**BENCHMARKS**

Benchmarks are brief communications that describe helpful hints, shortcuts, techniques or substantive modifications of existing methods.

**MS-IRS PCR: A Simple Method for the Isolation of Microsatellites**

Microsatellites (MS) are excellent genetic markers because of their prevalence in eukaryotic genomes, ease of typing by PCR and high degree of polymorphism. MS-based genetic maps of the human and mouse genomes have been constructed with average inter-marker interval sizes of 1.6 cM and 0.42 cM, respectively (1,6). While these maps are essential resources for gene mapping, their resolution may not be sufficient for positional cloning of disease genes or other genes of interest.

The first approach used for the isolation of MS repeats was based on the screening of cloned DNA fragments with radioactively labeled repeat-specific oligonucleotides, followed by sequencing of hybridizing clones. This approach is time consuming and labor intensive, especially when large numbers of clones have to be analyzed. A PCR-based technique, vectorette PCR, has also been adapted for the isolation of MS (4). This method involves the generation of a vectorette-ligated pool of genomic DNA inserts, followed by PCR amplification using MS and vectorette-specific primers to amplify the unique sequences flanking the repeat. While considerably more efficient than hybridization-based screening, vectorette PCR still relies on the construction of several representative vectorette libraries using different restriction enzymes. In addition, because yeast genomic DNA also contains MS repeats, the analysis of yeast artificial chromosomes (YACs) requires pulsed-field gel purification, which can be a major rate limiting step of the procedure.

Here, we describe a novel PCR-based method for the isolation of MS from genomic DNA clones. Our approach makes use of the fact that, in addition to MS, mammalian genomes contain various species-specific repeats known as interspersed repetitive sequences (IRS), including Alu in humans and B1 and B2 in the mouse. Using a combination of primers corresponding to MS and IRS sequences, it is possible to amplify genomic regions between the two types of elements (Figure 1). The resulting MS-IRS products are sequenced and, based on the unique sequence obtained, primers are generated to allow the extension of the sequence to the other side of the MS by inverse PCR (see Reference 7 for a schematic diagram of inverse PCR).

A major advantage of this technique is the species specificity provided by the IRS primers used in the initial amplification. This specificity permits the analysis of unpurified YAC clones in the presence of yeast genomic DNA. We have applied this technique to isolate novel, polymorphic MS from a mouse YAC clone from the critical chromosomal region of the fatty liver dystrophy (fld) mutation.

MS-IRS PCR was performed using...
a panel of eight anchored dinucleotide repeat pools [CA1: (AC)₁₀,C/G/T; CA2: (CA)₁₀,A/G/T; CA3: (GT)₁₀,A/C/T; CA4: (TG)₁₀,G/T; GA1: (AG)₁₀,C/G/T; GA2: (GA)₁₀,A/C/T; GA3: (CT)₁₀,A/G/T; GA4: (TC)₁₀,A/C/G] (9) and an equimolar mixture of 5 primers specific for conserved regions of the mouse B1 [AGTTCCAGGACAGCCAGGG (3), TACACAGAGAAACCCTGTCTC (8) and CTGGAACTCACTCTGAAGAC (2)] and B2 [TCTTCTGGAGTGTCT-GAAGA and GACTGCTCTCCGA-AGTGCC (2)] repeats. Fifty nanograms of total yeast genomic DNA from YAC-containing clones were used as the template for amplification in 50 µL 1× reaction buffer, pH 8.7, containing 1.5 mmol/L MgCl₂, 200 mmol/L dNTPs, 30 pmol of the MS primer pool, 50 pmol of the IRS primer mix and 2.5 U Taq DNA polymerase (Qiagen, Valencia, CA, USA). Reaction mixtures were denatured at 96°C for 1 min, followed by 21–25 cycles of amplification consisting of 94°C/30 s, 55°C/30 s and 72°C/2 min in a Model PTC-100™ Thermal Cycler (MJ Research, Watertown, MA, USA). PCR products were separated in 0.8% SeaKem agarose gels, gel purified (QIAquick; Qiagen) and the isolated fragments cloned by TA-cloning (Invitrogen, Carlsbad, CA, USA). Unique inter-repeat sequences were determined by cycle sequencing and used to design outward facing primers for inverse PCR (Figure 1, p1 and p2).

A template for inverse PCR was generated by digesting 1 µg of YAC-containing yeast genomic DNA with HinfI, Sau3AI, FokI and AvaII restriction enzymes separately. After purification on QIAquick spin columns (Qiagen), samples were diluted to 5 ng/mL concentration and ligated. The four ligations were then combined and 1 µL (5 ng) of the mixture was used for inverse PCR (35 cycles of 94°C for 40 s, 55°C for 40 s and 72°C for 2 min). Inverse PCR products were cloned and sequenced as described above, and the unique sequence flanking the MS was used to design a third primer for mapping and polymorphism analysis (Figure 1, p3). MS polymorphisms were determined between the BALB/cByJ and CAST/EiJ mouse strains by gel electrophoresis using 4% MetaPhor® agarose (FMC BioProducts, Rockland, ME, USA).

Amplification from a 600 kb YAC clone using MS and IRS-specific primer pools resulted in several amplified fragments (Figure 2). PCR in the absence of MS primers revealed undesired inter-IRS products, while amplification of a yeast control template facilitated the recognition of yeast-derived products. As summarized in Table 1, we cloned 20 PCR fragments that appeared specific for the presence of the

Table 1. Summary of the MS-IRS PCR Experiment on a YAC

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer-pool</th>
<th>Structure</th>
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</thead>
<tbody>
<tr>
<td>D12Lip1</td>
<td>GA2</td>
<td>(GA)₂₂(GGAAA)₂₀(GAAA)₁₅</td>
</tr>
<tr>
<td>D12Lip2</td>
<td>GA2</td>
<td>(GA)₃₀</td>
</tr>
<tr>
<td>D12Lip3</td>
<td>GA2</td>
<td>(GA)₁₉</td>
</tr>
<tr>
<td>D12Lip4</td>
<td>GA2</td>
<td>(GA)₃₀</td>
</tr>
<tr>
<td>D12Lip5</td>
<td>GA2</td>
<td>(GT)₁₅(GA)₂₈</td>
</tr>
<tr>
<td>D12Lip6</td>
<td>CA1</td>
<td>(CA)₂₄</td>
</tr>
<tr>
<td>D12Lip7</td>
<td>CA4</td>
<td>[(CA)ₙ(CT)ₘ]₅</td>
</tr>
<tr>
<td>D10Lip1</td>
<td>CA1</td>
<td>(CA)₁₈</td>
</tr>
</tbody>
</table>

₃3 clones did not contain sufficient length of unique sequence between repeats to design primers for inverse PCR; inverse PCR failed for 4 clones.

D10Lip1 was mapped to chromosome 10 indicating that the YAC is chimeric.

Figure 2. MS-IRS PCR products generated from a mouse YAC clone. The MS primer pools used are indicated at the bottom of the figure (i.e., CA1, CA2, etc.), corresponding to sequences given in the text. A control PCR with the IRS primer mix alone was applied to the gel in 3 positions to facilitate size comparisons. In addition to the YAC-containing template (Y), a yeast control template (C) was also amplified with all primer combinations. Representative bands specific for the presence of the MS primer and the YAC template (Y) are indicated with white dots.

MS primers and were absent from the yeast control. Sequence analysis of these clones indicated that 15 contained MS. Inverse PCRs corresponding to 8 of these clones provided the sequence of the entire MS and flanking region.

Among the MS repeats we identified, five were simple (GA)\text{n} or (CA)\text{n} tandem repeats, while three had a more complex repeat structure (Table 1). Using specific primers for each MS (Figure 1, p1 and p3), we mapped back all 8 MS to the original YAC (Table 1). This result confirms that all the isolated MS are derived from the YAC and not from yeast genomic DNA. Finally, we tested the MS repeats for polymorphism between the BALB/cByJ and CAST/EiJ mouse strains, and determined that all were polymorphic. Data relating to these polymorphisms have been submitted to the Mouse Genome Database (www.informatics.jax.org; D12Lip17, MGI Accession IDs 1342092–1342098). The identification of these new polymorphic MS repeats in a BALBxCAST cross segregating the \textit{fld} mutation was instrumental in reducing the critical genomic region and in the subsequent positional cloning of the underlying gene (5).

In summary, we developed a simple PCR-based procedure for the identification of novel MS from YACs and other large-insert clones [e.g., bacterial artificial chromosome (BAC) and P1 artificial chromosome (PAC)]. Although we have demonstrated the use of the technique by isolating several polymorphic markers from a mouse YAC clone, the procedure could be easily adapted to human DNA by substituting the B1/B2-specific primers with primers derived from the human \textit{Alu} repeat. A major advantage of the new method over existing ones is its species specificity, which eliminates the need to gel-purify YACs and enables analysis from total yeast DNA preparations.

REFERENCES


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Jack Phan, Karen Reue and Miklós Péterfy
University of California and West Los Angeles Healthcare Center
Los Angeles, CA, USA

Denaturing Gradient Gel Electrophoresis Resolves Virus Sequences Amplified with Degenerate Primers


Denaturing gradient gel electrophoresis (DGGE) is a versatile technique for studying genetic variation in populations. The technique is based on the separation (melting) of double-stranded (ds) DNA as a function of temperature and concentration of denaturant (i.e., urea and/or formamide) (9). As a result of the formation of molecules with complex structure, partial separation of strands inhibits the electrophoretic mobility of dsDNA. Resolution of dsDNA fragments of the same length that differ in sequence occurs by running the DNA in a polyacrylamide gel that has a continuously increasing concentration of denaturant. Because dsDNA molecules that differ in sequence partially melt at different concentrations of denaturant, rates of migration are inhibited at different positions in a denaturing gradient gel. Furthermore, the attachment of a G+C-rich sequence (i.e., GC clamp) to one end of the dsDNA fragments that are to be separated increases the sensitivity of DGGE. The G+C-rich region creates a high melting temperature domain that allows detection of single base pair substitutions in lower melting domains (11). Attachment of this GC clamp to DNA fragments is easily achieved by the incorporation of a GC-rich region into one of the primers used for PCR (10,11). This modification has become standard in most applications of DGGE.

Commonly, PCR with non-degenerate primers with a GC clamp is used to amplify short DNA fragments ranging from 150–400 bp in length (5,7,8,16). While use of a GC clamp greatly enhances the sensitivity of DGGE and allows the detection of single base pair changes, it may not always be advantageous or necessary. In this report, we demonstrate that DGGE can be used to resolve longer PCR products without the use of a GC clamp primer. In addi-