In Situ Hybridization Protocols

Contents

CHAPTER 8

FISH of *Alu*-PCR-Amplified YAC Clones and Applications in Tumor Cytogenetics

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1. Introduction

Yeast artificial chromosomes (YACs) *(1)* containing human inserts of up to 1 megabase (Mb) length have been mapped by fluorescence *in situ* hybridization (FISH) (for review *see* ref. *2*). If total yeast clone DNA is used as a probe, an excess of yeast DNA (approx 98%) is labeled in addition to the human sequences (approx 2%). This excess labeling not only leads to wastage of expensive labeling reagents, but contributes to background in *in situ* hybridization experiments. The hybridization efficiency is often less than satisfactory in metaphase spreads, whereas signals in interphase nuclei generally cannot be evaluated. Cutting out the YAC band from a pulsed-field gel and purification of the DNA is time-consuming and yields only rather limited amounts of a probe.

To overcome these problems an *Alu*-PCR approach *(3)* has been optimized for the selective generation of human DNA sequences from YAC-containing yeast strains with complexities sufficient for FISH experiments *(4)*. Two oligonucleotide primers, termed CL1 and CL2, have been established, which bind to the highly conserved 5' and the 3' ends of the approx 300-bp long, interspersed *Alu* repeat DNA block *(4,5)*. Provided that the distance between two adjacent *Alu* blocks does not exceed a few kilobases (kb), this primer pair allows the amplification of DNA sequences located between any two *Alu* blocks independent of their orientation.

Using this approach, we have tested more than 150 YAC clones containing some 100 kb up to 1 Mb of human inserts from various regions of the human genome, including both R- and G-bands. The large majority of these clones yielded specific, fluorescent signals both in metaphase spreads and interphase nuclei. Signal intensities are generally comparable with those observed using chromosome-specific repetitive DNA probes and can easily be detected using standard fluorescence microscopy. This approach facilitates the rapid mapping of YAC clones and is ideally suited for chromosome analysis at all stages of the cell cycle, e.g., in tumor cytogenetics *(6)* (*see* Notes 1 and 2).

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2. Materials

1. For *Alu*-PCR, YAC DNA may be prepared by three different methods:
   a. Purified high-molecular-weight genomic yeast clone DNA (100 ng). This DNA is prepared as described by Green and Olson *(7)*.
   b. Agarose plugs containing yeast cell DNA with the YAC of interest. For the growth of yeast strains and the preparation of chromosome-sized DNA in agarose for pulsed-field gel electrophoresis, the protocols published by Brownstein et al. and Burke et al. are used *(1,8)*. Low-melting-point agarose (ultrapure) purchased from Gibco-BRL/Life Technologies (Gaithersburg, MD) is recommended for plug preparation. Note that agarose plugs are stored routinely in EDTA (0.5*M*) at 4°C. However, since the presence of EDTA inhibits *Taq* polymerase and affects the Mg^{2+} concentrations, which are critical for primer specificity, EDTA should be carefully avoided in PCR assays. Plugs are therefore washed two times for 30 min each in 1*M* Tris-HCl, pH 8.0, and equilibrated in PCR-buffer (10 m*M* Tris-HCl, pH 8.4, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.001% gelatin) for 30 min at room temperature. Plugs are further equilibrated overnight in fresh PCR-buffer at 4°C. After melting of the plug for 5 min at 75°C, approx 150 ng of genomic yeast cell DNA is added to the *Alu*-PCR assay. Melted plug material can be stored at 4°C and reused for several months.
   c. For rapid testing, a very small amount of yeast cells (barely visible on a sterile pipet tip or a toothpick) can be picked directly from clones grown on plates or filters. The cells are diluted in 1.5 mL of distilled water, and aliquots of up to 20 µL are added to the PCR assay without any further treatment. This approach, however, provides less reproducible amplification yields than methods a and b.
2. Disposable gloves.
3. Set of microliter pipets (e.g., Gilson).
4. 1.5-mL Eppendorf tubes with screw caps.
5. 10X PCR-buffer: 100 m*M* Tris-HCl, pH 8.4, 500 m*M* KCl, 15 m*M* $MgCl_2$, 0.01% gelatin. The buffer is stable for at least 3 mo at –20°C.
6. dNTP-mix. Stock solution containing all four dNTPs at a concentration of 25 m*M* is prepared from nucleotides (100-m*M* solutions) purchased from Boehringer Mannheim (Mannheim, Germany) and stored at –20°C for several weeks.
7. *Thermus aquaticus* DNA polymerase (Native *Taq*; Perkin-Elmer/Cetus, Norwalk, CT).
8. Oligonucleotide primers. CL1 (5' TCC CAA AGT GCT GGG ATT ACA G 3') and CL2 (5' CTG CAC TCC AGC CTG GG 3') primers are dissolved in a vol sufficient to yield a 25-µ*M* solution, and stored at –20°C.
9. Paraffin oil (light mineral oil).
10. Thermocycler 60 (BioMed, Theres, Germany).
11. Seakem ME agarose (FMC Bioproducts, Rockland, ME).
12. 50X TAE: 2*M* Tris-acetate, pH 8.0, 0.05*M* EDTA.
13. 1% Ethidium bromide.
14. DNA gel electrophoresis apparatus.
15. 0.5*M* $MgCl_2$, 5*M* NaCl, and 99% ethanol for precipitation of DNA.

16. 20 mg/mL RNase A. Boil for 10 min and store at –20°C.
17. 10% Pepsin. Store at –20°C.
18. 1% Formaldehyde solution: Mix 2.7 mL of 37% acid-free, buffered formaldehyde (Merck, Rahway, NJ) with 97.3 mL of PBS containing 50 m*M* $MgCl_2$.
19. Phospate buffered saline (PBS): 8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 1 g $Na_2HPO_4 \cdot 2H_2O$, 0.15 g $NaH_2PO_4 \cdot H_2O$. Add distilled water to 1 L and adjust pH to 7.4 with HCl.
20. 20X SSC: 3*M* NaCl, 0.3*M* sodium citrate, pH 7.0.
21. Ethanol series: 70, 90, and 99%.
22. Cot-1 DNA (BRL/Life Technologies) with a size range of 200–500 bp.
23. Salmon sperm DNA. This should be sheared or DNase digested to an average size of approx 500 bp (Sigma, St. Louis, MO).
24. Hybridization buffer: 4X SSC, 20% dextran sulfate.
25. 0.2 mg/mL DAPI (4,6-diamidino-2-phenylindole-dihydrochloride).
26. 1 mg/mL Propidium iodide.
27. Antifade buffer. Mix 10 mL of *p*-phenylendiamine solution (100 mg *p*-phenylendiamine-dihydrochloride in 10 mL of PBS), pH 8.0, and 90 mL of glycerine. Store at –20°C.

3. Methods

In the following protocol, we have put a greater emphasis on the generation of *Alu*-PCR probes and refer the readers to other chapters in this volume (e.g., Chapters 4, 7, and 10) for a detailed description of the *in situ* hybridization procedure.

3.1. Performing the Polymerase Chain Reaction (PCR)

1. For each PCR, prepare the following reaction mix in a 1.5-mL Eppendorf tube:
   a. 10 µL of 10X PCR-buffer;
   b. 1 µL of dNTP-mix;
   c. 1 µL of *Alu*-primer CL1;
   d. 1 µL of *Alu*-primer CL2;
   e. Double distilled water to a final vol of 100 µL, taking into account the calculated vol for 150 ng of genomic yeast clone DNA;
   f. 150 ng of genomic yeast clone DNA; and
   g. 5 U of *Taq* DNA polymerase. Mix gently.
2. Overlay the reaction mix with 50 µL of paraffin oil. Note that for most thermocyclers an overlay of paraffin oil is essential. In the case of the Thermocyler 60 (BioMed), paraffin oil can be omitted.
3. Using the BioMed machine, perform the following cycles:
   a. 97°C for 3 min;
   b. 97°C for 1 min;
   c. 37°C for 30 s;
   d. 72°C for 6 min;
   e. Repeat steps b–d for a total of 30 cycles; and
   f. 72°C for 4 min.
4. Store amplified DNA until further use at 4°C (up to 1 mo) or at –20°C for more extended periods.

3.2. Analyzing the Reactions

1. Prepare a 100-mL agarose gel (1.2%). Bring the solution to a boil in a microwave oven until all agarose has dissolved. Cool the solution to 55°C, add ethidium bromide (final concentration of 0.5 µg/mL), and pour onto a gel tray.
2. Remove the overlay of oil from the reaction mix. This is done by adding 50 µL of chloroform, mixing and centrifuging for 10 s. The oil dissolves in the $CHCl_3$ (bottom layer). Remove the reaction mix (top layer) with a microliter pipet. Alternatively, use a microliter pipet to directly take up the reaction mix below the oil layer. Remove oil adhering to the pipet tip with a Kimwipe tissue paper. Note that any remaining oil will interfere with the migration of the DNA through the gel.
3. Load a 10-µL aliquot from the PCR mixture on the gel and electrophorese at 120 V until sufficient separation of bands between 50 bp and 5 kb is achieved.
4. Watch the gel on a UV transilluminator.

An appropriate gel shows a series of distinct bands. Size and intensity of the individual bands are highly reproducible for each YAC clone. Beside distinct bands, a smear of amplification products ranging up to 8 kb can be detected. Overlapping YAC clones should show bands with apparently identical positions and intensities, while other bands are distinctly different (*see* Note 3).

Figure 1 shows an example of a typical agarose gel with *Alu*-PCR-amplified sequences of eight YAC clones containing human inserts between 150 and 790 kb that belong to a YAC contig previously established from the cystic fibrosis gene at band 7q31 *(9)*.

3.3. Probe Labeling

1. Ethanol precipitate PCR products by adding 1/25 vol 5*M* NaCl, 1/50 vol 0.5*M* $MgCl_2$, and 2.5 vol 99% ethanol. Leave at –80°C for 30 min.
2. Centrifuge in an Eppendorf centrifuge for 20 min.
3. Resuspend the pellet in double distilled water. The DNA can now be used for nick translation.
4. Label the DNA probe with biotin-11-dUTP or digoxigenin-11-dUTP by nick translation as described by Lichter and Cremer *(10)*. Alternatively, for labeling with biotin-11-dUTP, use the nick translation kit of Gibco-BRL/Life Technologies (Gaithersburg, MD) following the instructions of the supplier (*see* Note 4).
5. Separate labeled probe from unincorporated nucleotides by standard gel filtration method such as using a spin column.

3.4. Pretreatment of Chromosome Preparations

The quality and pretreatment of chromosome preparations is particularly important for FISH of *Alu*-PCR-amplified YAC clone sequences. We follow a protocol established by J. Wiegant (University of Leiden, The Netherlands) *(11)*, which involves pretreating chromosome preparations with RNase A (100 µg/mL 2X SSC; 37°C for 1 h) and pepsin (50 µg/mL 0.01*M* HCl; 37°C for 10 min), and postfixing in 1% formaldehyde at room temperature for 10 min and dehydrating in an ethanol series of 70, 90, and 99% for 3 min each.

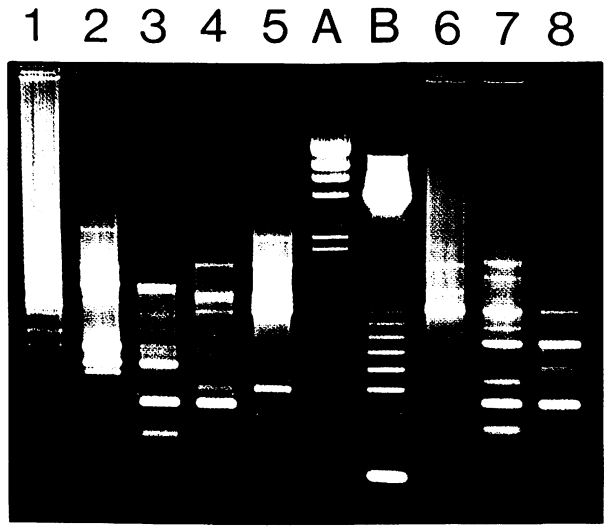
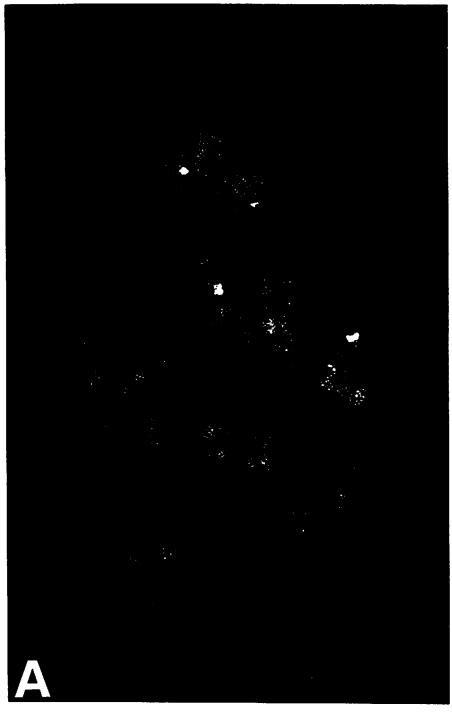


Fig. 1. Agarose gel (1.2%) of *Alu*-PCR-amplified YAC clones of the CF contig *(9)*. Lane 1: *Alu*-PCR amplification of a melted agarose plug containing the YAC yCF-1/7/5–R (790 kb). Lanes 2–8: *Alu*-PCR-amplified YAC clone DNA of yKM19-3 (150 kb), yCF-10 (240 kb), yCF-7 (240 kb), yCF-5 (280 kb), yW30–5 (260 kb), yJ311-3 (340 kb), and yJ311-1 (350 kb), respectively. Marker lane A: λ/*Hind* III; marker lane B: 123-bp ladder. Overlapping YAC clones show bands with apparently identical position and intensity, while other bands are distinctly different. Reproduced by permission from *Genomics* **13,** 827.

3.5. Hybridization and Probe Detection

Chromosomal *in situ* suppression (CISS-) hybridization *(12–14)* is carried out as described in detail by Lichter and Cremer *(10)*, with the following modifications:

1. For each slide (18 × 18 mm hybridization area), combine:
   a. 100–150 ng of labeled *Alu*-PCR-amplified DNA-probe;
   b. Between 5 and 50 µg of unlabeled Cot-1 DNA (*see* Note 5); and
   c. 10 µg salmon sperm DNA.
2. Add 1/20th vol of 3*M* sodium acetate and 2 vol of ethanol. Mix well and leave at –80°C for 30 min.
3. Centrifuge in an Eppendorf centrifuge for 30 min at 4°C.
4. Discard the supernatant and wash the pellet by adding 400 µL of 70% ethanol.
5. Spin for 5 min.
6. Discard the supernatant and lyophilize in a SpeedVac.
7. Resuspend the dried pellet in 5 µL of deionized formamide and 5 µL of hybridization buffer. Vortex vigorously for several minutes.
8. Refer to other chapters in this volume (e.g., Chapters 4, 7, and 10) for denaturation and preannealing of the probe, denaturation of the chromosome preparations, hybridization, washing, and detection procedures.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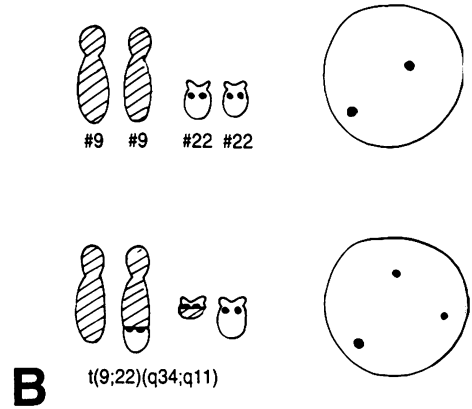
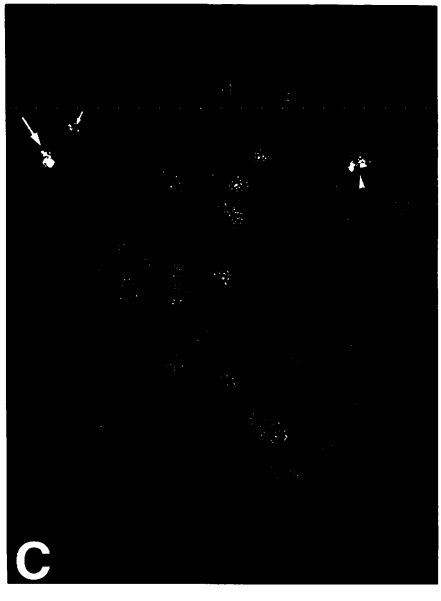

BCR-YAC clone
#9 #9 #22 #22
B
t(9;22)(q34;q11)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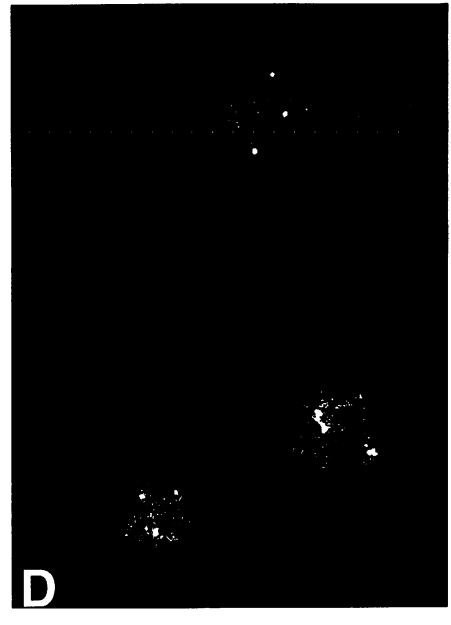
D

9. After CISS-hybridization and probe detection, counterstain chromosome preparations with 0.2 µg/mL DAPI and/or 1 µg/mL propidium iodide (depending on the fluorochromes used for detection) and mount in fluorescence antifade buffer. Photomicrographs are taken with a Zeiss (Oberkochen, Germany) photomicroscope III equipped for epifluorescence, or a Zeiss Axiophot. Agfachrome 1000 RS color slide films are used. A typical result is shown in Fig. 2A.

4. Notes

1. Applications: *Alu*-PCR-generated probes from YAC clones containing inserts from unique sites of the human genome provide ideal tools for the analysis of specific numerical and structural chromosome aberrations. They can be used with high reliability in patients with various hematologic diseases at all stages of the cell cycle. For example, YAC clones have been applied to visualize specific structural chromosome aberrations in peripheral blood cells from patients with myelodysplasia exhibiting the deletion del(5)(q13q34), and acute promyelocytic leukemia (ANLL M3) with the translocation t(15;17)(q22;q21) *(6)*. Figure 2C,D shows a metaphase spread (C) and interphase nuclei (D) from a patient with chronic myeloid leukemia (CML) exhibiting the translocation t(9;22)(q34;q11) after FISH with the biotinylated *Alu*-PCR-amplified sequences of YAC clone D107F9. This clone spans the breakpoint cluster region (BCR) on chromosome 22.
2. Large YACs and YAC contigs of increasing size *(15)*, including YACs spanning and flanking tumor-specific breakpoints *(6,16–19)*, are rapidly becoming available for each band of the human chromosome complement. While many YAC clones show signals restricted to one specific chromosome band, others show signals on different chromosome subregions, indicating that these subregions contain sequence homologies or that a given YAC clone contains several YACs or (more likely) a chimeric YAC.
3. The yield of *Alu*-PCR amplification products that can be expected for a given YAC depends on the sequence, number, and distance of *Alu* elements in the human insert. The following procedure can be applied for YACs from *Alu*-poor

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Fig. 2 *(opposite page)*. (**A**) Normal human lymphocyte metaphase spread and adjacent interphase nucleus after fluorescence *in situ* hybridization of biotinylated *Alu*-PCR products of YAC clone A168H4. This 550-kb YAC contains the protooncogene c-raf-1. Probe detection is performed with avidin-FITC. Chromosomes are counterstained with propidium iodide. Specific signals are seen on 3p25. Two specific signals are also detected in the adjacent interphase nucleus. One hundred consecutively evaluated metaphase spreads showed the expected signals on both homologs and both chromatids. In addition, 96% of 400 evaluated interphase nuclei showed two clearly separated signals. (**B**) Scheme showing the expected outcome of a FISH experiment using YAC clone D107F9 to chromosomes and interphase nuclei of a normal patient (upper row) and of a patient with chronic myeloid leukemia (CML) exhibiting the translocation t(9;22) (q34;q11) (lower row). This 215-kb YAC spans the breakpoint cluster region (BCR) on chromosome 22. (**C**) Metaphase spread of a patient with CML exhibiting the translocation t(9;22)(q34;q11) after FISH of biotinylated *Alu*-PCR products of YAC clone D107F9. Probe detection is performed with avidin-FITC. Chromosomes are counterstained with propidium iodide. Signals can be seen on both chromatids of the normal chromosome 22 (large arrow), the Philadelphia chromosome (small arrow), and the derivative chromosome 9 (9pter→9q34::22q11→22qter) (arrowhead). (**D**) Three interphase nuclei of the same patient.

regions that give very little *Alu*-PCR products and accordingly yield only weak fluorescent signals after FISH. The YAC is first separated from the yeast chromosomes by pulsed-field gel electrophoresis (PFGE) in a low-melting-point agarose gel. After cutting out the respective band, universal DNA amplification protocols are applied to overcome the problems of the limited YAC DNA amounts present in the PFGE band. For this purpose, an aliquot (5–50 ng) of the melted agarose piece containing the YAC is applied to a PCR assay using the degenerate oligonucleotide primer MW-6 (DOP-PCR) *(20)* (*see* Chapter 3). This approach provides amplified YAC DNA probes that yield strong, specific signals on human metaphase spreads and in interphase nuclei *(27)*.

4. The size of labeled probe should ideally be smaller than 500 bp and larger than 100 bp. A predominant probe size at 250–300 bp seems optimal in our hands for efficient FISH with low background. Biotin-11-dUTP or digoxigenin-11-dUTP can also be incorporated directly during the PCR assay. However, many amplification products are larger than required for optimal FISH and should be posttreated with appropriate concentrations of DNase I.
5. For each YAC clone, the optimal amount of unlabeled Cot-1 DNA should be determined empirically. Use of excessive amounts of Cot-1 DNA do not impair the results of CISS-hybridization, but can create a financial burden. When simultaneously hybridizing *Alu*-PCR products of multiple YAC clones in one experiment (*see* Note 6), the amount of Cot-1 DNA does not have to be increased proportionally but can remain almost the same.
6. *Alu*-PCR-amplified products from a series of YACs can be combined as a multiplex probe for FISH to yield reproducible signals on a number of chromosome bands simultaneously. Using various pools of *Alu*-PCR-amplified YACs, new types of colored chromosome banding patterns, called chromosomal bar codes, can be obtained on human chromosomes *(22)*. Owing to their high hybridization efficiency, such multiplex probes can be implemented for diagnostic applications as a complementary tool to conventional banding. Chromosomal bar codes can be tailored to specific diagnostic goals in clinical and tumor cytogenetics and can help to improve automated chromosome analysis.

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