Megabase DNA Isolation  
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Megabase-size DNA isolation from plants

To construct large insert DNA libraries in BAC and YAC vectors, methods must be developed to isolate very high molecular weight DNA - megabase-size DNA - from plants. To isolate such DNA, protoplasts or nuclei must first be embedded in agarose plugs or microbeads. The agarose acts as a solid yet porous matrix which allows for the diffusion of various reagents for DNA purification and subsequent manipulations while preventing the DNA from being sheared (Schwartz and Cantor, 1984). Microbeads are preferred over plugs because the use of beads increases the surface area surrounding the tissue sample by approximately 1000 fold thereby allowing for more efficient and rapid diffusion of chemicals and enzymes into and out of the agarose beads (Cook, 1984, Overhauser and Radic, 1987, Wing et al, 1993). Once embedded, the protoplasts or nuclei are lysed and proteins degraded in the presence of 0.5 M EDTA, 1% sarcosyl, and 0.1-1.0 mg/ml of proteinase-K at 50 C (Schwartz and Cantor, 1984). After cell lysis and protein degradation, the remaining DNA is suitable for enzymatic modifications.

Most protocols for the isolation of megabase-size DNA from plants utilize the protoplast method (Cheung and Gale, 1990, Ganal, et al, 1989, Honeycutt, et al, 1992, Sobral, et al, 1990, van Daelen, et al, 1989, Wing, et al, 1993, Woo et al, 1995). Although the protoplast method yields megabase-size DNA of high quality, the process is costly and labor intensive. For example, to prepare protoplasts from tomato, young leaves are manually feathered with a razor blade before being incubated for 4-5 hours with cell wall degrading enzymes (Ganal and Tanksley, 1989). With sorghum, Woo et al (1995) found the best way to generate high yields of protoplasts for megabase-size DNA isolation is to rub carborundum on both sides of the leaves with a paintbrush, 50 strokes/side, before a 4-5 hour incubation with cellulysin. Thus the amount of time before embedding in agarose can be between 7-9 hours, depending on the amount of leaf material being processed. Furthermore, since each plant species requires a different set of conditions to generate protoplasts the method will only work if a high yielding protoplast method has been developed for a given plant species.

Some investigators have tried with varying degrees of success to prepare megabase-size DNA from nuclei (Hatano, et al, 1992). Zhang et al (1995) have recently developed a nuclei method that works well for several divergent plant taxa. Fresh or frozen tissue is homogenized with a blender or mortar and pestle, respectively. Nuclei are then isolated and embedded as above. The quality of the DNA is as good as DNA prepared from protoplasts, is often more concentrated, and was shown to contain lower amounts of chloroplast DNA. The primary advantage of method is that it is economical and not as labor intensive as the protoplast method. The amount of time required to isolate nuclei and embed in agarose routinely takes
Preparation of Megabase-size DNA from Plant Nuclei

A. Plant materials

Plant leaves or whole plants of divergent species, including grasses, legumes, vegetables, and trees can be used as materials for preparation of megabase-sized DNA by this method. The tissue can be either frozen in liquid nitrogen and stored in a -70°C freezer or kept fresh on ice before use.

B. Reagents

1. 10 x homogenization buffer (HB) stock: 0.1 M Trizma base, 0.8 M KCl, 0.1 M EDTA, 10 mM spermidine, 10 mM spermine, final pH 9.4-9.5 adjusted with NaOH. The stock is stored at 4°C.

2. 1 x HB: A suitable amount of sucrose is mixed with a suitable volume of 10x HB stock. The final concentration of sucrose is 0.5 M and HB stock is 1x. The resultant 1 x HB is stored at 4°C. Before use, b-mercaptoethanol is added to 0.15%. 20% Triton X-100 in 1 x HB: Triton X-100 is mixed with 1 x HB without b-mercaptoethanol to 20%. The solution is stored at 4°C.

Wash Buffer (1 x HB plus 0.5% Triton X-100): Prepared by mixing 1 x HB without b-mercaptoethanol with Triton X-100 and stored at 4°C. Before use, b-mercaptoethanol is added to 0.15%.

Lysis buffer: 0.5 M EDTA; pH 9.0-9.3, 1% sodium lauryl sarcosine, and 0.1 mg/ml proteinase K. The proteinase K powder is added just before use.

C. Preparation of intact nuclei

For homogenization of the plant tissue, two methods can be used.

1A. Homogenization of Fresh Tissue

a. Wash about 15 g of fresh tissue with tap water, and if necessary, cut into suitable pieces for homogenization with a kitchen blender (Osterizer 10 Speed Blender).
b. Homogenize the tissue in 200 ml ice cold 1 x HB plus b-mercaptoethanol in the kitchen blender at speed 4 or "puree" for 10 to 30 seconds.
c. Filter the homogenate into an ice cold 250 ml centrifuge bottle through two layers of cheesecloth and one layer of miracloth. Retrieve the remaining nuclei by squeezing the homogenate with gloved hands.
d. Add 5 ml 1 x HB plus 20% Triton X-100 (the final concentration of Triton X-100 is 0.5%) to each bottle (200 ml 1 x HB buffer), gently mix the contents, and incubate on ice for 20 minutes.

1B. Homogenization of Frozen Tissue

a. Grind about 15 g of the frozen tissue into a powder in liquid nitrogen with a mortar and pestle and immediately transfer into an ice cold 500 ml beaker containing 200 ml ice-cold 1 x HB plus b-mercaptoethanol and 0.5% Triton X-100.
b. Gently swirl the contents with a magnetic stir bar for 10 minutes on ice and filter into an ice-cold 250 ml centrifuge bottle as above.
2. Pellet the homogenate prepared by either of the above two methods by centrifugation with a fixed-angle rotor at 1,800 g at 4 C for 20 minutes.

3. Discard the supernatant fluid and add approximately 1 ml of ice cold wash buffer.

4. Gently resuspend the pellet with assistance of a small paint brush soaked in ice cold wash buffer. After the pellet is resuspended, add an additional 30 ml of ice cold wash buffer.

5. To remove the particulate matter remaining in the suspension, filter the resuspended nuclei into a 50 ml centrifuge tube through two layers of miracloth by gravity.

6. Centrifuge the contents at 57 g (500 rpm), 4 C for 2 minutes to remove intact cells and tissue residues.

7. Transfer the supernatant fluid into a fresh centrifuge tube and pellet the nuclei by centrifugation at 1,800 g, 4 C for 15 minutes in a swinging bucket centrifuge.

8. Wash the pellet 1 to 2 additional times by resuspension in wash buffer followed by centrifugation at 1,800 g, 4 C for 15 minutes.

9. After the final wash, resuspend the pelleted nuclei in a small amount (about 1 ml) of 1 x HB without b-mercaptoethanol with a paint brush. Count the nuclei, if possible, under a phase contrast microscope and adjust the nuclei concentration to approximately 4 x 10^7 nuclei/ml with addition of the 1 x HB without b-mercaptoethanol, and store on ice.

**D. Embedding the nuclei in agarose plugs and microbeads**

1. Prepare a 1% LMP agarose solution in 1 x HB without b-mercaptoethanol and store in a 45 C water bath.

2A. Embedding the nuclei in agarose microbeads
   a. Warm 20ml of light mineral oil in a 50 ml Falcon tube to 45 C in a water bath (about 15 minutes)
   b. Pour 150 ml of ice cold 1 x HB without b-mercaptoethanol into a 500 ml beaker and place the beaker in an ice water bath on top of a magnetic stir plate. Vigorously swirl the solution using a magnetic stir bar.
   c. Place a 500 ml flask into a 45 C water bath and prewarm for at least 5 minutes.
   d. Prewarm the nuclei to 45 C in a water bath (about 5 minutes).
   e. Mix the prewarmed nuclei suspension with an equal volume of 1% LMP agarose in 1 x HB without b-mercaptoethanol kept in a 45 C water bath, pour into the prewarmed 500 ml flask, and add 20 ml of prewarmed light mineral oil at 45 C.
   f. Shake the contents of the flask vigorously for 2-3 seconds and immediately pour into the 500 ml beaker containing the swirling 150 ml of ice cold 1 x HB without
b-mercaptoethanol. Continue to swirl the contents for 5-10 minutes on ice. This allows for the agarose microbeads to be more uniform in size.
g. Harvest the agarose microbeads by centrifugation at 1,200 g, 4 C for 15-20 minutes in a swinging bucket centrifuge.
h. Discard the supernatant fluid and resuspend the microbeads in 5-10 volume of lysis buffer.

2B. Embedding the nuclei in agarose plugs
a. Mix the nuclei with an equal volume of 1% low-melting point (LMP) agarose using a cut off pipet tip.
b. Prewarm the nuclei to 45 C in a water bath (about 5 minutes) before being embedded in agarose.
c. Aliquot the mixture into ice cold plug molds on ice with the same pipette tip, 100ml per plug. When the agarose is completely solidified, transfer the plugs into 5-10 volumes of lysis buffer.

3. Incubate the agarose microbeads or plugs in the lysis buffer for 24-48 hours at 50 C with gentle shaking.

4. Wash the plugs or the beads once in 0.5 M EDTA, pH 9.0-9.3 for one hour at 50 C, once in 0.05 M EDTA, pH 8.0 for one hour on ice, and store in 0.05 M EDTA, pH8.0, at 4 C.

E. Digestion of DNA Embedded in Agarose Microbeads

1. Before use, wash the agarose microbeads or the plugs containing HMW DNAs three times in 10 - 20 volumes of ice cold TE (10 mM Tris-HCl, pH8.0, 1 mM EDTA pH 8.0) plus 0.1 mM phenylmethyl sulfonyl fluoride (PMSF) and three times in 10 - 20 volumes of ice cold TE on ice, one hour each wash. The washed beads can be stored at 4 C for several months without significant degradation.

2. For digestion of agarose beads, mix the followings in a sterile microcentrifuge tube:
ddH2O 25 ml
Beads 50 ml*
10 x enzyme buffer 10 ml
40 mM spermidine 10 ml

Mix the beads well before pipetting and pipet the beads with a cut-off tip. The amount of beads to be digested is dependent on the concentration of DNA embedded in the agarose beads.

3. Incubate on ice for one hour, add 5 ml (approximately 10 units/mg DNA) of restriction enzyme for complete digestion and add 5 ml of each enzyme dilution series for partial digestion.
4. Incubate on ice for additional 30 minutes to allow the enzyme access to the DNA in the agarose beads and then transfer the reaction mixture to the recommended temperature for enzyme activity.

5. For complete digestion, incubate the reaction for at least 3 hours at the appropriate temperature. For partial digestion, incubate the beads for one hour.

6. Stop the reaction by adding 1/10 volume of 0.5 M EDTA, pH 8.0 and then place on ice or store at 4°C.

F. PFGE analysis and Southern blotting

1. Load the digested HMW DNA in agarose microbeads into an 1% agarose gel in 0.5 x TBE with a cut-off tip and seal the beads in position with the same molten agarose as the gel.

2. Perform pulsed-field gel electrophoresis as described below.

3. Stain the gel with ethidium bromide for 20 minutes, destain for one hour, photograph, and nick the DNA in the gel with 60 mJoules of UV light (254 nm) using the GS Gene Linker (BioRad, USA).

4. Blot the DNA in the gel onto Hybond-N+ membrane (Amersham, USA) with 1.5 M NaCl, 0.5 M NaOH.

Set up the prereaction as follows:

a. Label four 1.5 ml tubes and pipet 50 ml of the beads with a cut off pipette tip into each tube. Be sure to mix the microbeads solution well before pipeting.

b. Make a cocktail with 4.5 x 15 ml H2O, 6.5 x 10 ml 10 x reaction buffer and 4.5 x 10 ml 40 mM spermidine, and then aliquot 45 ml of the cocktail to each tube containing the microbeads. (A single restriction digestion has the following composition.)

   Megabase-size DNA in microbeads 50 ml
   10X HindIII reaction buffer 10 ml
   BSA (10 mg/ml) 1.0 ml
   SPD (40mM) 10 ml
   ddH2O 24 ml

   (10X HindIII reaction buffer: 60 mM Tris-HCl, 60 mM MgCl2, 0.5 M NaCl, 10 mM DTT, pH 7.5)

c. Incubate the microbeads without the restriction enzyme on ice for 20 minutes.

d. Dilute the HindIII enzyme serially ranging from 0 to 20 units in 1 X HindIII reaction
buffer.
tube 1-0 u/5 ml;
tube 2-1 u/5 ml;
tube 3-2 u/5 ml;
tube 4-4 u/5 ml.

e. Add 5 ml of HindIII to each reaction and incubate on ice for additional 20 minutes to allow the enzyme to diffuse into the microbeads.

f. Incubate the reactions in a 37 C water bath for 5 minutes and stop the reaction by addition of 1/10 volume of 0.5 M EDTA, pH 8.0 on ice.

g. Analyze the digests on a 1.0% agarose CHEF gel at 6.0 V/cm, with a 90 second pulse, for 20 hours, at 11 C running in 0.5x TBE buffer.

h. Select the amount of HindIII giving the best digest and set up the digestion reaction in a large scale by setting up the number of reactions you need as above (Do not increase the total volume of a reaction more than 100 ml).

G. An Example: Rice

Set up the prereaction as follows:

1. Label five 1.5 ml tubes, mix well the microbeads in the storage tube, and pipet 100 ml of the beads with a cut off pipette tip into each tube. Be sure to mix the microbeads solution well before pipeting.

2. Make a cocktail with 5.5 x 68 ml H2O, 5.5 x 20 ml 10 x reaction buffer and 5.5 x 10 ml 40 mM spermidine, and then aliquot 98 ml of the cocktail to each tube containing the microbeads. (A single restriction digestion has the following composition.)
   Megabase-size DNA in microbeads 100 ml (5-7 ug)
   10X BamHI reaction buffer 20 ml
   SPD (40mM) 10 ml
   ddH2O 68 ml

3. Incubate the mixture on ice for one hour and then add 2 ml dilutions of Bam HI enzyme in 1 x reaction buffer:
tube 1-0 u/ml;
tube 2-1 u/ml;
tube 3-2 u/ml;
tube 4-4 u/ml,
tube 5-8 u/ml.

4. Incubate on ice for additional 30 minutes, then transfer into a 37 C water bath, and incubate for one hour.
5. After one hour of digestion, transfer the reactions onto ice immediately and then add 20 ml of 0.5 M EDTA, pH 8.0 to each tube to stop the reaction. Keep the digests on ice before analyzing by pulsed-field gel electrophoresis (PFGE).

H. Electrophoretic analysis of partial digestions

1. Analyze the digests on a 1.0% agarose CHEF gel at 6.0 V/cm, with a 90 second pulse, for 20 hours, at 11 C running in 0.5x TBE buffer.
   a. Prepare 2 L 0.5 x TBE and pour into the CHEF buffer chamber and start the cooling system to cool the TBE down to 11 C.
   b. Prepare an 1% pulsed-field gel (120 ml) in 0.5 x TBE and keep 1-5 ml of the molten agarose in a 65 C water bath for later use.
   c. When the gel is completely solidified, remove the comb carefully, disassemble the gel mold, and load the gel as follows:
      
      (1) During storage of the digests on ice, the beads fall on the bottom of the tube. Carefully remove the supernatant with a pipet, and load the beads into a well of the gel using a cut off pipette tip.
      
      (2) Seal the beads in the well with the molten agarose kept in the 65 C water bath in step 4.
      
      (3) Insert a small slice of an agarose plug that contains lambda DNA as a concatemer for a molecular weight standard. Seal the marker in place with the remaining molten agarose.
   d. When the TBE in the CHEF buffer chamber cools down to 11 C, place the loaded gel with the gel-pouring plate in the CHEF buffer chamber and leave at least 15 minutes to equilibrate the temperature of the gel.
   e. Run the gel at 11 C, 90 sec., and 150 V for 20 hours.
   f. Stain the gel with ethidium bromide, photograph, and determine the optimal partial digestion condition for BAC cloning. In this experiment, the partial digestion conditions that show a majority of restricted DNA fragments from 200 to 400 kb in size on the pulsed-field gel will be selected for large scale partial digestion of HMW DNA to be used for the construction of the BAC libraries. They may include the optimal condition, and one-step above and below the optimal condition.

2. As above, perform a 10-fold scale partial digestions with Bam HI or 6-fold scale partial digestion with HindIII under conditions: one step above, optimal, and one-step below. After the large scale digestions are stopped, the reactions can be checked by PFGE using a small portion of each reaction while majority of the reaction is stored at 4 C or directly used for the first size selection.

I. Size selection of HMW DNA

After partial digestion, the DNA must be size-selected to remove the smaller DNA
fragments that can compete more effectively than the larger DNA fragments for vector ends and which can also be more efficiently transformed. The two most common size-selection techniques are sucrose gradient centrifugation (Burke and Olson, 1991) and pulsed-field gel electrophoresis (Albersten, et al, 1990, Birren and Lai, 1993). Most cosmid libraries have been constructed with DNA selected on sucrose gradients. All but one of the plant YAC and BAC libraries have used pulsed-field gel electrophoresis for size selection. The notable exception is one of the A.thaliana YAC library constructed by Ward and Jen (1990) who used sucrose gradients. Pulsed-field gel electrophoresis is primarily used for size selection because the DNA is usually more concentrated than DNA isolated on sucrose gradients; therefore, it is easier to concentrate DNA from agarose gels without substantial loss in yield and size. Additionally, 5-20% gradients do not efficiently resolve DNA above 100 kbs. Cosmid libraries can be constructed with DNA size-selected on sucrose gradients because the yields are good in the size range of 25 to 40 kb.

Procedure
A. First size selection
   i. Prepare a 1% LMP agarose CHEF gel using 1x TAE as the electrophoresis buffer.
   
   ii. Prepare 2 liters of 1X TAE electrophoresis buffer and place in CHEF gel apparatus and cool to 11 C.

   iii. Load partially digested DNA with size markers (lambda concatemers and/or yeast chromosomes) on either side of the digests into a 1% low melting point agarose gel using a cut off pipet tip. Seal the wells with 1% agarose.

   iv. Run the CHEF gel at 6.0 V/cm, with a 90 second pulse, for 20 hours, at 11 C.

   v. Cut both sides of the gel including the size markers and part of the DNA lanes with a clean microscope coverslip. Remove the center of the gel and keep in a CHEF gel box at 11 C.

   vi. Stain, destain, and photograph the gel sides with a ruler, side-by-side.

   vii. Cut DNA ranging from 300 to 500 kb from the unstained portion of the gel by measured migration ligation or for a second size selection (see below).

B. Second size selection

   1. Melt the gel piece cut from first size selection gel at 67 C for 10 minutes, pipet the solution into the wells of a second size selection gel prepared as above (1% LMP agarose in 1xTAE), with a cut off pipet tip, and wait for 5 minutes until it solidifies.

   2. Load size markers on the either side of the digests with a known quantity of
uncut lambda DNA for estimating the concentration of size selected DNA.

3. Remove the small trapped DNA from the first size selection by PFGE with the following settings: 4.0 V/cm, 5 second pulse, 10-13 hours, at 11 C, in 1x TAE buffer.

4. Stain and photograph the sides of the gel as above and excise the compressed DNA band from the unstained portion of the CHEF gel.

5. Wash the gel piece containing DNA with TE (1 ml), 2-3 times, on ice in a 1.5 ml microfuge tube, and store at 4 C.

C. Ligation of the size-selected DNA fragments into pBeloBAC11

1. Wash the LMP agarose gel slices containing the size-selected DNA fragments on ice with 1.0 ml ice-cold TE three times, 10 minutes each time.

2. After the last wash, remove the solution completely, melt the gel slice at 68 C for 10-15 minutes, and transfer to a 45 C water bath to equilibrate the temperature of the molten agarose to 45 C (5-10 minutes).

3. Estimate the volume of the molten agarose and digest the agarose with 1 unit of GELase (Epicentre, USA) per 100 mg of gel slice. Incubate at 45 C for 1 hour.

   Alternatively, add 1/10 volume of prewarmed 10 x beta-agarase reaction buffer (supplied by the manufacturer) at 40 C and 1 unit of b-agarase per 150 ml molten agarose, invert the tube several times very gently, and incubate at 40 C for 1 hour.

   Note: you should always pipet the naked HMW DNA with a cut off pipet tip very gently or the DNA will be physically sheared.

4. After digestion, estimate concentration of selected DNA by the following procedure:
   a. Prepare a 1% agarose/TBE gel.
   b. Load lambda DNA of known concentration as standard.
   c. Load 10 ml aliquots of each sample into the gel.
   d. Electrophorese at 50 volts for 30-50 minutes.

5. When the agarose is completely digested, add the vector DNA at a molar ratio of 5-10 vector DNA : 1 source DNA and incubate at 55 C for 10 minutes.

6. Cool the mixture to room temperature (10-15 minutes), and add 1/10 volume of 10 x ligation buffer supplied by the manufacturer and 4 units of T4 DNA ligase per 100 ml reaction containing approximately 40 ng source DNA.

   Size selected DNA (approx. 25-50 ng) x ml
   Dephosphorylated pBeloBAC 11 x ml (in a molar ratio of 1 (insert DNA) to 5-10 (vector DNA))
10X T4 ligase buffer 10 ml
T4 DNA ligase (1 unit/ml) 4 ml
ddH2O x ml
Total 100 ml
(10X T4 DNA ligase buffer: 660 mM Tris-HCl, pH 7.6, 66 mM MgCl2, 100 mM DTT, 660 mM ATP)

7. Remove 20 ml of the reaction very gently with a cut-off pipet tip and mix with 1 ml 0.5 ug/ml Hind III-digested lambda DNA for ligation tests.

8. Incubated the reaction and the test ligation reaction at 16 C overnight.

9. Analyze the test ligation reaction on an 1% agarose gel using the unligated Hind III-digested lambda DNA as a control. Ligation of a majority of the smaller fragments of Hind III-digested lambda DNA into larger fragments as demonstrated on the gel indicates a successful ligation of the source DNA into the BAC vector. This ligation is now ready for E. coli transformation by electroporation.