System for PCR Identification of cDNA Ends (SPICE)

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We have devised a method, system for polymerase chain reaction (PCR) identification of cDNA ends (SPICE), for retrieving clones from λ cDNA libraries (Figure 1). SPICE resembles the process. A method described by Li and Nicholas (8) incorporates new features to enhance sensitivity and speed the cloning of fragments containing the 5′ and 3′ cDNA sequences, joined at the PCR primers. This DNA can be used directly for automated sequencing from the primer sites in the phagemid.

SPICE differs from other iPCR strategies (3–6,8,10,11) in several ways. It allows the screening of existing λ cDNA libraries, provided they can be bulk-excised [e.g., λ Zap® (Stratagene, La Jolla, CA, USA) and λ ZipLox (Life Technologies, Gaithersburg, MD, USA)]. It therefore represents an alternative to λ plaque screening, rather than an alternative to rapid amplification of cDNA ends (RACE) (7). SPICE is free from extra steps present in other iPCR strategies (3–6,8,10,11), such as the ligation of adapter oligonucleotides or the subcloning of separate PCR products into sequencing vectors—both 5′ and 3′ ends are recovered together in one step in a single clone ready for sequencing. Finally, we have found the addition of GC-Melt™ (CLONTECH, Palo Alto, CA, USA) greatly improves the success of this protocol, often allowing a clone to be recovered in a single PCR step without primer nesting.

The SPICE method consists of first excising a λ cDNA library. We used libraries constructed in the Zap® II vector (Stratagene), excised following the manufacturer’s protocol with VCSM13 helper phage to yield cDNA libraries in the pBluescript® phagemid (Stratagene). Two long, outward-oriented primers (25–35 nucleotides (nt), 10 pmol each) were used for LA-iPCR on 100 ng phagemid library DNA using the KlenTaq Polymerase Mix, with the supplied KlenTaq reaction buffer (both from CLONTECH) and GC-Melt at a final concentration of 1 M. Dimethyl sulfoxide (DMSO) was found to offer no benefit (not shown). The PCR also contained 250 μM (final) of each dNTP, 1 μL KlenTaq Polymerase Mix and water to 50 μL in a 0.2-mL thin-wall PCR tube (Stratagene) overlaid with 40 μL light mineral oil. PCR was performed in a Model 2400 Thermal Cycler (PE Biosystems, Foster City, CA, USA) with a two-step program of 99°C for 35 s and 68°C for 8 min, for 35 cycles. If a band migrating on an agarose gel at >3 kb (larger than pBluescript) was not obtained in the first round of PCR, 5 μL of the PCR were used in a second round of PCR with nested, outward-oriented primers. The band was excised from the gel and phosphorylated with T4 Polynucleotide Kinase (New England Biolabs). The PCR product was run on an agarose-ethidium bromide gel. The frequency of phagemids with β-actin clones was estimated based on a starting frequency of 0.5% in the λ library, as determined by plaque screening. The primers used for β-actin were TGGCTTCTAGACCAATGAAAAGATCAAGATCT and CCTTATCGTCCCAGTTGGTTACAATGCCG.

The product is visualized on an agarose gel at >3 kb (larger than pBluescript) was not obtained in the first round of PCR, 5 μL of the PCR were used in a second round of PCR with nested, outward-oriented primers. The band was excised from the gel and phosphorylated with T4 Polynucleotide Kinase (New England Biolabs). The PCR product was run on an agarose-ethidium bromide gel. The frequency of phagemids with β-actin clones was estimated based on a starting frequency of 0.5% in the λ library, as determined by plaque screening. The primers used for β-actin were TGGCTTCTAGACCAATGAAAAGATCAAGATCT and CCTTATCGTCCCAGTTGGTTACAATGCCG.
Kinase (Life Technologies) in forward reaction buffer plus 10 mM ATP for 45 min at 37°C. The DNA was then precipitated, resuspended in ligation buffer and incubated overnight at 12°C with 5 U T4 DNA Ligase (Boehringer Mannheim, Indianapolis, IN, USA). The ligation reaction was used to transform E. coli, and several colonies were screened by mini-prep and restriction with EcoRI to check the size of cDNA inserts. Although the PCR product seen on the gel often appears as a discrete band, it usually yields several positive clones of slightly different lengths, probably representing different clones in the library. The clones were then auto-sequenced (Model L-4000; LI-COR, Lincoln, NE, USA) from the T3 and/or T7 primer sites in pBluescript.

The addition of GC-Melt enhanced the sensitivity of the technique for all cDNAs recovered, regardless of the G:C content of the target sequence. Figure 2 shows rat β-actin, which we used to optimize the system, retrieved from a lung cDNA library. GC-Melt raised the sensitivity of this β-actin LA-iPCR roughly 10-fold. The clone was found to contain 273 nt of sequence upstream and 1135 nt downstream of the PCR primer sites (thus complete except for the first 261 nt) and probably represents the most abundant species in the library. By plaque screening, this clone the corresponding full-length human homologue of this gene from an intestinal carcinoma library (unpublished). Finally, we cloned the 1.7-kb, full-length human homologue of a yeast RNA-binding protein, by single-round SPICE, using primers based on a short published expressed sequence tag (EST) sequence (unpublished data).

REFERENCES


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