Improved Method for the Construction of Cosmid Sublibraries from Yeast Artificial Chromosomes

BioTechniques 22:264-268 (February 1997)

We describe here a simple and rapid procedure for the construction of cosmid sublibraries from human chromosomal fragments cloned in yeast artificial chromosomes (YACs). Subcloning of human DNA fragments in cosmids is increasingly important for the analysis of the human genome. The ultimate goal of many genome projects is the identification of disease-associated genes. Various techniques have been described for the identification of genes from chromosomal or chromosomal regions, including the use of CG-islands, hybridization selection and exon trapping (2,4,5). The availability of DNA fragments cloned in cosmid vectors is a necessary prerequisite for the efficient application of several gene identification strategies, most notably of exon trapping, which was crucial for the identification of numerous disease genes (e.g., neurofibromatosis I) (7). Since YACs represent frequently the starting material in positional cloning projects, the subcloning into cosmids is frequently the bottleneck of these experiments. Although the subcloning is theoretically an easily performed experiment, in practice, this step is time-consuming and frequently inefficient.

Our new method differs from previously reported approaches in that a modified preparative pulsed field electrophoresis (PFGE) gel is used for high resolution separation of YAC-DNA, and partial digestion of YAC-DNA takes place directly in the excised and melted agarose slice. The procedure minimizes contamination of YAC-DNA with yeast genomic DNA and reduces shearing forces. This results in the generation of optimal length fragments for efficient cosmid cloning (Figure 2A). The new method yields hundreds of human-specific cosmid clones after a single plating and hybridization step (Figure 2B). Comparable approaches are less efficient and more time-consuming (1,6).

To demonstrate the usefulness of our method, we analyzed the YAC YmetH1 (852D1), which retains a human insert of 870 kb. The YAC was isolated from the Centre d’Etude du Polymorphisme Humain (CEPH) Mega YAC library as previously described (3). A schematic presentation of the procedure is shown in Figure 1.

To prepare yeast DNA in agarose plugs, yeast cells containing the YACs were embedded in 1% low-melting-point (LMP) agarose (SeaPlaque® GTG; FMC BioProducts, Rockland, ME, USA) at a concentration of 3 × 10⁸ cells/plug (plug volume: 90 μl). PFGE was used to isolate the YAC. Before the isolation, it is, however, necessary to determine the exact position of the YAC DNA using analytical PFGE.

Analytical PFGE I was performed at 200 V with switching times ranging from 50–90 s for 22–24 h in 1% Rapid Agarose™ (Life Technologies, Gaithersburg, MD, USA) using the CHEF-DR®II gel apparatus (Bio-Rad, Hercules, CA, USA).

Subsequently, the YAC was recovered using preparative “slot” PFGE, which was modified as follows: Since preparative PFGE with LMP agarose gives a very poor resolution, PFGE was run with standard PFGE agarose (Rapid Agarose). To isolate the YAC-DNA, a preparative slot of 5-mm width was left in the gel at the predetermined position of the YAC DNA and filled with 1% LMP agarose. A preparative comb formed the slot while pouring the gel. The plugs containing the yeast cells were cut into thirds of which 7 were loaded into a single long slot at the start position of the pulse field gel (PFGE). The modified preparative PFGE was run under the same condition as the analytical PFGE. Following electrophoresis, a portion of the gel was stained with ethidium bromide to verify the position of the YAC DNA in the LMP agarose slot. LMP agarose slices were excised from the unstained portion of the gel with a spatula and stored in 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6) at 4°C. An agarose slice of 100 μl contains approximately 50–100 ng YAC DNA according to the size of the YAC.

Partial digestion of the YAC DNA was performed to obtain DNA fragments of suitable size for subcloning.
fragments between 20–50 kb. Following equilibration of the gel slice for 1 h in 10 mL ddH₂O and for 2 h in 10 mL of restriction buffer (4°C), the agarose plugs (100 µL) were melted in 1.5-mL reaction tubes for 15 min at 70°C. To control the size of the undigested DNA, 10 µL of the melted agarose are removed with a cut-off pipet tip and saved for PFGE. After cooling to 37°C, partial restriction was performed using the enzyme MboI (MBI Fermentas, St. Leon-Rot, Germany). In detail, melted agarose slices (90 µL each) were restricted with a different concentration of the restriction enzyme (e.g., 0.2, 0.15, 0.1 U/slice). The restriction enzyme was dissolved in 10 µL reaction buffer, gently mixed with the melted agarose (not vortex mixed) and incubated for 1 h at 37°C. Inactivation of the enzyme was accomplished in 20 min at 70°C. The melted agarose was removed using GELase (Epicentre Technologies, Madison, WI, USA). The DNA/agarose solution was cooled to 4°C for 10 min, digested with 2 U of gelase for 1 h at 45°C and finally cooled to room temperature. The correct size of genomic fragments was critical for subcloning. To control the partial restriction digest, an aliquot of 10 µL was removed for PFGE.

For analytical PFGE II, the aliquots of the melted agarose slices, which were saved before and after the restriction digest, are size-fractionated by PFGE using the same parameters as for the preparative PFGE except for a runtime of 16 h. Entire λDNA (49 kb) and λDNA digested by HindIII (23 kb) were used as markers at a concentration of 100 ng per lane. Ethidium bromide staining of the gel permitted an approximate size estimation of the DNA fragments. To allow more accurate size determination of the digested human fragments, the DNA was transferred onto a membrane and hybridized with a mixture of 32P-labeled Cot-1 DNA (100 ng; Life Technologies) and 32P-labeled λDNA (0.5 ng). A representative autoradiograph of the human fragments on the analytical PFGE is shown in Figure 2A.

To dephosphorylate pooled DNA, DNA digests, which retained human fragments between 20–50 kb (as shown in Figure 2A, lanes 7 and 9) were pooled and further processed. Using cut-off pipet tips, DNA was extracted by phenol and chloroform and precipitated with 1 vol 5 M NH₄ acetate and 4 vol ethanol. The precipitated DNA was dried under a vacuum and resuspended in 10 µL TE. Before cloning, the DNA was treated by alkaline phosphatase. In detail, partially digested YAC DNA (100–400 ng) was incubated with 2 U of shrimp alkaline phosphatase (Amer-sham, Arlington Heights, IL, USA) for 1 h at 37°C in a total volume of 100 µL. The enzyme was inactivated by incubation for 20 min at 70°C. The DNA was phenol/chloroform extracted, ethanol-precipitated and resuspended in 10 µL TE.

For cloning purposes, the DNA was ligated into the double Cos site SuperCoI vector (Stratagene, La Jolla, CA, USA), using the rapid ligation kit of

**Figure 1.** Flow chart of the YAC subcloning procedure. Modifications are bold.
Figure 2. Distribution of human genomic fragments after partial restriction digest of YAC DNA and identification of primary human-specific cosmids clones. (A) The figure shows the autoradiograph of a Southern blotted analytical PFGE gel. PFGE parameters were as described in the text, and the human DNA was detected with 32P-labeled human repetitive DNA (Cot-1). The marker DNA in lane 1 (100 ng of 23-kb λ fragment) and lane 2 (100 ng of λDNA) were detected using a minimal amount of labeled λDNA (0.5 ng) as a probe. Lane 3 contained genomic DNA of the untreated yeast recombinant YmetH1 (fourth of a plug) to demonstrate size and position of the intact YAC (870 kb). Lanes 4, 6, 8 and 10 contained YAC DNA of separate preparatively isolated agarose plugs without restriction digest (+). Lanes 5, 7, 9 and 11 contained the same DNA after digestion with different concentrations of MboI (0.25, 0.125 and 0.0625 U, respectively) for 1 h (+). Digested DNA in lanes 7 and 9 showed accurate size distribution of the preparative gel and minimization of the shearing procedure. (B) The primary colonies of the complete library were replica plated onto a Duralose-UV membrane. The membrane was processed for storage and DNA isolation.

Boehringer Mannheim (Mannheim, Germany). The limited amount of genomic DNA fragments required “small volume” ligation. The DNA concentration in the reaction mixture should be at least 200 ng/µL to favor concatemere formation. In brief, the dephosphorylated YAC DNA (for optimal results use 200–300 ng in 10 µL TE), and an equal amount of prepared SuperCos1 vector was mixed and vacuum dried in a small reaction tube (0.2 mL). DNA was resuspended directly with the components of the ligation mixture without ligase in a final volume of 2.5 µL. T4 DNA ligase (1 U) was added, gently mixed and incubated for 15 min at room temperature. The complete ligation reaction was used for packaging.

Packaging, infecting and plating on single membranes were performed. Packaging was done according to the manufacturer’s instructions (Gigapack® II XL-Kit; Stratagene). Transfected host cells were plated onto a single 140-mm Duralose-UV™ membrane (Stratagene), which was placed on the surface of an LB-kanamycin (100 µg/mL) plate. Plating resulted in 850 single primary colonies.

For replica plating and isolation of human specific clones, the primary colony pattern of the master filter was transferred to a fresh membrane as described in the Stratagene protocol (6). The membrane was prepared for hybridization and probed against human repetitive sequences (Cot-1 DNA) to identify human specific clones. Figure 2B shows a representative autoradiograph of the colony filter with 250 human-specific cosmids clones identified. Single positive clones could be directly isolated from the master plate without further plating steps and processed for storage and DNA isolation.

Because of the improved resolution of the preparative gel and minimization of DNA shearing, the described procedures will prove useful for the rapid and efficient subcloning of large genomic fragments from YACs into cosmid vectors.

REFERENCES


This work has been supported by a grant from the Deutsche Forschungsgemeinschaft (Me 917/2-2). Address correspondence to Eckart U. Meese, University of the Saar Medical School, Department of Human Genetics, 66421 Homburg/Saar, Germany.

Received 6 May 1996; accepted 9 July 1996.

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