Catch-linker+PCR Labeling: A Simple Method to Generate Fluorescence In Situ Hybridization Probes from Yeast Artificial Chromosomes

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A simple and efficient method to generate hapten-labeled DNA fragments from a trace amount of YAC DNA isolated by PFGE is described. After agarase digestion of the gel slice containing the resolved YAC recombinant, the purified DNA is digested with *Sau3AI* and a compatible CL oligonucleotide duplex ligated on. A probe is generated by PCR amplification using a primer complementary to the CL with a single biotin moiety incorporated at the 5' end. When used as a FISH probe, this material yields mapping results superior to *Alu*-PCR or whole YAC labeling methods and allows sensitive detection of chimerism.

The large insert size of yeast artificial chromosomes (YACs) has made them the method of choice for cloning and mapping large genomic regions. With the increasing dependence on YACs, however, a number of problems associated with their use have come to light and must be addressed. Principal among these is the high level of chimerism present in several widely used YAC libraries.⁽¹⁻³⁾ Fluorescence in situ hybridization (FISH) is a convenient and powerful complementary technique to YAC cloning and has the potential to detect chimerism. A number of methods have been described for direct or indirect labeling of YAC recombinants for use in FISH analysis, but current methods are tedious, insensitive, and/or selective with respect to the fraction of the insert labeled.

Labeling total yeast DNA is simple but requires large amounts of DNA and is insensitive. Diverse PCR-based approaches, such as *Alu*-PCR⁽⁴⁾ and degenerate oligonucleotide-primed PCR (DOP-PCR),⁽⁵⁾ have been developed to facilitate the isolation of discrete internal YAC fragments to generate probes, but all involve tedious efforts to eliminate uneven amplification of the YAC insert and none is comprehensive. Difficulties in obtaining probes representing the entire insert of the YAC also limit the identification of potentially important information within the large clone and restrict the ability to detect chimerism.

Here, we present a simple and efficient method to generate hapten-labeled DNA probes that represent essentially

the entire YAC insert using a trace amount of YAC DNA isolated by pulsed field gel electrophoresis (PFGE). This technique generates DNA probes that can be directly used for FISH with high sensitivity and very low background.

MATERIALS AND METHODS

YAC DNA Preparation

YAC DNA was isolated in a gel slice by preparative PFGE in 1% low melting temperature agarose using minimal ethidium bromide and UV light exposure.⁽⁶⁾ An equal volume of 0.1 M NaCl was added, and the sample was melted by heating to 70°C for 15 min, then allowed to cool at room temperature for 30 sec. During this 70°C incubation, the sample was mixed vigorously and centrifuged briefly three or four times. One unit of β -agarase I (New England Biolabs) per 50 μ l sample volume was added and the reaction incubated at 37°C for 3 hr. YAC DNA was precipitated by the addition of one-tenth volume of 2 M sodium acetate (pH 5.5) and two volumes of ethanol. After chilling at -20°C for 1 hr, the DNA was recovered by 15 min of centrifugation. The DNA pellet was resuspended in 5 μ l of *Sau3AI* buffer and digested with 1 unit of *Sau3AI* enzyme at 37°C for 1 hr. The enzyme was inactivated by heating at 70°C for 15 min.

Catch-linker preparation

Oligonucleotides employed were A (5'-CCGAATTCTAGAGTCGACC-3') and B (5'-GATCGGTCGACTCTAGAATTCGG-

3'). Four micrograms of oligonucleotide B was 5'-phosphorylated by treatment with 25 units of T4 polynucleotide kinase (BRL) in 50 μ l at 37°C for 30 min. The oligonucleotide B was recovered by CHCl_3 /isoamyl alcohol extraction, ether extraction, and ethanol precipitation. Based on an estimated recovery efficiency of 25%, 1 μ g of oligonucleotide A was mixed with 1 μ g of the kinase-treated oligonucleotide B in 10 μ l of 10 mM Tris-HCl, 5 mM MgCl_2 (pH 7.4). The mixture was placed in a 200-ml water bath at 70°C and allowed to cool to room temperature over ~1 hr. This produced 200 ng/ μ l of AB duplexed catch-linker (CL) that can be frozen and stored indefinitely.

CL to YAC Ligation

On ice, ~20 ng of the processed YAC DNA (as judged by the initial pulsed field gel) was mixed with 100 ng of duplexed CL in a 10- μ l ligation reaction employing 2 units of T4 DNA ligase (Boehringer Mannheim). After incubation at 16°C overnight, the ligase was heat inactivated at 70°C for 15 min.

PCR amplification and labeling

Oligonucleotide bio-A was synthesized with a single biotin moiety incorporated at the 5' end of oligonucleotide A during synthesis. Using 600 ng of oligonucleotide A-bio as a primer, 1- μ l aliquots of the ligation reaction were amplified in PCR reactions of 50 μ l volume using 1 unit of *Taq* polymerase (Boehringer Mannheim) and an OmniGene thermal cycler (Hybaid). PCR conditions were as follows: For 35 cycles using tube temperature control, the denaturation steps were 93°C for 15 sec; the annealing steps were 54°C for 30 sec; and the extension steps were 72°C for 1 min (cycles 1–10), 2 min (cycles 11–20), and 4 min (cycles 21–35). Ten microliters of the PCR product was examined by electrophoresis on a 1.5% agarose gel. The remainder of the PCR reaction was ethanol precipitated and resuspended at a concentration of 20 ng/ μ l in TE.

Alternative preparation of FISH probes

Yeast DNA containing a human YAC recombinant was isolated by a standard

method.⁽⁷⁾ *Alu*-PCR was performed according to Breen et al.⁽⁴⁾ Both total yeast DNA and *Alu*-PCR products were biotin-labeled by nick translation.

FISH

Chromosomal in situ suppression hybridization was performed as described previously.⁽⁸⁾ Briefly, the chromosomal DNA was denatured by incubation of the slides in 0.1 M glycine buffer (pH 12.5), at room temperature for 3 min followed by dehydration in 70% ethanol and 99.5% ethanol at –20°C and then air-dried. The hybridization mixture containing biotin-labeled DNA probes (6 ng/ μ l for CL+PCR or *Alu*-PCR products, and 100 μ g/ μ l for total yeast DNA), 500 ng/ μ l of Cot-1 DNA (BRL), 1.5 $\times$ SSC, 10% dextran sulfate, and 50% formamide were denatured by heating at 78°C for 5 min, chilled on ice, and placed at 37°C for 15 min to allow annealing of repeated DNA sequences and thus suppress their hybridization to chromosomal homologs. Thirty microliters of the hybridization mixture was added to each prewarmed (37°C) slide under a 22 $\times$ 22-mm coverslip, which was sealed with rubber cement. The hybridization was carried out at 37°C overnight in a humid chamber.

Following hybridization, the slides were washed three times in 50% formamide/2 $\times$ SSC, once in 2 $\times$ SSC, and once in PN buffer [100 mM sodium phosphate buffer at pH 8.5, with 0.1% NP-40 (Pharmacia)] at 45°C for 5 min each, and then incubated in 5% nonfat dry milk in PN buffer (PNM) at 37°C for 10 min. Signal detection was performed by incubation in FITC-avidin DCS (Vector Laboratories) diluted to 1:100 in PNM at room temperature for 20 min. The signal was amplified with 5 μ g/ml of biotinylated anti-avidin antibody D (Vector Laboratories) followed by a second layer of 1:100 FITC-avidin DCS (Vector Laboratories) at room temperature for 20 min each. The successions of antibody incubations were separated by washing twice with PN buffer at room temperature for 2 min each. After the final wash, the slides were mounted by a fluorescent anti-fade solution, VectaShield (Vector Laboratories), containing 0.5 μ g/ml of propidium iodide. Chromosome preparations were examined under a Leitz microscope equipped with a Bio-Rad MRC-600 confocal laser scanning system.

Results and Discussion

ICI yac 36IF5, a human YAC recombinant with an insert size of ~600 kb, was identified by hybridization of gridded filters with a microdissection clone, MD220, which we had mapped previously to the long arm of chromosome 11.⁽⁹⁾ Figure 1 shows the comparisons of FISH results using probes for ICI yac 36IF5 labeled by three different meth-

FIGURE 1 Partial metaphases hybridized with ICI yac 36IF5. Probe DNAs are prepared by three different methods: (A) (CL) + PCR labeling of ICI yac 36IF5; (B) total yeast DNA of ICI yac 36IF5 labeled by nick translation; and (C) pooled *Alu*-PCR products for ICI yac 36IF5 followed by nick translation. Arrows indicate FITC signals of ICI yac 36IF5 on chromosome band 11q14.3-q21. This YAC is chimeric and gives minor signals on chromosome band 5p14 (arrowheads). Cross-hybridization of yeast rRNA genes onto acrocentric chromosomes was also seen by the total yeast method (double arrowheads).

ods: (A) CL plus PCR (CL+PCR); (B) total yeast DNA containing a YAC labeled by nick translation; and (C) *Alu*-PCR followed by nick translation.

Arrows indicate FITC signals of ICI yac 36IF5 on chromosome band 11q14.3-q21. This YAC is chimeric as demonstrated by the minor signal on chromosome band 5p14 (arrowheads). The FISH results from both CL+PCR, and the total yeast methods showed clean signals without background noise (Fig. 1A,B). However, YAC DNA consists of only about one-tenth to one-fiftieth of total yeast DNA; therefore, the total yeast method requires a large amount of yeast DNA to compensate for the amount of YAC DNA necessary for FISH. In addition, the vast majority of labeled DNA is the yeast genomic DNA, and this causes cross-hybridization of conserved sequences such as rRNA onto human chromosomes (Fig. 1B: double arrowheads).

All three labeling methods correctly localize ICI yac 36IF5 to 11q14.3-q21. The study, however, illustrated several points that limit the usefulness of total yeast or *Alu*-PCR and favor the CL+PCR method.

1. *Alu*-PCR labeling is accompanied by a high background noise that may obscure or falsely indicate chimerism (Fig. 1C).
2. Although both CL+PCR and total yeast labeling give low background noise, the amount of material required for total yeast labeling is large and the sensitivity is low. As little as 2 ng of YAC will generate by CL+PCR sufficient probe for 10 FISH analyses.
3. Labeling of total yeast DNA but not CL+PCR results in labeling of human homologs of yeast sequence, most notably rRNA genes (Fig. 1B, double arrowheads).
4. The success of *Alu*-PCR (or related methods) but not CL+PCR is limited to human recombinants and is heavily dependent on the local distribution of repeated DNA sequence motifs that serve to prime PCR.
5. Because all ends of restriction fragments are converted to priming sites for PCR in CL+PCR, but not other methods, essentially the complete YAC DNA is directly available without sequence bias for

FISH, comprehensive cloning, and further molecular analysis by other techniques.

Because the DNA fragment size range after CL+PCR technique (200–700 bp; data not shown) is optimal for FISH without further digestion with DNase I, hapten labeling of DNA fragments during the PCR reaction is preferred. The conventional way of labeling DNA during PCR is to substitute 1 of the 4 nucleotides in the reaction mixture with a labeled one such as bio-*n*-dUTP or DIG-11-dUTP. This requires a high concentration of labeled nucleotide, and is therefore costly. On the other hand, the labeling efficiency by CL+PCR using a biotinylated primer without additional labeled nucleotide in the PCR reaction is adequate both for the standard signal detection procedure for FISH (Fig. 1A) and for filter hybridization (data not shown). Our experiences with CL+PCR have shown the method to be extremely reliable and sensitive (12 YACs tested and all successful). Estimated from the results of contig construction, the size of chimeric fragment of ICI yac 36IF5 mapped on chromosome 5 is <50 kb. Furthermore, once the YAC clone is confirmed to be valuable for further analysis, the amplified DNA fragments by CL+PCR can be subcloned into suitable vector, using the internal *EcoRI* site on the linker adapter or the *Sau3AI* of the original ligation site.

We conclude that the CL+PCR labeling technique offers valuable advantages over current methods for high-resolution YAC mapping and the detection of chimerism by FISH analysis.

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