ISOLATION OF NUCLEAR DNA FROM PLANTS

Ya-Long Guo --- take from Peterson et al (1997, 2000) and QIAGEN® Genomic-tip Protocols, a little change.

Peterson DG, Boehm KS, Stack SM. 1997. Isolation of milligram quantities of DNA from tomato (*Lycopersicon esculentum*), plant containing high levels of polyphenolic compounds. Plant Mol. Biol. Reptr., 15, 148-153.

Peterson DG, Tomkins JP, Frisch DA, Wing RA, Paterson AH (2000) Construction of plant bacterial artificial chromosome (BAC) libraries: An illustrated guide. Journal of Agricultural Genomics 5: www.ncgr.org/research/jag.

This is an extremely robust protocol that has been used to isolate highly-pure milligram quantities of nuclear DNA from a wide variety of plants including pine, tomato, juniper, cypress, sorghum, maize, soybean, cycad, fern, prickly pear cactus, cabbage, and cotton. It is specially designed for plants in which polyphenols are a problem, although it has provided good results for every plant species on which it has been tested. If properly conducted, this protocol provides nuclear DNA that (a) has little or no visible coloration, (b) possesses a spectrophotometric (A 260-A 320)/(A 28 -A 320) value > 1.7, (c) has a mean fragment length between 50 and 100 kb, and (d) is readily digestible with *Hind*III or other common restriction endonucleases. Of note, the protocol can be used to isolate DNA from adult plant leaves as well as younger tissues (including seedlings). The protocol works best if the tissue is fresh. It generally does not work well with frozen tissue or lyophilized tissue (these processes commonly rupture organelles including nuclei). If one does not have access to fresh tissue, leaves/seedlings can be sent from a remote location *via* overnight mail. For best results, the tissue should be wrapped in damp paper towels, sealed in plastic bags, and shipped on ice.

Preparing nuclei suitable for BAC library construction can be one of the most difficult steps in making a plant BAC library. The predominant problems involved in trying to isolate plant nuclear DNA are ones that animal researchers do not typically encounter. For example, (a) plant cell walls must be physically broken or enzymatically digested without damaging nuclei, (b) chloroplasts must be separated from nuclei and/or preferentially destroyed, an important process since copies of the chloroplast genome may comprise the majority of DNA within a plant cell, (c) volatile secondary compounds such as polyphenols must be prevented from interacting with the nuclear DNA, and (d) carbohydrate matrices that often form after tissue homogenization must be prevented from trapping nuclei. While it would be ideal if there were a nuclear DNA isolation protocol that worked for all plant species, the biochemical and morphological diversity

within the plant kingdom make development of such a protocol unlikely (Loomis 1974, Peterson et al. 1997). Below we present two quite different nuclear DNA isolation protocols that we have used to construct BAC libraries from plants. **OPTION X** is a promising technique that has only recently been used in BAC library construction. **OPTION Y** (or prototypes of this protocol) has been used in the construction of BAC libraries for several years.

OPTION X is an adaptation of a procedure originally designed for isolating milligram quantities of highly pure nuclear DNA from tomato (Peterson et al. 1997, 1998). It has several features that make it well suited for use in BAC library construction as well as other molecular biology applications: (a) Prior to homogenization, tissues are treated with ether to make nuclei more friable. Ether treatment markedly increases the yield of nuclei (Watson and Thompson 1986; our observations). (b) Homogenization is performed using a simple kitchen blender. (c) The nuclear isolation buffer (MEB) is designed to deal with several common problems in plant nuclear DNA extraction. First of all, the buffer contains 2-methyl-2,4-pentanediol (MPD), a compound that helps stabilize nuclei and prevents their premature lysis. Nuclear yield using MEB is > 10 times that obtained using sucrose-based buffers (Peterson et al. 1997 and our observations). The buffer also contains the antioxidants β- mercaptoethanol, sodium diethyldithiocarbamate, and sodium metabisulfite. These compounds limit the oxidation of polyphenols. In their oxidized forms, polyphenols covalently bind to DNA turning it brown and making it useless (Katterman and Shattuck 1983; Guillemaut and Maréchal-Drouard 1992). Polyvinylpyrrolidone in the buffer adsorbs polyphenolic compounds preventing them from interacting with DNA (Loomis 1974). (d) The low pH of the buffer (pH 6.0) serves to inhibit polyphenol oxidation. (e) After homogenization, addition of Triton X-100 to a concentration of 0.5% results in preferential lysis of chloroplasts and mitochondria. The presence of divalent cations (Mg^{2+}) in the MEB prevents nuclei from being lysed by the Triton X-100 (Watson and Thompson 1986). (e) Nuclei are separated from most debris by centrifugation through a Percoll gradient. Further low-speed centrifugation steps are used to remove some, if not most, of the starch grains that typically pellet with nuclei. (f) Throughout the protocol, nuclear preparations are examined using a light microscope. This allows the investigator to visually assess nuclear concentration and purity.

We have used **OPTION X** to isolate nuclei and nuclear DNA from numerous plant species (*e.g.*, sorghum, sugarcane, grape, cotton, loblolly pine, prickly-pear cactus, fern, peanut, Leyland cypress). The protocol's usefulness in BAC library construction was first demonstrated in August 1999 when it was used to isolate DNA that subsequently was used to construct a 9X library for *Gossypium raimondii* (Peterson et al., in preparation). This success quickly was followed by construction of a 16X library for grape (*Vitis vinifera*) (Tomkins et al., in preparation) and a 9.2X library for *Gossypium hirsutum* (Acala Maxxa) (Tomkins et al., in preparation). Using **OPTION X**, agarose plugs containing nuclear DNA of suitable size and restrictability for BAC library construction have been generated for peanut (*Arachis hypogaea* 'Florunner') and tomato (*Solanum lycopersicum*). The peanut plugs are currently being used in BAC library construction.

OPTION X has produced megabase-sized, restrictable DNA from all dicots in which it has been tested, but whether it will be useful in isolating megabase-sized DNA from monocots is still uncertain. Several attempts at isolating high molecular weight DNA from *Sorghum bicolor* using **OPTION X** yielded fragments too small (< 100 kb) for BAC cloning, presumably due to partial digestion of the DNA by endogenous nucleases. Attempts to limit nuclease activity by removing Mg^{2+} (a DNase cofactor) from the nuclear isolation buffers (either directly or indirectly) resulted in premature nuclear lysis. However, addition of the nuclease inhibitors EGTA (6 mM) and L-lysine-HCl (200 mM) (see Liu and Wu 1999) to the nuclear isolation buffers permitted isolation of sorghum DNA fragments about 800 kb in length without a noticeable decrease in nuclear yield. Further research on the use of **OPTION X** to isolate megabase-sized DNA from monocots is in progress.

Another potential drawback of **OPTION X** is that it is designed for extraction of nuclei from relatively large quantities (ca. 500 g) of fresh tissue. Whether the protocol can be scaled-down to accommodate smaller quantities of tissue and/or used to isolate high molecular weight DNA from frozen tissue has yet to be tested.

MATERIALS

(1) *MEB (MPD-based Extraction Buffer*) (MADE DAY OF EXPERIMENT)
1.0 M 2-methyl-2,4-pentanediol (Aldrich, cat. no. 11,210-0), 10 mM PIPES-KOH, 10 mM $MgCl_2$, 2% polyvinylpyrrolidone (PVP) (Sigma, cat. no. PVP-10), 10 mM sodium metabisulfite, 5 mM β-mercaptoethanol, 0.5% sodium diethyldithiocarbamate (Sigma, cat. no. D-3506; store at -20°C with dessicant), pH 6.0. Prepare MEB on the day of the extraction, and chill the buffer to 4°C before starting the isolation. To make 3 L of MEB (enough for a typical experiment), place the following into a 4 L plastic beaker:

- 354.54 g of 2-methyl-2,4-pentanediol (Aldrich, cat. no. 11,210-0)
- 9.07 g of PIPES-KOH
- 6.10 g of $MgCl_2 \cdot 6H_20$
- 5.70 g of sodium metabisulfite
- 15 g of sodium diethydithiocarbamate (Sigma, cat. no. D-3506; store at -20°C with dessicant)
- 2 L of distilled water
- 1.2 ml of β-mercaptoethanol
- 6.6 g EGTA
- Mix using a stir bar
- Add 60 g of PVP (Sigma, cat. no. PVP-10) a little at a time to prevent formation of PVP clumps
- Add water or ice until the total volume is slightly less than 3 L
- Add highly concentrated HCl to the medium until the pH is 6.0. If the pH falls below 6.0, add 1N NaOH until the pH is 6.0. Place the mixture in a refrigerator

(2) *MPDB (2-methyl-2,4-pentanediol buffer)* (MADE DAY OF EXPERIMENT)
0.5 M 2-methyl-2,4-pentanediol (Aldrich, cat. no. 11,210-0), 10 mM PIPES-KOH, 10 mM $MgCl_2$, 0.5% Triton X-100, 10 mM sodium metabisulfite, 5 mM β-mercaptoethanol, pH 7.0. To make 50 ml of MPD buffer (enough for a typical experiment), place the following into a 100 ml beaker:

- 2.95 g of 2-methyl-2,4-pentanediol
- 0.15 g of PIPES-KOH
- 0.10 g of $MgCl_2$
- 0.10 g of sodium metabisulfite
- 0.25 ml of Triton X-100
- 19.5 µl of β-mercaptoethanol
- Bring the total volume to 50 ml with MBG water. Add NaOH until the final pH is 7.0. Place the solution in the refrigerator

(3) *Cheesecloth*

(4) *Miracloth*

(5) *100X TE (Tris-EDTA) stock buffer*: 1.0 M Tris base (also known as THAM), 0.1 M EDTA. To make 2 L, place 242.28 g of Tris-base and 58.44 g of EDTA (or 74.44 g of EDTA disodium salt: dihydrate) in a 4 L beaker. Add distilled water to 1.5 L. Adjust pH with concentrated HCl to yield a solution that is pH 7.0. Add distilled water to a final volume of 2 L. Place the 100X TE in glass bottles.

(6) *1X TE (non-sterile)*: Dilute the 100X TE stock solution 1:100 with distilled water.

(7) *70% v/v and 100% v/v ethanol* (-20°C)

(8) *Proteinase K* (molecular biology grade)

(9) *RNaseA/T stock solution*

(10) *Methylene blue* (1.0% w/v - aqueous)

(11) *Kitchen blender*

(12) *Light microscope*: The microscope should be capable of bright-field and/or phase-contrast illumination and have a total magnification power of at least 200x.

(13) microscope slide and coverglass

(14) *Percoll* (Amersham Pharmacia, cat. no. 17-0891-01): Percoll consists of silica particles (15-30 nm diameter) coated with PVP. Percoll is non-toxic and virtually chemically inert. When spun in a centrifuge, Percoll forms a density gradient within the

range of 1.0-1.3 g/ml. Gradients are isoosmotic throughout.

(15) Qiagen Genomic-tip 100/G cat no 10243

(16) Lysis buffer 2–8°C or room temp.
20 mM ETDA
10 mM TRIS-Cl, pH 7.9
1% Triton X-100
500 mM Guanidine-HCl
200 mM NaCl

(17) Buffer QBT (Equilibration Buffer) 2–8°C or room temp.
750 mM NaCl
50 mM MOPS pH 7.0
15% isopropanol
0.15% Triton X-100

(18) Buffer QC (Wash Buffer) 2–8°C or room temp.
1.0 M NaCl
50 mM MOPS pH 7.0
15% isopropanol

(19) Buffer QF (Elution Buffer) 2–8°C or room temp.

1.25 M NaCl
50 mM Tris·Cl pH 8.5
15% isopropanol

METHODS

DAY 1 - *Preparation*

(1A) Place 4000 ml of 1X TE in each of three polypropylene (or glass) beakers. The 1X TE is prepared from the 100X TE stock solution (see MATERIALS). To produce 4000 ml of 1X TE from the 100X TE stock solution, simply place 40 ml of stock solution in each beaker and add distilled water until the final volume in each is 4000 ml. Place these beakers of 1X TE in a refrigerator.

(1B) Place 2 L of ether in a refrigerator or freezer if it is not already stored in the cold.

(1C) Make sure 1.0% methylene blue solutions are prepared and ready for use. Also be sure to make up RNase A and RNase T1 stock solutions if they have not already been

prepared (see instructions in MATERIALS).

(1D) DO NOT MAKE UP THE MEB OR MPDB UNTIL THE DAY OF THE EXPERIMENT (these solutions lose their potency if stored for more than a few hours at 4 °C). In our experience, storing MEB and MPDB in a freezer also results in a loss of effectiveness.

DAY 2 - *Homogenization, filtration, extraction, and dialysis*

Prepare MEB and MPDB as described in the MATERIALS section. Ideally, the medium is prepared before any tissue is harvested (*i.e*., the fresher the tissue, the better the results).

1. This nuclear DNA isolation method can be used to isolate DNA from adult plants and seedlings. Collect flowering material, after removing a leaf, it is immediately submerged in a 1x TE. After collected leaves (200 – 400 g), place in 3 L of ice cold MEB.

2. Homogenize leaves/seedlings in the MEB using a kitchen blender (highest speed attainable for 30 seconds). Squeeze the homogenate through 3 layers of cheesecloth, and then filter the resulting filtrate through 1 layers of miracloth. If possible, let this second filtration occur by gravity only (*i.e.*, no squeezing).

3. Add Triton X-100 to the filtrate to a final concentration of 0.5% v/v. Fill four 500 ml centrifuge bottles with the mixture and spin at 800 x *g* for 20 min at 4°C. Decant supernatants, place the remaining homogenate in the bottles, and centrifuge as described above (this reduces the number of bottles one has to wash at the end of the experiment).

4. Resuspend each pellet in 4 ml of MPDB, and pool suspensions. Mix a small drop of the suspension with an equal volume of 1.0% methylene blue on a microscope slide. Add a coverglass and examine the slide by phase-contrast and/or bright-field microscopy. If all has gone well, the mixture should contain numerous dark-blue stained nuclei, no intact cells, and no mitochondria or chloroplasts. Starch grains (visible by phase-contrast microscopy) should stain little, if at all, with methylene blue.

5. Place 7.5 ml of Percoll in a 50 ml polypropylene centrifuge tube. Add MPDB (12.5) to a final volume of 20 ml to produce “37.5% Percoll”. Layer the suspension of nuclei onto the 37.5% Percoll bed. Centrifuge the gradient in a swinging bucket rotor at 650 x *g* for 60 minutes (4°C). Nuclei are relatively dense structures and normally form a pellet (along with large starch grains) at the bottom of the centrifuge tube. Less dense materials (*e.g.*, most cell debris, organelles, plastid DNA) remain in the supernatant.

! Note 7.2: On occasion, some or all of the nuclei will not pellet. This can occur if the batch of Percoll is more viscous than normal and/or cell debris has formed a semi-solid barrier within the gradient preventing passage of nuclei. Thus it is important not to discard the supernatant unless you are confident that the nuclei are in the pellet. It is prudent to look at a drop of the supernatant under a microscope (see step 4) before discarding it. If the supernatant has several distinct layers, examine a drop from each layer before discarding that layer. If nuclei are found in the supernatant (no matter what the cause), the simplest solution is to add 5-10 ml of MPDB, vortex the mixture, and spin the tube at 650 x g for an additional 30 minutes. If the nuclei still have not pelleted, dilute the contents further and spin again (though, in our experience, this has never been necessary).

6. Decant the supernatant, and gently resuspend the pellet in 0.5 ml of MPDB. Examine a drop of the suspension under the microscope as described in step 4 (see FIGURE 7.1). Add 30 ml of MPDB, and transfer the nuclear suspension to a 50 ml polypropylene centrifuge tube.

7. Centrifuge at 300 x *g* for 10 min, and then turn the centrifuge up to a speed equivalent to 650 x *g*. Allow the tube to spin for an additional ten minutes. Discard the supernatant.

8. The resulting pellet may or may not contain two or more distinct layers. If there is a single layer, proceed to step 11. If not, do steps 9 and 10.

9. If more than one layer is present, add two ml of MPDB and gently vortex the tube until the upper-most layer is resuspended. Pour this suspension into its own 15 ml centrifuge tube leaving the rest of the pellet in the first tube.

10. Repeat step 9 until all of the original pellet layers are segregated into different tubes. Prepare a slide from the contents of each tube (see step 4). Based on the microscopy results, pick the tube(s)/layer(s) that contains the most nuclei and the least debris. Discard all other tubes.

11. Add 10 ml of MPDB to the nuclei and mix by gentle inversion. Centrifuge the suspension at 650 x *g* for 10 minutes, and carefully decant the supernatant.
! Note 7.3: Highly pure nuclei do not form a very hard pellet and may slide out of the tube if the supernatant is discarded too forcefully.

12. repeat 11 again.

13. Gently tap the tube so that the nuclei become resuspended in the residual MPDB left in the tube. Place the tube containing the nuclei on ice.

The following step we used the QIAGEN® Genomic-tip Protocols

1. Add 60mL of lysis buffer (prewarmed to 39 degree), and divided it into two 50 ml tubes, and quickly invert to resuspend in the buffer. It is very important that the powder is evenly resuspended. So shake as much as it takes to do this but no more. Bring the volume in tube up to 27.5 mL with lysis buffer. Incubate at 39 C for 15 minutes, mixing gently several times.

2. Add RNAse A/T1 to the sample so that their final concentration in the solution are 50 units/ml and 50 ug/ml, respectively, mix gently. Incubate at 38°C for 1/2 h (invert several times gently after the first 15 minutes).

3. Make the solution 150 ug/ml Proteinase K. Incubate at 55°C for 2 h (waterbath). Invert gently several times every 30 minutes. At this point you have a big green mess.

4. Transfer to 50 ml, spin at 8,000 rpm for 15 minutes (at 4°C) (this step is just to pellet the insoluble material that was not digested by the proteinase k).

5. Equilibrate tip G-100 with 4 ml buffer QBT (takes about 5 min).

6. Pour supernatant onto tip 100 and watch it go through. You should add a little bit, watch it go through, and add a little bit more. This step can take a long time.

7. Wash the column 3 times with 7.5 ml buffer QC prewarmed to 55˚C .

8. Elute DNA with 7 ml prewarmed buffer QF (55°C) into a 15 ml falcon tube.

9. Precipitate by adding 5 ml 2-propanol, invert immediately, and rock slowly until DNA appears (it is not always visible). Make absolutely sure the sample is well mixed.

10. Spin at 5000 g for 40 minutes to pellet DNA.

11. Wash pellet with 70% EtOH, spin again 20 min, immediately discard supernatant and air-dry pellet.

12. Air-dry and Resuspend in 1 x TE (pH = 8.0) overnight at 4° C. Flick the next morning to make sure it is in solution. Store at -20° C.