Preparation of YAC End Fragments from the Whitehead/MIT Mouse YAC Library pRML Vectors

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Yeast artificial chromosome (YAC) libraries containing large genomic DNA inserts are valuable resources for producing long-range physical maps of mammalian genomes. Numerous YAC libraries have been constructed for both human and mouse genomes. Since the majority of these libraries have used the pYAC4 vector (1), methodology for YAC clone characterization in this vector has been well documented. In contrast, the mouse YAC library recently produced at the Whitehead/Massachusetts Institute of Technology (MIT) Center for Genome Research (Cambridge, MA, USA) was constructed using a different vector system consisting of two separate plasmids, pRML1 (left centric arm) and pRML2 (right acentric arm) (4). The Whitehead/MIT library provides the largest inserts (average insert size of 820 kb) and greatest coverage of the mouse genome (10-fold coverage) available in any mouse YAC library to date. Additionally, clones from this library are being used to construct a comprehensive physical map anchored to the mouse genetic map (2). Thus, the Whitehead/MIT mouse YAC library has become the library of choice for many applications, e.g., the isolation of genes by positional cloning approaches. However, techniques for characterization of clones in the pRML vectors have not been well described in the literature. Methodology for the generation of YAC end fragments that can be cloned and sequenced is particularly lacking. Below, we present a method

Table 1. Oligonucleotide Primers for Amplification of YAC End Sequences

<table>
<thead>
<tr>
<th>Primers for amplification of specific YAC end sequences</th>
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<tr>
<td><strong>pRML1 (left arm) primers</strong></td>
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<tr>
<td>→ → 5’-CTCATGGTTCACGCTTATCATC-3’</td>
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<tr>
<td>← ← 5’-CTGTTGCTGGAAGTCG-3’</td>
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<tr>
<td>PCR cycling parameters: 63°C–53°C touchdown</td>
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<tr>
<td><strong>pRML2 (right arm) primers</strong></td>
</tr>
<tr>
<td>→ → 5’-GAATCCTAGTGCAATTG-3’</td>
</tr>
<tr>
<td>← ← 5’-CTGGTGCTGCTGACAGGC-3’</td>
</tr>
<tr>
<td>PCR cycling parameters: 59°C–49°C touchdown</td>
</tr>
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</table>

**Example primers:**

- 5′-CTCATGGTTCACGCTTATCATC-3′
- 5′-CTGTTGCTGGAAGTCG-3′
- 5′-GAATCCTAGTGCAATTG-3′
- 5′-CTGGTGCTGCTGACAGGC-3′

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Benchmarks

for cloning end fragments from YACs in pRML vectors using inverse polymerase chain reaction (PCR). The resulting YAC end fragment sequences are useful for designing PCR primers to develop new sequence tagged sites, to screen libraries to build YAC contigs and to map YAC ends in radiation hybrid panels for detecting chimerism.

Inverse PCR is an efficient method for isolation of the ends of YAC inserts, because it allows amplification of DNA segments that lie outside the boundaries of known sequences (6,7). The YAC is first digested into small fragments with a restriction enzyme that cleaves both the vector and the insert at positions near the cloning site. The resulting fragments are diluted and ligated under conditions that favor intramolecular circularization of individual fragments. YAC sequences corresponding to the end of the YAC insert are then selectively amplified using PCR primers derived from vector sequences (in inverse orientation) that flank the insert sequence in the circularized fragments (see Reference 5 or 6 for schematic diagrams of inverse PCR). For production of YAC end fragments from the Whitehead/MIT library, we designed distinct PCR primer pairs for isolation of ends adjacent to the pRML1 and pRML2 vectors based on sequence information provided by Research Genetics (Huntsville, AL, USA). Figure 1 shows a schematic representation of inverse PCR products that are amplified by these primer pairs.

YAC clones from the Whitehead/MIT library were obtained from Research Genetics, and DNA was isolated from yeast spheroplasts (5). A 500-ng amount of yeast DNA containing the YAC of interest was digested with Sau3AI (New England Biolabs, Beverly, MA, USA) in a volume of 20 µL at 37°C for 2 h. Samples were extracted with phenol/chloroform and then ethanol-precipitated. Pellets were resuspended in water, and 25–100 ng were used in a circularization reaction performed in a volume of 20 µL with the Rapid DNA Ligation Kit (Boehringer Mannheim, Indianapolis, IN, USA) according to manufacturer's instructions. End fragments were subsequently amplified in a PTC-200™ Thermal Cycler (MJ Research, Watertown, MA, USA) from 1 µL of the ligation reaction using PCR primers and cycling conditions listed in Table 1. Amplification reactions contained 0.2 mM of each dNTP, 0.25 µg each primer, 0.2 µL Advantage® KlenTaq LA Polymerase Mix (CLONTECH Laboratories, Palo Alto, CA, USA), and 1x KlenTaq PCR Buffer (40 mM Tricine-KOH, pH 9.2 at 25°C, 15 mM

Figure 1. Schematic representation of inverse PCR products from pRML vectors. pRML YAC vector DNA sequences are shown in light and dark gray. Dark gray highlights are the sequences that correspond to pRML1 and pRML2 forward and reverse primer sets (Table 1). Mouse genomic DNA is cloned within the EcoRI sites at positions 283 and 257 for pRML1 and pRML2, respectively. After Sau3AI digestion and recircularization, the Sau3AI site at positions 145 (pRML1) and 192 (pRML2) of the vector is ligated back to the most proximal Sau3AI site located in the mouse genomic DNA insert. The pRML primer sets are then used to amplify the intervening DNA consisting of mouse genomic insert and short regions of flanking pRML vector sequence.
Benchmarks

potassium acetate, 3.5 mM magnesium acetate, 75 µg/mL bovine serum albumin) in a total volume of 50 µL. Other Taq polymerases and their corresponding buffers have also been used with similar success (i.e., AmpliTaq® DNA Polymerase; PE Biosystems, Foster City, CA, USA, and Taq DNA Polymerase; Qiagen, Chatsworth, CA, USA). A “touchdown” thermal cycling program with dynamic annealing temperature was used for each set of primers to reduce nonspecific amplification (3). The cycling program for pRML1 primers was as follows: denaturation at 92°C for 30 s, annealing at 63°C for 40 s and extension at 72°C for 1 min. This cycling was repeated for a total of 20 cycles with a reduction in annealing temperature of 0.5°C per cycle bringing the final annealing temperature to 53°C. An additional 15 cycles were carried out with a static annealing temperature of 53°C. Cycling conditions for pRML2 primers were similar, except that the starting and ending annealing temperatures were 59° and 49°C, respectively. Negative PCR controls consisting of nonligated samples were amplified in parallel. Resulting PCR products were analyzed by agarose gel electrophoresis, which showed that end fragment products typically ranged from 200–500 bp (not shown). PCR products were purified using Wizard® PCR Preps (Promega, Madison, WI, USA), ligated into the PCR®2.1 TA Cloning Vector (Invitrogen, Carlsbad, CA, USA) and sequenced using primers corresponding to pCR2.1 vector sequences flanking the insert site (i.e., standard T7 and M13-reverse primers).

The cloned inverse PCR products contained the expected pRML vector arm sequences flanking unique sequence that presumably represents contiguous genomic DNA (Figure 1). To confirm that these unique sequences are those that occur adjacent to the vector arms in the intact YAC clones, PCR was performed with an insert-specific primer (Table 1) coupled with a pRML primer. As shown in Figure 2a, these primer pairs amplified the parent YAC, producing a product of the expected size, but did not amplify unrelated YAC or mouse genomic DNA templates. Additionally, to verify that the cloned inserts correspond to unique mouse genomic sequences, which can be used as sequence-tagged sites, PCR was performed with primer pairs derived from insert sequences. As shown in Figure 2b, the expected products were amplified from the parent YAC and from mouse genomic DNA but not from unrelated YACs.

These results demonstrate that the pRML primers reported in Table 1 permit specific and reliable amplification of insert sequences adjacent to the vector cloning site and should be broadly applicable for characterization of clones from the Whitehead/MIT mouse YAC library. We have used this technique with a success rate of >80% for end fragment isolation. The resulting end fragment clones can be used as

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Figure 2. Verification of YAC end clones by PCR. YAC clones 223C9, 460D12 and 198H3 from the Whitehead/MIT mouse YAC library (4) were obtained from Research Genetics. YAC end fragments were generated as described in the text. R refers to right-arm end clones and L to left-arm end clones (e.g., 223R, 460L, 198L and 198R). PCR primer sequences are listed in Table 1, and PCR conditions were as described in the text with a 63–53°C cycling program. Amplification products were analyzed on 4% Metaphor Agarose (FMC BioProducts, Rockland, ME, USA) and detected by staining with ethidium bromide. A 50-bp DNA marker (Life Technologies, Gaithersburg, MD, USA) was used as a molecular size standard (M). (a) PCR performed with an insert-specific primer coupled with a pRML primer demonstrates cloning of insert sequences adjacent to YAC vectors. In each case, the forward pRML1 or pRML2 vector primer was paired with the reverse YAC-specific primer (listed in Table 1) as indicated under “Primer Set”. Amplification produced the expected product from the corresponding parent YAC template (denoted by italics); however, no product was produced from unrelated YACs, C57BL/6J mouse genomic DNA (B6) and the water control (—). (b) PCR performed with YAC-specific primers demonstrates the establishment of sequence tagged sites in mouse genomic DNA. Amplification with YAC-specific forward and reverse primers produced the expected products from the parent YAC (italics) and B6 genomic DNA, but no product from both unrelated YACs and water controls.
hybridization probes or sequenced to design PCR primers. These markers are useful in screening YAC or bacterial artificial chromosome (BAC) libraries to develop contigs and to characterize YACs for chimeraism by mapping the ends in backcross panels or radiation hybrid panels.

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Isolation of pcDNAI-Based Mammalian Expression Vectors from Escherichia coli Strain MC1061/P3


Expression of cloned DNA sequences in mammalian systems is becoming an increasingly important tool in all areas of biological and biochemical research. This process allows one to determine what features of the cloned sequence are important for proper function in mammalian systems and to dissect these regions on a molecular level. Many mammalian expression vectors are currently available, each with its own advantages and limitations. The mammalian expression vector pcDNAI (Invitrogen, Carlsbad, CA, USA) and its derivatives have been widely used for cloning, expression and analysis of genes in higher eukaryotes—especially when plasmid rescue is required. pcDNAI contains the supF gene rather than a selectable antibiotic resistance gene, a feature that keeps this vector small enough to be useful for expression library construction. The supF gene encodes a tRNA that suppresses amber mutations. Thus, pcDNAI must be propagated in a host that carries an amber mutation in an antibiotic resistance gene. Although pcDNAI has been superseded by a derivative that contains the ampicillin-resistance gene (pcDNAI.1/Amp), a variety of cDNA li-

Figure 1. Comparison of DNA integrity by gel electrophoresis. DNA samples isolated from overnight cultures as indicated were run on a 0.8% agarose gel in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) in the presence of 1 µg EtdBr/mL at 90 V (ca. 50 mA) for 45–60 min at room temperature. Photographs of the gels were taken using Polaroid® Type 57 Film (Polaroid, Cambridge, MA, USA) under UV illumination and reproduced using UMax VistaScan Version 2.28 (UMax Data Systems, Hsin Chu, Taiwan, ROC) and Adobe Photoshop® (Adobe Systems, San Jose, CA, USA). The migration of maker DNA (lambda DNA cut with HindIII) is shown for each gel. Lanes 1 and 2, 1/25th of total pcDNAI isolated from 1.5 mL of an overnight culture by alkaline lysis (4); lanes 3 and 4, 1/25th of total pcDNAI isolated from 1.5 mL of an overnight culture by alkaline lysis (5); lanes 5 and 6, entire pcDNAI sample isolated from a 1.5 mL of an overnight culture by direct lysis in PCI; lanes 7 and 8, 1/30th of the total pcDNAI-βgal isolated from 1.2 mL of an overnight culture by alkaline lysis (4) with PCI extraction step added; lane 9, 1/30th of the total pcDNAI-βgal isolated from 1.2 mL of an overnight culture by alkaline lysis (4) with PCI extraction step added; lane 10, 1/30th of the total pcDNAI-βgal isolated from 1.2 mL of an overnight culture by alkaline lysis (4). The DNA samples in lanes 2, 4, 6 and 12 were digested with BamHI to linearize pcDNAI. The DNA samples in lanes 1, 3, 5 and 11 were digested with HindIII, producing 4.0-, 3.1- and 0.9-kb fragments from pcDNAI. The DNA in lane 10 was undigested, indicating that the DNA preparation yielded primarily covalently closed circular (supercoiled) DNA.