0 μL, PCR Primer (Clontech) (10 μM); 1.0 μL, 50X Advantage cDNA Polymerase Mix (Clontech). Thermocycling was performed in a GeneAmp® 9600 (Perkin Elmer) with the following conditions: 95°C, 2 min; 30X(95°C, 15 s; 68°C, 3 min); 68°C, 3 min; hold at 4°C. PCR products were separated on a 1.0% agarose gel in 1X TAE with 0.5 μg/μL ethidium bromide. Generally, PCR products from the first round of enrichment appeared as a smear in the gel (Figure 1). In this case, additional rounds of enrichment were conducted until a darker region or, preferably, a band was distinguishable in the gel of the PCR products. The region of gel containing the band (or, if further enrichment was necessary, an approximately 1000-bp region spanning the

anticipated mRNA size) was excised, and the DNA was purified using the QIAquick Gel Extraction Kit (Qiagen Inc.) and resuspended in 10 mM Tris-HCl (pH 8.5). A portion of the purified DNA was cloned (see below) and/or a portion was used as the target in another round of enrichment.

Gel purified products were ligated into pGEM® T Easy (Promega, Madison, WI, USA) according to manufacturer's instructions and transformed into competent DH5α *E. coli* cells. To screen colonies for the desired inserts, sterile toothpicks were used to pick colonies. Each toothpick was first touched to fresh LB + ampicillin plate to make a copy of the selected colony and then dipped into a separate tube filled with 50 microliters of dH_2O to make colony dilutions for PCR. The colony dilutions were stored at -20°C until needed for PCR and the LB + ampicillin plates were incubated at 37°C for 4 hours before storing at 4°C.

To screen colonies for insert size, the following reagents were used for colony PCR: 5.0 μL, colony dilution; 8.7 μL, dH_2O; 2.0 μL, 10X *Taq* Polymerase Buffer (15 mM in $MgCl_2$) (Promega); 0.2 μL, dNTPs (20 mM each); 2.0 μL, forward LM13 (10 μM); 2.0 μL, reverse LM13 (10 μM); 0.1 μL, *Taq* Polymerase (5 Units/μL) (Promega). Thermocycling was done in a GeneAmp® 9600 (Perkin Elmer) with the following conditions: 95°C, 2 min; 30X(94°C, 15 s; 65°C, 30 s; 72°C, 2 min); 72°C, 5 min; hold at 4°C. PCR products were separated on an agarose gel (1%, 1X TAE, 0.5 μg/mL ethidium bromide) alongside appropriate molecular size markers and visualized by UV illumination (Figure 2A).

Probes derived from the original fragment were used to identify desirable clones from background sequences by dot southern hybridization (1). Radioactive or

nonradioactive labeling can be used. To screen colonies with radiolabeled EST probes, samples of colony PCR DNA (from PCR with forward plus reverse LM13), unlabeled probe DNA (positive control), and unlabeled DNA from another differential display clone (negative control) were normalized so equal amounts of DNA could be spotted on a membrane. Volumes were adjusted to 30 μL with TE (pH 8.0), and DNAs were denatured by adding 20 μL of 1.0 M NaOH and 1.0 μL of 0.5 M EDTA and heating to 95°C for 5 min. Samples were chilled on ice for 5 minutes, and then 5 μL of each sample was spotted onto Hybond™ N+ (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and UV crosslinked. Fifty nanograms of EST DNA was used to make ^{32}P-labeled probes with Ready-To-Go DNA labeling beads (Amersham Pharmacia Biotech) according to manufacturer's instructions. Blots were prehybridized (7% SDS, 1% BSA, 0.25 M $NaPO_4$ (pH 7.2), 1.0 mM EDTA) for 3 hours at 65°C and hybridized in fresh buffer at 65°C for 12 to 18 hours (4). Each blot was washed 6 times with the following conditions: 1) RT, 2X SSC, 0.1% SDS, 15 min; 2) RT, 2X SSC, 0.1% SDS, 30 min; 3) 42°C, 0.2X SSC, 0.1% SDS, 15 min; 4) 42°C, 0.2X SSC, 0.1% SDS, 30 min; 5) 60°C, 0.2X SSC, 0.1% SDS, 30 min; 6) 60°C, 0.2X SSC, 0.1% SDS, 30 min. Blots were exposed to a phosphorimaging plate for 10 minutes. Screens were read with a BAS1800 (software v1.0) and images were manipulated with ImageGauge (v2.54) (Fuji Photo Film Co., Ltd., Kanagawa, Japan). Positive clones were identified by comparing the signal intensity from each spot versus the positive and negative controls (Figure 2D). Sequencing of inserts revealed regions homologous to the biotinylated probe indicating that the clones identified in this screen were indeed 'full-length' versions of the original probe.

In cases where sequence information for the original probe was available so EST-specific primers could be synthesized, PCR was used to identify colonies bearing the desired 'full-length' sequence. The colony PCRs consisted of: 5.0 μL, colony dilution; 8.7 μL, dH_2O; 2.0 μL, 10X *Taq* Polymerase Buffer (15 mM in $MgCl_2$) (Promega); 0.2 μL, dNTPs (20 mM each); 2.0 μL, forward OR reverse M13 primer (10 μM); 2.0 μL, EST-specific primer (10 μM); 0.1 μL, *Taq* Polymerase (5 Units/μL) (Promega). PCRs with both primer combinations (EST-specific plus *forward* LM13 and EST-specific plus *reverse* LM13) were done for each colony. Thermocycling and electrophoresis was identical to that for insert size screening. These results also distinguish clones whose sequence is related to the original probe from background colonies (Figures 2B & 2C).

On occasion, we noticed that screening colonies by hybridization could identify positives that were not revealed by PCR (data not shown). In all cases of this apparent contradiction, the insert in question was smaller than the average positive insert. This suggests that these smaller positive inserts did not have priming sites for the EST-specific primer, but did bear enough homology to the probe to be selected during enrichment and identified as a positive during screening via dot southern hybridization.

We have presented a simple method that allows desirable clones to be quickly isolated from a SMART™ cDNA or a similar cDNA pool that is susceptible to PCR amplification from terminal primers. The method can be completed in a few working days and is reliable and accurate. As opposed to using the biotinylated probe approach for selecting cDNAs that have previously been cloned into plasmid vectors (8), which requires library construction and synthesis of 3 separate oligonucleotides, the method described here requires amplifiable cDNA, a PCR-generated biotinylated probe and only

one EST-specific oligonucleotide if colonies will be screened by PCR or no EST-specific primer if colonies will be screened by dot southern hybridizations. As with other enrichment protocols (5,8), multiple rounds of enrichment are occasionally necessary in order to isolate a less abundant sequence. The method permits the longest clones in a screen to be identified and selected and provides for isolation of 'full length' cDNAs without sequence information. Furthermore, by using more than one probe per hybridization, we have also been able to select for multiple cDNAs simultaneously (data not shown).

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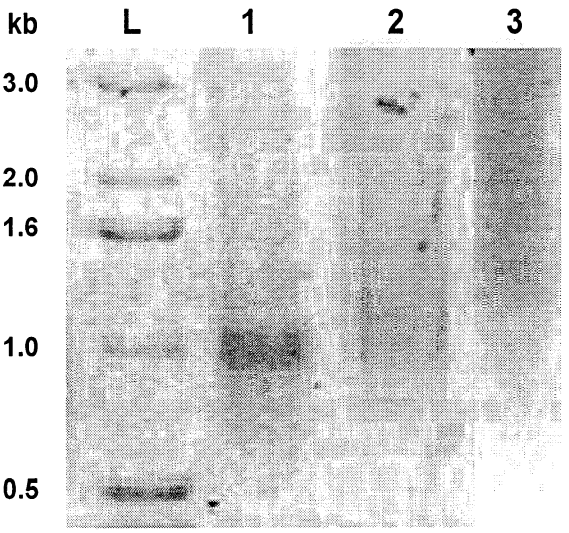
Figure 1. Electrophoresis and photography of PCR products to demonstrate the enrichment process. Lane 1 is amplified eluate following 2 rounds of enrichment. Lane 2 is amplified eluate following 1 round of enrichment. Lane 3 is the original, unenriched cDNA. The enrichment process is illustrated by comparing lanes 1, 2, and 3. After 1 round of enrichment (lane 2), PCR products are mostly a smear with some darkening around 1.0-kb, and the majority of the products have shifted to a smaller molecular size versus the smear from the unenriched cDNA (lane 3). After the second round of enrichment (lane 1), a band is clearly distinguishable above the background smear.

Figures 2A–D. An example of screening 6 secondary enrichment colonies for insert size and cDNA of interest. Colony number (1-6) is listed across the top of the figure. **A:** Electrophoresis and photography of products from colony PCRs with both vector primers (LM13-forward and LM13-reverse) determines insert size. **B & C:** Electrophoresis and photography of products from colony PCRs with (**B**) an EST-specific primer and the LM13-forward primer and (**C**) an EST-specific primer and the LM13-reverse primer identifies positive colonies. Note that the plasmid from colony 6 does not yield a PCR product when an EST-specific primer is used. **D:** Dot southern hybridization of colony PCR DNAs 1-6, positive control DNA, and negative control DNA hybridized against a radiolabeled probe. Note that cDNA from colony 6 does not hybridize with the probe. Colony PCRs and dot hybridizations indicate that colonies 1-5 contain the cDNA of interest.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3.0
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1.6
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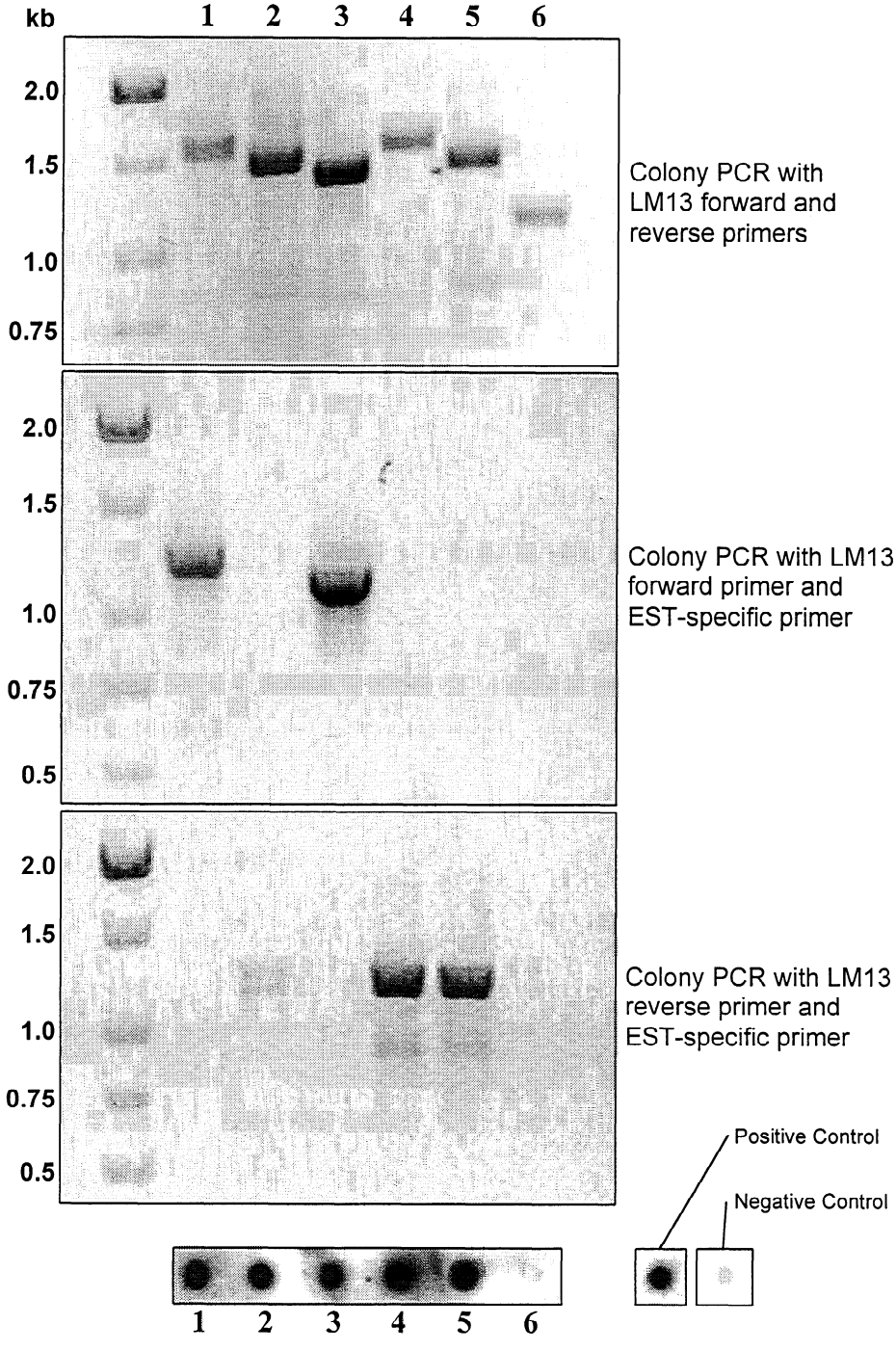
kb
1 2 3 4 5 6
2.0
1.5
1.0
0.75
Colony PCR with LM13 forward and reverse primers
2.0
1.5
1.0
0.75
0.5
Colony PCR with LM13 forward primer and EST-specific primer
2.0
1.5
1.0
0.75
0.5
Colony PCR with LM13 reverse primer and EST-specific primer
Positive Control
Negative Control
1 2 3 4 5 6