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Protocols

Isolation of Bacterial Artificial Chromosome DNA by Means of Improved Alkaline Lysis and Double Potassium Acetate Precipitation

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Abstract. This work describes an easy alkaline lysis method for isolatating bacterial artificial chromosome (BAC) DNA in sufficient quantity and quality for further manipulation without the need to use a kit. The method starts with 10 mL of culture and, by alkaline lysis only, renders up to 150 ng of DNA per milliliter of culture. This BAC DNA was successfully digested with restriction enzymes, sequenced, and subjected to PCR.

Key words: BAC, DNA isolation, DNA precipitation, FIGE, sodium acetate

Abbreviations: BAC, bacterial artificial chromosome; Fd-GOGAT, ferredoxin dependent glutamate synthase (EC 1.4.7.1); FIGE, field inversion gel electrophoresis.

Introduction

The bacterial artificial chromosome (BAC) vector can accommodate DNA inserts ranging from 30-300 kb; 130 kb is the average. The resulting clones are stable and rarely chimeric, which makes this vector suitable for constructing large-insert DNA libraries for further sequence assembling in contigs in order to construct a physical map of a large genome or genome region (Zhang and Wu, 2001; She, 2003). In consequence, many large-insert DNA libraries have been developed for a large number of plant species (Lijavetzky et al., 1999; Vanhouten and MacKenzie, 1999; Song et al., 2000; Luo et al., 2001; Georgi et al., 2002; Vilarinhos et al., 2003), and the complete nucleotide sequence of several BAC vectors is known (Wang et al., 1997). An additional advantage of BACs is that they are proven templates for direct end-sequencing (Kelley et al., 1999).

Analysis of large-insert bacterial clones is essential for characterization and utilization of large-insert DNA libraries. They can be isolated by use of a standard alkaline lysis protocol (Sambrook and Russell, 2001) or by alkaline lysis followed by further purification with a commercial kit (Patocchi et al., 1999; Luo et al., 2001) or with cesium chloride gradient centrifugation (Lijavetzky et al., 1999). Because plasmids larger than 15 kb are difficult to isolate by these methods

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because they are susceptible to physical damage (Thomas et al., 1988), BAC clones of a library containing large inserts would provide low yields by use of such protocols. New methods adapted to BAC DNA have been described (Sinnett et al., 1998; Zhang, 2000). The present report describes a method that improves the DNA yield and quality starting with low culture volumes and without the use of any kits.

Materials and Methods

Isolation of BAC DNA

1. Centrifuge a BAC culture of 10 mL of Luria-Bertani medium (10 g bacto-tryptone, 5 g bacto-yeast extract, and 10 g NaCl) and 12.5 µg/mL chloramphenicol at 19,800*g* at 4°C for 5 min.
2. Suspend the pellet in 400 µL of 10 mM EDTA (pH 8) by use of a vortex.
3. Incubate the solution on ice for 5 min.
4. Add 800 µL of a freshly prepared solution of 0.2 N NaOH and 1% *N*-laurylsarcosine SLS and suspend the cells very gently by inversion until the lysis is visible.
5. Incubate on ice for 4 min.
6. Add 600 µL of 2 M potassium acetate and mix carefully by inversion until a white precipitate appears.
7. Place on ice for at least 5 min and centrifuge at 13,800*g* for 15 min at 4°C.
8. Transfer the supernatant to a new tube and add an equal volume of 2-propanol; mix gently by swirling.
9. Centrifuge at 3440*g* for 15 min, decant the supernatant, and drain the pellet.
10. Resuspend the DNA pellet quickly and gently in 180 µL of 10:50 TE (10 mM Tris HCl [pH 7.6], 50 mM EDTA).
11. Add 90 µL of 7.5 M potassium acetate and keep the solution at –80°C for 30 min.
12. Thaw the tube and centrifuge it at 3220*g* for 10 min at 4°C.
13. Draw off the supernatant and discard the pellet. Add 2.5 vol of 95% ethanol to the supernatant and precitpite it at –20°C for 30 min.
14. Centrifuge at 15,000*g* for 5 min at 4°C and suspend the pellet in 100 mL of 50:50 TE (50 mM Tris HCl [pH 7.6], 50 mM EDTA).
15. Add 2 µL of 10 mg/mL DNase-free RNase A and incubate the solution at 37°C with shaking at 200 rpm for 30 min.
16. Add 1 vol of phenol-chloroform (1:1) and mix gently.
17. Centrifuge for 5 min at 3200*g* at room temperature.
18. Transfer the aqueous phase to a new tube and then add 1 vol of chloroform. Mix by pipetting up and down with a pipette with cut tips.
19. Centrifuge the mixture for 5 min at 3200*g* at room temperature and draw off the supernatant.
20. Precipitate DNA with 1 vol of 2-propanol and centrifuge it for 5 min at 3200*g* at room temperature.
21. Wash the DNA pellet with 500 µL of 70% ethanol and dry it for 5-10 min.

22. Suspend the pellet in 20 μL of TE by gently pipetting up and down with a pipette with cut tips and place at 4°C overnight to ensure complete dissolution of DNA.

DNA electrophoresis

Electrophoresis of DNA molecules under 15 kb (which includes the BAC vector) was carried out with conventional methods in 0.8% agarose gels (Sambrook and Russell, 2001). BAC clones were electrophoresed in 0.8% agarose gels under field inversion to separate high-molecular-weight bands. A FIGEMapper (BioRad) was used under the following conditions: migration in 0.33× Tris-borate-EDTA at 4 V/cm and 11°C for 16 h, with an initial pulse time of 0.1 s and a final pulse time of 3.5 s. The gels were stained with ethidium bromide and photographed.

Further DNA manipulations

Restriction enzyme reactions were performed as described by Sambrook and Russell (2001), using 2 μL of BAC DNA (~500 ng) and 3 U of the appropriate restriction enzyme.

DNA of BAC clones was sequenced by means of the conventional dideoxynucleotide method (Sambrook and Russell, 2001) adapted to separation in an Applied Biosystems ABI PRISM 310 Genetic Analizer. The reactions were primed with standard T7 (5′-TAATACGACTCACTATAGGGCGA-3′) and SP6 (5′-GATTACGCCAAGCTATTTAGGTGACACTATAGAATA-3′) sequencing primers.

Polymerase chain reactions (PCRs) were done in a Thermal Cycler (Perkin-Elmer) for 30 cycles at 91°C for 60 s, 60°C for 60 s, and 72°C for 90 s with the primers GOGAT1 (5′-TGATATCACCTCCTCCACATC-3′) and GOGAT2 (5′-TGCTCCAGTGCCCCCATCATG-3′) to amplify a ferredoxin-dependent glutamate synthase (Fd-GOGAT) sequence of 200 bp of the pine genome.

Results

We have compared the yields of our protocol with those of others and found that the protocol described by Zhang (2000) did not provide a sufficient amount of DNA. The method exclusively based on an alkaline lysis (Sinnett et al., 1998) with resuspension of DNA in 20 μL, instead of the 50 μL prescribed in the original technique, permitted a yield of approximately 10 ng of DNA per milliliter of culture, which was too low for current use (Figure 1A). However, when BAC DNA from 6 distinct clones was isolated by using the present method and 2 μL of each preparation was electrophoresed (Figure 1B), we obtained a higher yield (up to 150 ng/mL of culture) without any visible bacterial genomic DNA. With use of commercial kits, the genomic DNA was too predominant (data not shown).

The BAC DNA obtained with the protocol described herein can be easily cut by 3 U of restriction enzymes such as *Pst*I (Figure 2A) and *Not*I (Figure 2B), indicating that the protocol is suitable for further analysis of the DNA. *Not*I digestion was used to determine the average size of inserted DNA into the BAC vector, which was 107 kb (Bautista et al., unpublished data).

We have also tested whether this DNA can be used as template for PCR. We used the primers GOGAT1 and GOGAT2 (Bautista et al., unpublished data) to

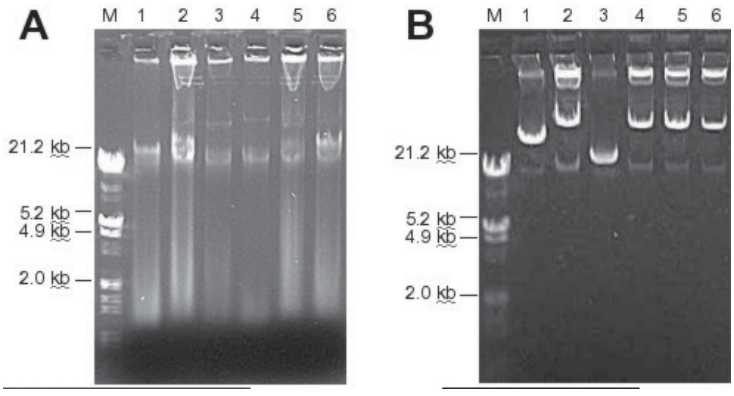

Figure 1. Results of electrophoresis of minipreparations of bacterial artificial chromosome (BAC) DNA obtained by different protocols. (A) Electrophoresis of 20 µL of BAC DNA from 6 different minipreparations (lanes 1-6) obtained by the method described by Sinnet et al. (1998). (B) Electrophoresis of 2 µL of BAC DNA from 6 different minipreparations (lanes 1-6) obtained by the optimized method described herein. M, molecular weight marker (sizes indicated at left).

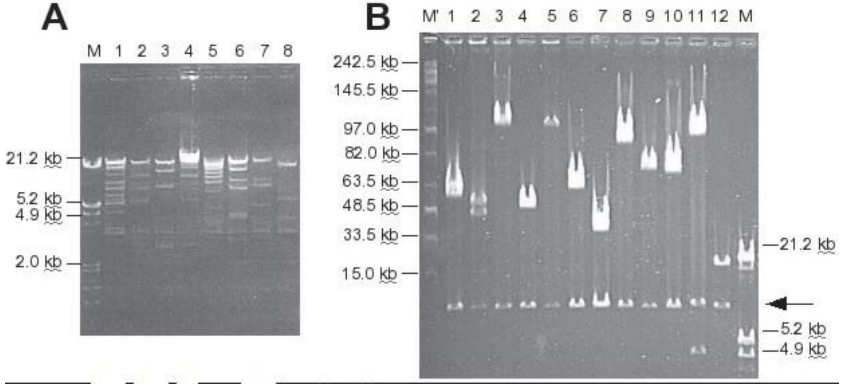

Figure 2. Enzymatic digestion of bacterial artificial chromosome (BAC) DNA isolated from 8 different clones (lanes 1-8). (A) BAC DNA cleaved with *Pst*I and subjected to standard electrophoresis; M, molecular weight marker (sizes indicated at left). (B) BAC DNA cleaved with *Not*I and separated by field inversion gel electrophoresis (FIGE). The arrow indicates the cloning vector (7.3 kb); M′ is the MidRange I size marker for pulsed field gel electrophoresis (New England Biolabs).

amplify a DNA fragment of 200 bp with different substrates (Figure 3): a BAC clone containing the Fd-GOGAT gene isolated in our laboratory, genomic DNA of *Pinus pinaster,* and Fd-GOGAT cDNA (García-Gutiérrez et al., 1995). The 200-bp fragment was amplified in all cases, indicating that the protocol provides DNA suitable for PCR analysis.

Finally, we tested to end-sequence a BAC clone, taking advantage of the presence of the T7 and SP6 promoters in the BAC vector. The sequences from the T7 side were similar to those of a callus tomato protein (EMBL accession number

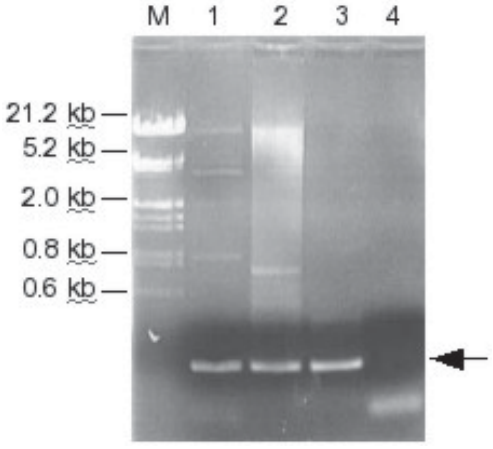

Figure 3. PCR analysis of a clone containing GOGAT cDNA (lane 1), *Pinus pinaster* genomic DNA (lane 2), and the bacterial artificial chromosome (BAC) clone containing the GOGAT gene (lane 3) by use of the primers GOGAT1 and GOGAT2. Lane 4, negative control without any DNA. The arrow indicates the 200-bp amplified DNA fragment.

BI422964), whereas the sequence from the SP6 side was similar to that of a pine root xylem protein (EMBL accession number BQ291368).

Discussion

The alkaline lysis procedure relies on a differential precipitation step in which high-molecular-weight DNA is precipitated and the relatively small plasmids remain in the supernatant (Birnboim and Doly, 1979). The further precipitation with lauryl sulfate and potassium acetate leads to the trapping of large denatured plasmids, which cannot renature to the extent that the small plasmids do.

The results presented here indicate that our protocol results in better performance than those previously described (Sinnett et al., 1998; Zhang, 2000). The improvements can be explained first by the quick suspension of cells in 10 mM EDTA instead of TE, which is the usual solution of plasmid minipreparations. The rapid and complete suspension was important so that the lysis could occur in an efficient manner.

Second, prolonged exposure of the coiled DNA to the alkali gives rise to its irreversible denaturalization (Sambrook and Russell, 2001). For that reason, we decide to use SLS instead of sodium dodecyl sulfate as detergent because SLS was reported to preserve large plasmids in the presence of potassium salt (Thomas et al., 1988), and we decided to vary the time of incubation in the solution of NaOH because we found that incubations longer than 4 min in cold severely diminished the yield (data not shown).

Third, the potassium acetate precipitation step cleaned the BAC DNA from cell debris originated during the lysis (Chen et al., 2002). Furthermore, DNA precipitations are ordinarily performed with cold ethanol, whereas we used 2-propanol at room temperature because it precipites lower amounts of salts.

Overall, the protocol described herein is simple and provides a high yield, hence offering a productive method for the isolation of high-quality BAC DNA suitable for numerous applications, including restriction analysis, sequencing, and PCR.

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