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An Improved Method for Plant BAC Library Construction

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Summary

Large genomic DNA insert-containing libraries are required as critical tools for physical mapping, positional cloning, and genome sequencing of complex genomes. The bacterial artificial chromosome (BAC) cloning system has become a dominant system over others to clone large genomic DNA inserts. As the costs of positional cloning, physical mapping, and genome sequencing continuously decrease, there is an increasing demand for high-quality deep-coverage large insert BAC libraries. In our laboratory, we have constructed many high-quality deep-coverage large insert BAC libraries including arabidopsis, manocot and dicot crop plants, and plant pathogens. Here, we present the protocol used in our laboratory to construct BAC libraries.

Key Words

BAC, library, method, pCUGIBAC1, plant

1. Introduction

Large genomic DNA insert-containing libraries are essential for physical mapping, positional cloning, and genome sequencing of complex genomes. There are two principal large insert cloning systems that are constructed as yeast or bacterial artificial chromosomes (YACs and BACs, respectively). The YAC cloning ***(1)*** was first developed in 1987 and uses *Saccharomyces cerevisiae* as the host and maintains large inserts (up to 1 Mb) as linear molecules with a pair of yeast telomeres and a centromere. Although used extensively in the late 1980s and early 1990s, this system has several disadvantages ***(2,3)***. The recombinant DNA in yeast can be unstable. DNA manipulation is difficult and inefficient. Most importantly, a high level of chimerism, the clon-

From: *Methods in Molecular Biology, vol. 236: Plant Functional Genomics: Methods and Protocols*
Edited by: E. Grotewold © Humana Press, Inc., Totowa, NJ

ing of two or more unlinked DNA fragments in a single molecule, is inherent within the YAC cloning system. These disadvantages impede the utility of YAC libraries, and subsequently, this system has been gradually replaced by the BAC cloning system introduced in 1992 ***(4)***.

The BAC cloning uses a derivative of the *Escherichia coli* F-factor as vector and *E. coli* as the host, making library construction and subsequent downstream procedures efficient and easy to perform. Recombinant DNA inserts up to 200 kb can be efficiently cloned and stably maintained in *E. coli*. Although the insert size cloning capacity is much lower than that of the YAC system, it is this limited cloning capacity that helps to prevent chimerism, because the inserts with sizes between 130–200 kb can be selected, while larger inserts, composed of two or more DNA fragments, are beyond the cloning capacity of the BAC system or are much less efficiently cloned.

In 1994, our laboratory was the first to construct a BAC library for plants using *Sorghum bicolor* ***(5)***. Since then, we have constructed a substantial number of deep coverage BAC libraries, including *Arabidopsis* ***(6)***, rice ***(7)***, melon ***(8)***, tomato ***(9)***, soybean ***(10)***, and barley ***(11)*** and have provided them to the community for genomics research ([http://www.genome.arizona.edu] and [http://www.genome.clemson.edu]).

The construction of a BAC library is quite different from that of a general plasmid or λ DNA library used to isolate genes or promoter sequences by positive screening. Megabase high molecular weight DNA is required for BAC library construction. Because individual clones of the BAC library will be picked, stored, arrayed on filters, and directly used for mapping and sequencing, a BAC library with a small average insert size and high empty clone (no inserts) rate will dramatically increase the cost and labor for subsequent work. Usually, a BAC library with an average insert size smaller than 130 kb and empty clone rate higher than 5% is unacceptable. These strict requirements make BAC library construction much more difficult than the construction of a general DNA library.

As the costs of positional cloning, physical mapping, and genome sequencing continuously decrease, so increases the demand for high-quality deep-coverage large insert BAC libraries ***(12)***. As a consequence, we describe in this chapter how our laboratory constructs BAC libraries.

Several protocols have been published for the construction of high quality plant and animal BAC libraries ***(13–18)***, including three from our laboratory ***(16–18)***. We improved on these methods in several ways ***(8)***. First, to easily isolate large quantities of single copy BAC vector, pIndigoBAC536 (*see* **Note 1**) was cloned into a high copy cloning vector, pGEM-4Z. This new vector, designated pCUGIBAC1 (**Fig. 1**), replicates as a high copy vector and can be isolated in large quantity using standard plasmid DNA isolation methods. It

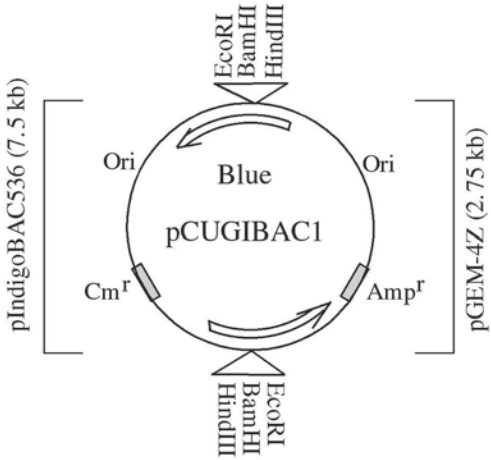

Fig. 1. pCUGIBAC1. Not drawn to scale.

retains all three unique cloning sites (*Hin*dIII, *Eco*RI, and *Bam*HI), as well as the two *Not*I sites flanking the cloning sites, of the original pIndigoBAC536. Second, to improve the stability of megabase DNA and size-selected DNA fractions in agarose, as well as digested dephosphorylated BAC vectors, we determined that such material can be stored indefinitely in 70% ethanol at –20°C and in 40–50% glycerol at –80°C, respectively.

The vector has been distributed to many users worldwide, and the high molecular weight DNA preservation method, established by Luo et al. ***(8)***, has been extensively used by colleagues and visitors and shown to be very efficient ***(18)***. These improvements and protocols described here save on resources, cost, and labor, and also release time constraints on BAC library construction.

2. Materials, Supplies, and Equipment

2.1. For pCUGIBAC1 Plasmid DNA Preparation

1. pCUGIBAC1 (http://www.genome.clemson.edu).
2. LB medium; 10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl.
3. Ampicillin and chloramphenicol (Fisher Scientific).
4. Qiagen plasmid midi kit (Qiagen).
5. Thermostat shaker (Barnstead/Thermolyne).

2.2. For BAC Vector pIndigoBAC536 Preparation

2.2.1. For Method One

1. Restriction enzymes (New England Biolabs).
2. HK phosphatase, Tris-acetate (TA) buffer, 100 m*M* $CaCl_2$, ATP, T4 DNA ligase (Epicentre).

3. Agarose and glycerol (Fisher Scientific).
4. 10× Tris-borate EDTA (TBE) and 50× Tris-acetate EDTA (TAE) buffer (Fisher Scientific).
5. 1 kb DNA ladder (New England Biolabs).
6. Ethidium bromide (EtBr) (10 mg/mL).
7. λ DNA (Promega).
8. Water baths.
9. CHEF-DR III pulse field gel electrophoresis system (Bio-Rad).
10. Dialysis tubing (Spectra/Por2 tubing, 25 mm; Spectrum Laboratories).
11. Model 422 electro-eluter (Bio-Rad).
12. Minigel apparatus Horizon 58 (Whatman).
13. UV transilluminator.

2.2.2. For Method Two

1. Restriction enzymes and calf intestinal alkaline phosphatase (CIP) (New England Biolabs).
2. 0.5 *M* EDTA, pH 8.0.
3. Absolute ethanol, agarose, and glycerol (Fisher Scientific).
4. T4 DNA ligase (Promega).
5. 10× TBE and 50× TAE buffer (Fisher Scientific).
6. 1 kb DNA ladder.
7. EtBr (10 mg/mL).
8. λ DNA.
9. Water baths.
10. CHEF-DR III pulse field gel electrophoresis system.
11. Dialysis tubing (Spectra/Por2 tubing, 25 mm).
12. Model 422 electro-eluter.
13. Minigel apparatus Horizon 58.
14. UV transilluminator.

2.3. For Preparation of Megabase Genomic DNA Plugs from Plants

1. Nuclei isolation buffer (NIB): 10 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA, pH 8.0, 100 m*M* KCl, 0.5 *M* sucrose, 4 m*M* spermidine, 1 m*M* spermine.
2. NIBT: NIB with 10% Triton® X-100.
3. NIBM: NIB with 0.1% β-mercaptoethanol (add just before use).
4. Low melting temperature agarose (FMC).
5. Proteinase K solution: 0.5 *M* EDTA, 1% N-lauroylsarcosine, adjust pH to 9.2 with NaOH; add proteinase K to 1 mg/mL before use.
6. 50 m*M* phenylmethylsulfonyl fluoride (PMSF) (Sigma) stock solution (prepared in ethanol or isopropanol).
7. $T_{10}E_{10}$ (10 m*M* Tris-HCl and 10 m*M* EDTA, pH 8.0) and TE (10 m*M* Tris-HCl and 1 m*M* EDTA, pH 8.0).
8. Mortars, pestles, liquid nitrogen, 1-L flasks, cheese cloth, small paintbrush, and Pasteur pipet bulbs.

9. 50-mL Falcon® tubes (Fisher Scientific) and miracloth (Calbiochem-Novabiochem).
10. Plug molds (Bio-Rad).
11. GS-6R centrifuge (Beckman).
12. Model 230300 Bambino hybridization oven (Boekel Scientific).

2.4. For Preparation of High Molecular Weight Genomic DNA Fragments

2.4.1. For Pilot Partial Digestions

1. Restriction enzymes and BSA (Promega).
2. 40 m*M* Spermidine (Sigma) and 0.5 *M* EDTA, pH 8.0.
3. λ Ladder pulsed field gel (PFG) marker (New England Biolabs).
4. Agarose and 10× TBE.
5. EtBr (10 mg/mL).
6. Razor blades, microscope slides, and water baths.
7. CHEF-DR III pulse field gel electrophoresis system.
8. UV transilluminator.
9. EDAS 290 image system (Eastman Kodak).

2.4.2. For DNA Fragment Size Selection

1. Restriction enzymes and BSA.
2. 40 m*M* spermidine and 0.5 *M* EDTA, pH 8.0.
3. λ Ladder PFG marker.
4. Agarose and 10× TBE.
5. Low melting temperature agarose.
6. EtBr (10 mg/mL) and 70% ethanol.
7. Razor blades, microscope slides, water baths, and a ruler.
8. CHEF-DR III pulse field gel electrophoresis system.
9. UV transilluminator.
10. EDAS 290 image system.

2.5. For BAC Library Construction

2.5.1. For DNA Ligation

1. T4 DNA ligase and λ DNA.
2. Agarose and 1× TAE buffer.
3. EtBr (10 mg/mL).
4. Dialysis tubing (Spectra/Por2 tubing, 25 mm) or Model 422 electro-eluter.
5. Minigel apparatus Horizon 58.
6. UV transilluminator.
7. Water baths.
8. 0.1 *M* Glucose/1% agarose cones: melt 0.1 *M* glucose and 1% agarose in water, dispense 1 mL to each 1.5-mL microcentrifuge, insert a 0.5-mL microcentrifuge

into each 1.5-mL microcentrifuge containing 0.1 *M* glucose and 1% agarose, after solidification, pull out the 0.5-mL microcentrifuges.

2.5.2. For Test Transformation

1. DH10B T1 phage-resistant cells (Invitrogen).
2. SOC: 20 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 10 m*M* NaCl, 2.5 m*M* KCl, autoclave, and add filter-sterilized $MgSO_4$ to 10 m*M*, $MgCl_2$ to 10 m*M*, and glucose to 20 m*M* before use.
3. 100-mm diameter Petri dish agar plates containing LB with 12.5 μg/mL of chloramphenicol, 80 μg/mL of x-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-gal]) and 100 μg/mL of IPTG isopropyl-β-D-thiogalactoside or isopropyl-β-D thiogalactopyranoside.
4. 15-mL culture tubes.
5. Thermostat shaker.
6. Electroporator (cell porator; Life Technologies).
7. Electroporation cuvettes (Whatman).
8. 37°C incubator.

2.5.3. For Insert Size Estimation

2.5.3.1. For BAC DNA Isolation

1. LB with 12.5 μg/mL chloramphenicol.
2. Isopropanol and ethanol.
3. P_1, P_2, and P_3 buffers from plasmid kits (Qiagen).
4. 15-mL culture tubes.
5. Thermostat shaker.
6. Microcentrifuge.

2.5.3.2. For BAC Insert Size Analysis

1. *Not*I (New England Biolabs).
2. DNA loading buffer: 0.25% (w/v) bromophenol blue and 40% (w/v) sucrose in TE, pH 8.0.
3. MidRange I PFG molecular weight marker (New England Biolabs).
4. Agarose, 0.5× TBE buffer, and EtBr (10 mg/mL).
5. 37°C water bath or incubator.
6. CHEF-DR III pulse field gel electrophoresis system.
7. UV transilluminator.
8. EDAS 290 image system.

2.5.4. For Bulk Transformation, Colony Array, and Library Characterization

1. Freezing media: 10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl, 36 m*M* K_2HPO_4, 13.2 m*M* KH_2PO_4, 1.7 m*M* Na-citrate, 6.8 m*M* $(NH_4)_2SO_4$,

4.4% glycerol, autoclave, and add filter-sterilized $MgSO_4$ stock solution to 0.4 m*M*.
2. 384-well plates and Q-trays (Genetix).
3. Toothpicks (hand picking) or Q-Bot (Genetix).

3. Methods

3.1. Preparing pCUGIBAC1 Plasmid DNA

1. Inoculate a single well-isolated *E. coli* clone harboring pCUGIBAC1 in LB containing 50 mg/L of ampicillin and 12.5 mg/L of chloramphenicol and grow at 37°C for about 20 h with continuous shaking.
2. Prepare pCUGIBAC1 plasmid DNA using the plasmid midi kit according to the manufacturer's instruction, except that after adding solution P_2, the sample was incubated at room temperature for not more than 3 min instead of 5 min (*see* acknowledgments). Each 100 mL of culture yields about 100 μg of plasmid DNA when using a midi column.

3.2. Preparing BAC Vector, pIndigoBAC536

3.2.1. Method One

1. Set up 4–6 restriction digestions, each digesting 5 μg pCUGIBAC1 plasmid DNA (with *Hin*dIII, *Eco*RI, or *Bam*HI depending on which enzyme is selected for BAC library construction) in 150 μL 1× TA buffer at 37°C for 2 h. Check 1 μL on a 1% agarose minigel to determine if the plasmid is digested.
2. Heat the digestions at 75°C for 15 min to inactivate the restriction enzyme.
3. Add 8 μL of 100 m*M* $CaCl_2$, 1.5 μL of 10× TA buffer, and 5 μL of HK phosphatase, and incubate the samples at 30°C for 2 h.
4. Heat the samples at 75°C for 30 min to inactivate the HK phosphatase.
5. Add 6.4 μL of 25 m*M* ATP, 5 μL of 2 U/μL T4 DNA ligase, and 1.3 μL of 10× TA buffer, incubate at 16°C overnight for self-ligation.
6. Heat the self-ligations at 75°C for 15 min.
7. Combine the samples and run the combined sample in a single well, made by taping together several teeth of the comb according to the sample vol, on a 1% CHEF agarose gel at 1–40 s linear ramp, 6 V/cm, 14°C in 0.5× TBE buffer along with the 1 kb ladder loaded into the wells on the both sides of the gel as marker for 16–18 h.
8. Stain the two sides of the gel containing the marker and a small part of the sample with 0.5 μg/mL EtBr and recover the gel fraction containing the 7.5-kb pIndigoBAC536 DNA band from the unstained center part of the gel by aligning it with the two stained sides. Undigested circular plasmid DNA and non-dephosphorylated linear DNA that has recircularized or formed concatemers after self-ligation should be reduced to an acceptable level after this step. **Figure 2** shows a gel restained with 0.5 μg/mL EtBr after having recovered the gel fraction containing the 7.5-kb pIndigoBAC536 vector. The 2.8-kb band is the pGEM-4Z vector.

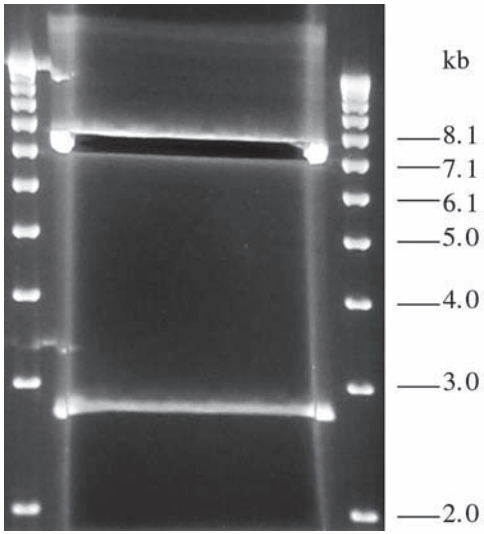

Fig. 2. Recovering linearized dephophorylated 7.5-kb pIndigoBAC536 vector from a CHEF agarose gel. See text for details.

9. Electroelute pIndigoBAC536 from the agarose gel slice in 1× TAE buffer at 4°C. Either dialysis tubing ***(19)*** or the Model 422 electro-eluter can be used ***(18)***.
10. Estimate the DNA concentration by running 2 μL of its dilution along with 2 μL of each of serial dilutions of λ DNA standards (1, 2, 4, and 8 ng/μL) on a 1% agarose minigel containing 0.5 μg/mL EtBr (for 10 min) and comparing the images under UV light, or simply by spotting a 1-μL dilution along with 1 μL of each of serial dilutions of λ DNA standards (1, 2, 4, and 8 ng/μL) on a 1% agarose plate containing 0.5 μg/mL EtBr and comparing the images under UV light after being incubated at room temperature for 10 min.
11. Adjust DNA concentration to 5 ng/μL with glycerol (final glycerol concentration 40–50%), aliquot into microcentrifuge tubes, and store the aliquots at –80°C. Use each aliquot only once.
12. Test the vector quality by cloning λ DNA fragments digested with the same restriction enzyme as used for vector preparation. Prepare a sample without the λ DNA fragments as the self-ligation control. For ligation, transformation, and insert check, follow the protocols in **Subheading 3.5**. for BAC library construction, except that inserts are checked on a standard agarose gel instead of a CHEF gel. Colonies from the ligation with the λ DNA fragments should be at least 100 times more abundant than those from the self-ligation control. More than 95% of the white colonies from the ligation with the λ DNA fragments should contain inserts.

3.2.2. Method Two

1. Set up 4–6 digestions, each digesting 5 μg pCUGIBAC1 plasmid DNA (with *Hin*dIII, *Eco*RI, or *Bam*HI depending on which enzyme is selected for BAC library construction) in 150 μL 1× restriction buffer at 37°C for 1 h. Check 1 μL on a 1% agarose minigel to see if the plasmid is digested.
2. Add 1 U of CIP and incubate the samples at 37°C for an additional 1 h (*see* **Note 2**).
3. Add EDTA to 5 m*M* and heat the samples at 75°C for 15 min.
4. Precipitate DNA with ethanol, wash it with 70% ethanol, air-dry, and add: 88 μL of water, 10 μL of 10× T4 DNA ligase buffer, and 2 μL of 3 U/μL T4 DNA ligase.
5. Incubate the samples at 16°C overnight for self-ligation. Then follow **steps 6–12** of Method One (**Subheading 3.2.1.**).

3.3. Preparing Megabase Genomic DNA Plugs from Plants (see [18] for alternatives) (see Note 3)

1. Young seedlings of monocotyledon plants, such as rice and maize, and young leaves of dicotyledon plants, such as melon, are used fresh or collected and stored at –80°C.
2. Grind about 100 g of tissue in liquid N_2 with a mortar and a pestle to a level that some small tissue chunks can be still seen (*see* **Note 4**).
3. Divide and transfer the ground tissue into two 1-L flasks, each containing 500 mL of ice-cold NIBM (1 g tissue/10 mL).
4. Keep the flasks on ice for 15 min with frequent and gentle shaking.
5. Filter the homogenate through four layers of cheese cloth and one layer of miracloth. Squeeze the pellet to allow maximum recovery of nuclei-containing solution.
6. Filter the nuclei-containing solution again through one layer of miracloth.
7. Add 1:20 (in vol) of NIBT to the nuclei-containing solution and keep the mixture on ice for 15 min with frequent and gentle shaking.
8. Transfer the mixture into 50-mL Falcon tubes. Centrifuge the tubes at 2400*g* at 4°C for 15 min.
9. Gently resuspend the pellets in the residual buffer by tapping the tubes or with a small paintbrush.
10. Dilute the nucleus suspension with NIBM and combine it into two 50-mL Falcon tubes. Adjust the vol to 50 mL with NIBM in each tube and centrifuge the tubes at 2400*g* at 4°C for 15 min.
11. Resuspend the pellets as in **step 9**. Dilute the nucleus suspension with NIBM and combine it into one 50-mL Falcon tube. Adjust the vol to 50 mL with NIBM and centrifuge it at 2400*g* at 4°C for 15 min.
12. Remove the supernatant and gently resuspend the pellet in approx 1.5 mL of NIB.
13. Incubate the nucleus suspension at 45°C for 5 min. Gently mix it with an equal vol of 1% low melting temperature agarose, prepared in NIB and pre-incubated

at 45°C, by slowly pipeting 2 or 3 times. Transfer the mixture to plug molds and let stand on ice for about 30 min to form plugs.
14. Tranfer <50 agarose plubs into each 50-mL Falcon tube, containing 40 mL of proteinase K solution, with a Pasteur pipet bulb.
15. Incubate the tubes in a hybridization oven (e.g., Model 230300 Bambino hybridization oven) at 50°C with a gentle rotation for about 24 h.
16. Repeat **step 15** with fresh proteinase K solution.
17. Wash the plugs, each time for about 1 h at room temperature with gentle shaking or rotation, twice with $T_{10}E_{10}$ containing 1 m*M* PMSF and twice with TE (40 mL each time for each 50-mL Falcon tube containing <50 plugs).
18. Store the plugs in TE buffer at 4°C (for frequent use) or rinse them with 70% ethanol and store in 70% ethanol (40 mL for each 50-mL Falcon tube containing <50 plugs) at –20°C (for long-term storage) (*see* **Note 5**).

3.4. Preparing High Molecular Weight Genomic DNA Fragments

3.4.1. Pilot Partial Digestions

1. Soak required number (e.g., 4 plugs) of TE-stored plugs in sterilized distilled water (more than 20 vol) for 1 h before partial digestion. For ethanol-stored plugs, transfer required number of 70% ethanol-stored plugs into TE buffer or directly into sterilized distilled water (more than 20 vol) at 4°C the day before use (*see* **Note 6**) and soak them in sterilized distilled water (more than 20 vol) for 1 h before partial digestion.
2. Dispense 45 μL of buffer mixture (24.5 μL of water, 9.5 μL of 10× restriction enzyme buffer, 1 μL of 10 mg/mL bovine serum albumin BSA, and 10 μL of 40 m*M* spermidine) into each of an ordered serial set (e.g., Nos. 1–8) of microcentrifuge tubes. Keep the microcentrifuge tubes on ice.
3. Chop each half DNA plug to fine pieces with a razor blade on a clean microscope slide (assume each half DNA plug has a vol of 50 μL) and transfer these pieces into a microcentrifuge tube containing 45 μL of restriction enzyme buffer on ice with a spatula. Mix by tapping and incubate on ice for 30 min.
4. Make serial dilutions of restriction enzyme (*Hin*dIII, *Eco*RI, or *Bam*HI, depending on which enzyme is selected for BAC library construction) with 1× restriction enzyme buffer (e.g., 0.4, 0.8, 1.2, 1.6, 2.0, and 2.4 U/μL).
5. Add 5 μL of one enzyme dilution to each of the microcentrifuge tube in **step 3**. Set up an uncut control, by not adding any enzyme, and a completely cut control, by adding 50–60 U of enzyme. Mix by tapping and incubate on ice for 30 min to allow for diffusion of the enzyme into the agarose matrix.
6. Incubate the microcentrifuge tubes in a 37°C water bath for 40 min.
7. Add 10 μL of 0.5 *M* EDTA, pH 8.0, to each microcentrifuge tube. Mix by tapping and incubate on ice for at least 10 min to terminate the digestions.
8. Prepare a 14 × 13 cm CHEF agarose gel by pouring 130 mL of 1% agarose (in 0.5× TBE buffer) at about 50°C into a 14 × 13 cm gel casting stand (Bio-Rad). Use two 15-well 1.5-mm-thick combs (Bio-Rad) bound together with tape for the samples. Set aside several milliliters of 1% agarose (in 0.5× TBE buffer) at 65°C.

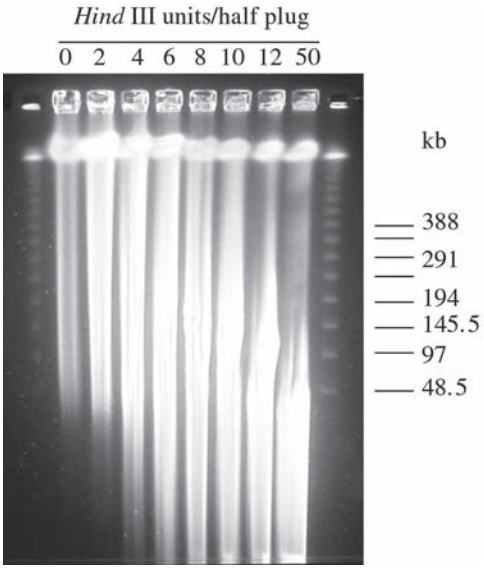

Fig. 3. Partial digestions of DNA plugs with serial dilutions of *Hin*dIII at 37°C for 40 min. DNA was separated on 1% CHEF agarose gel at 1–50 s linear ramp, 6 V/cm, 14°C in 0.5× TBE buffer for 20 h. The marker used is λ ladder PFG.

9. Load each sample from **step 7** into the center wells of the agarose gel with a spatula. Load the λ ladder PGF marker into the wells on the two sides of the gel. Seal the wells with the 1% agarose reserved at 65°C.
10. Run the gel at 1–50 s linear ramp, 6 V/cm, 14°C in 0.5× TBE buffer for 18–20 h.
11. Stain the gel with 0.5 μg/mL EtBr and take a photograph (*see* **Note 7**). **Figure 3** shows an example for the partial digestions of DNA plugs with serial dilutions of *Hin*dIII at 37°C for 40 min.

3.4.2. DNA Fragment Size Selection

1. Soak required number of plugs (e.g., 6 plugs) as in **Subheading 3.4.1.**, **step 1**.
2. Prepare a buffer mixture and dispense it into a set of microcentrifuge tubes (12 microcentrifuge tubes for 6 plugs) as in **Subheading 3.4.1.**, **step 2**.
3. Chop each half plug and treat the chopped plug pieces as in **Subheading 3.4.1.**, **step 3**.
4. Make the restriction enzyme dilution that produced the most DNA fragments in the range of 100–400 kb in the pilot partial digestion. For the batch of DNA plugs used in **Fig. 3**, 0.8 U/μL *Hin*dIII dilution (4 U of *Hin*dIII per half plug when 5 μL is used) was used for DNA fragment preparation.

5–7. Follow **Subheading 3.4.1.**, **steps 5–7**, except that 5 μL of the same enzyme dilution prepared in **step 4** is added to each of the microcentrifuge tubes in **step 3**.

8. Prepare a 14 × 13 cm CHEF agarose gel by pouring 130 mL of 1% agarose in

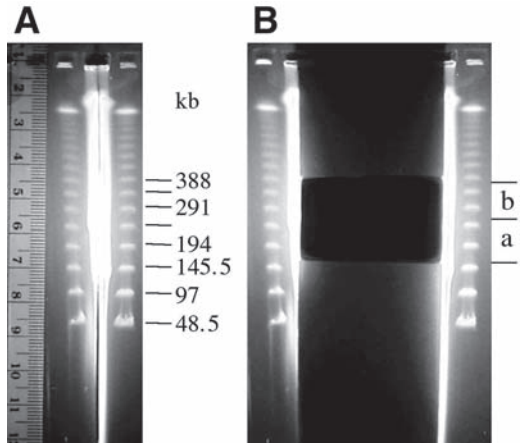

Fig. 4. An example for the first size selection of genomic DNA fragments. **(A)** Staining the two sides of the gel and taking a photograph with a ruler. **(B)** Recovering two gel fractions from the unstained center part of the gel corresponding to 150–250 and 250–350 kb located by a ruler.

0.5× TBE buffer at about 50°C into a 14 × 13 cm gel casting stand. Use a trimmed comb made by taping together several teeth of two 15-well 1.5-mm-thick combs to make a single well for the sample according to the sample vol.

9. Load the samples from **step 7** into the well with a spatula. Load the λ ladder PFG marker into the individual wells on the two sides of the gel. Seal the wells with 1% agarose in 0.5× TBE buffer maintained at 65°C.
10. Run the gel at 1–50 s linear ramp, 6 V/cm, 14°C in 0.5× TBE buffer for 18–20 h.
11. Stain the two sides of the gel containing the marker and a small part of the sample with 0.5 μg/mL EtBr and take a photograph with a ruler at one side (**Fig. 4A**).
12. Recover two gel fractions (first size-selected fractions: a and b) from the unstained center part of the gel corresponding to 150–250 and 250–350 kb located by a ruler (**Fig. 4B**).
13. Place the two gel fractions side by side (with a gap between them) on the top of a 14 × 13 cm gel casting stand with the orientation the same as in the original gel in **step 12**. Pour 130 mL of 1% agarose in 0.5× TBE at about 50°C into the gel casting stand to form a second gel encasing the two gel factions.
14. Run the gel at 4 s constant time, 6 V/cm, 14°C in 0.5× TBE buffer for 18–20 h.
15. Stain the two sides with 0.5 μg/mL EtBr, each containing a small part of one of the two first size-selected fractions, and the center part that contains the small parts of both first size-selected fractions. Take a photograph with a ruler at one side.
16. For each first size-selected fraction (a and b), recover two gel fractions (second size-selected fractions: a1 and a2, and b1 and b2) located by a ruler (**Fig. 5**). Gel fractions are used immediately or stored at –20°C in 70% ethanol (*see* **Note 5**).

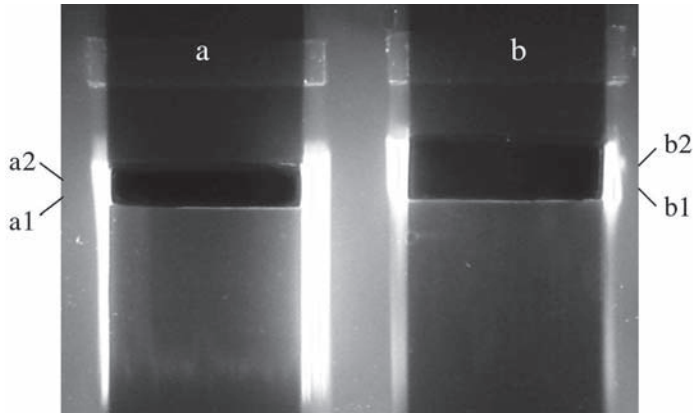

Fig. 5. An example for the second size selection of genomic DNA fragments.

3.5. BAC Library Construction

3.5.1. DNA Ligation

1. Transfer required amount of each 70% ethanol-stored fraction (e.g., one-third to one-half fraction) into 1× TAE buffer (more than 20 vol) at 4°C the day before use (*see* **Note 8**).
2. Electroelute high molecular weight genomic DNA at 4°C from fresh gel fractions or 1× TAE buffer soaked 70% ethanol-stored fractions in 1× TAE buffer. Either dialysis tubing ***(20)*** or Model 422 electro-eluter ***(18)*** can be used. Eluted DNA should be used as soon as possible (use it the same day it is eluted). Always use pipet tips with the tips cut off when manipulating high molecular weight genomic DNA to avoid mechanical shearing.
3. Estimate the DNA concentration by running 5 μL of the eluted DNA along with 2 μL of serial dilutions of λ DNA standards (1, 2, 4, 8, and 16 ng/μL) on a 1% agarose minigel containing 0.5 μg/mL EtBr (for 10 min) and comparing the images under UV light.
4. Set up ligations: in each microcentrifuge tube, add 4 μL of 5 ng/μL vector and 84 μL of DNA eluted in 1× TAE containing up to 200 ng of high molecular weight genomic DNA fragments. If the eluted DNA has a high concentration, dilute it with sterilized water. Incubate the vector–genomic DNA fragment mixture at 65°C for 15 min, cool at room temperature for about 10 min, and add 10 μL of 10× T_4 DNA ligase buffer and 2 μL of 3 U/μL T_4 DNA ligase. Incubate the ligations at 16°C overnight.
5. Heat the ligations at 65°C for 15 min to terminate the ligation reactions.
6. Transfer ligation samples into 0.1 *M* glucose/1% agarose cones (*see* **Subheading 2.5.1.**) to desalt for 1.5 h on ice ***(20)*** or transfer ligation samples onto filters (Millipore) floating on 5% polyethylene glycol (PEG)8000 in Petri dishes set on ice for 1.5 h as modified from Osoegawa et al. ***(15)***. Store the ligations at 4°C for not more than 10 d.

3.5.2. Test Transformation

1. Thaw ElectroMax DH10B T_1 phage-resistant competent cells on ice and dispense 16 μL into prechilled microcentrifuge tubes on ice. Precool the electroporation cuvettes on ice. Prepare SOC media and dispense 0.5 mL to each sterile 15-mL culture tube. Label the microcentrifuge tubes, cuvettes, and culture tubes coordinately.
2. Take 1 to 2 μL of ligated DNA from each ligation sample and mix it with the competent cells by gentle tapping.
3. Transfer the DNA/competent cell mixture from each microcentrifuge tube into precooled electroporation cuvettes. Electroporate on ice at 325 DC V with fast charge rate at a low resistance (4 kΩ) and a capacitance of 330 μF. We did not find a significant difference when different DC V between 300–350 V were applied.
4. Transfer the electroporated cells from each cuvette into sterile 15-mL culture tubes containing 0.5 mL SOC. Incubate the cultures at 37°C for 1 h with vigorous shaking.
5. Plate 20 and 200 μL of each culture on 100-mm diameter Petri dish agar plates containing LB with 12.5 μg/mL of chloramphenicol, 80 μg/mL X-gal, and 100 μg/mL IPTG. Incubate the plates at 37°C overnight.
6. Count the white colonies and determine the number of recombinant clones per microliter of ligation. This number, the genome size, and the required genome coverage will be considered to decide if the experiment should be continued. For example, 3 parallel 100 μL ligations of 100 white colonies/μL with the expected average insert size of 130 kb will result in about 9 genome coverages for rice (genome size is 430 Mbp), but only 1.56 genome coverages for maize (genome size is 2500 Mbp).

3.5.3. Insert Size Estimation

3.5.3.1. BAC DNA Isolation

Several automated methods, such as using an Autogen 740 (AutoGen) or using a Quadra 96 (TomTec) can be used to isolate BAC DNA. A manuscript for a detailed method for preparing BAC DNA with a Quadra 96 is in preparation by HyeRan Kim et al. Here we present a manual method adapted from the Qiagen method.

1. Randomly pick white colonies with sterilized toothpicks and inoculate each into 2 mL of LB containing 12.5 μg/mL chloramphenicol in a sterile 15-mL culture tube. Grow the cells at 37°C overnight with vigorous shaking.
2. Transfer each cell culture (about 1.5 mL) into a microcentrifuge tube and collect cells at 16,000*g* (at room temperature or 4°C) for 10 min; remove supernatant.
3. Add 200 μL of P_1. Mix the tubes with a vortex to resuspend pellets at room temperature.

4. Add 200 μL of P_2. Mix the contents gently but thoroughly by inverting the tubes 3 to 4 times. Stand the tubes at room temperature for not more than 3 min.
5. Add 200 μL of P_3. Mix the contents gently but thoroughly by inverting the tubes 3 to 4 times. Stand the tubes on ice for 15 min.
6. Centrifuge the samples at 16,000*g* (at room temperature or 4°C) for 30–40 min.
7. Carefully transfer about 550 μL of each supernatant to a new microcentrifuge tube containing 400 μL of isopropanol. Mix the contents gently.
8. Centrifuge the samples at 16,000*g* (at room temperature or 4°C) for 30 min.
9. Remove the supernatant. Add 400 μL of 70% ethanol and centrifuge the samples at 16,000*g* for 10 min to wash the DNA pellets.
10. Remove the supernatant carefully with a pipet. Air-dry the DNA pellets, and resuspend in 60 μL of TE buffer, pH 8.0.

3.5.3.2. BAC Insert Size Analysis

1. Dispense 11 μL of *Not*I digestion mixture (8.85 μL of water, 1.5 μL of 10× buffer, 0.15 μL of 10 mg/mL BSA, and 0.5 μL of 10 U/μL *Not*I) into each microcentrifuge tube or each well of a 96-well microtiter plate.
2. Add 4 μL of BAC plasmid DNA to each tube or each well. Spin the samples briefly. Incubate the samples at 37°C for 3 h. Dispense 3 μL of 6× DNA loading buffer ***(21)*** into each tube or each well. Spin the samples briefly.
3. Prepare a 21 × 14 cm CHEF agarose gel by pouring 150 mL of 1% agarose in 0.5× TBE buffer at about 50°C into a 21 × 14 cm gel casting stand. Use a 45-well 1.5-mm-thick comb for the samples.
4. Load DNA samples. Use MidRange I as the size marker.
5. Run the gel at 5–15 s linear ramp, 6 V/cm, 14°C in 0.5× TBE buffer for 16 h.
6. Stain the gel with 0.5 μg/mL EtBr. Take a photograph of the gel. Analyze the insert sizes.

3.5.4. Bulk Transformation, Colony Array, and Library Characterization

If the test colonies meet the requirement for average insert size and empty vector rate, transform all ligated DNA into ElectroMax DH10B T_1 phage-resistant competent cells. Pick individual colonies into wells of 384-well plates containing freezing media manually or robotically (Q-Bot) and characterize the BAC library by insert size analysis of random clones. Store the BAC library at –80°C.

4. Notes

1. pIndigoBAC536 has the same sequence as pBeloBAC11, except that the internal *Eco*R1 site was destroyed so that the unique *Eco*R1 site in the multiple cloning site can be used for cloning, and a random point mutation was selected for in the lac Z gene that provides darker blue colony color on X-gal/IPTG selection. The GenBank® accession number for pBeloBAC11 is U51113.
2. CIP is active in many different buffers.

3. Plug preparation is a critical part of the work for plant BAC library construction. Many failures are attributed to the plugs not containing enough megabase DNA. To increase the DNA content in plugs, more starting material can be used, and the resultant nuclei can be imbedded in fewer plugs. However, at least 25–35 plugs for each preparation are required for convenient subsequent manipulation. The same batch of plugs should be used for pilot partial digestion and scaled partial digestion for BAC library construction.
4. Do not grind the material to a complete powder, as novices in this field usually do. Overgrinding reduces the yield of nuclei dramatically.
5. Allow to stand at room temperature for about 30 min or at 4°C overnight before transferring to –20°C to avoid freezing the center part of the gel slices. Freezing causes high molecular weight DNA to shear.
6. If the 70% ethanol-stored plugs are needed to be used the same day, soak them in a large vol of sterilized distilled water (40 mL in a 50-mL Falcon tube) at room temperature for 3 h with gentle shaking and several changes of sterilized distilled water.
7. If the DNA in the completely cut control is not well digested (most of the DNA fragments should be below 50 kb after complete digestion), rewash the DNA plugs or use a different restriction enzyme. If a restriction condition to produce most of the DNA fragments in the range of 100–400 kb is not found, because of insufficient digestion or over digestion, repeat the pilot partial digestion with higher or lower enzyme concentrations respectively.
8. Similar to **Note 6**, if the 70% ethanol-stored fractions are needed to be used the same day, soak them in a large vol of 1× TAE buffer (40 mL in a 50-mL Falcon tube) at room temperature for 3 h with gentle shaking and several changes of 1× TAE buffer.

Acknowledgments

Jose Luis Goicoechea for BAC plasmid DNA preparation. We thank Dave Kudrna for his critical reading and suggestions.

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