

## **PULSED-FIELD GEL ELECTROPHORESIS OF *ARABIDOPSIS THALIANA* CHROMOSOMAL DNA**

**Gregory P. Copenhaver and Craig S. Pikaard**  
**Biology Department, Washington University**  
**Campus Box 1137, One Brookings Drive, St. Louis, MO 63130**

**Introduction:** Pulsed-field gel electrophoresis (PFGE) was developed in 1982 by Schwartz et. al. as a means of resolving very large DNA molecules [1]. PFGE can be used to separate DNA molecules from 10 kbp to approximately 10 Mbp and is an invaluable tool for genomic analysis. Contour-clamped Homogeneous Electric Field (CHEF) electrophoresis [2], a variety of PFGE, produces sharp, reproducible bands in straight lanes and can be used in a variety of applications including RFLP analysis of large restriction fragments, sizing and isolation of YACS and BACS, and direct visualization of small chromosomes. The following is a detailed, "cookbook" protocol for one and two-dimensional CHEF analyses of *Arabidopsis* DNA. We have used these protocols in several papers, referenced at the end [3, 4, 5]. For simplicity we have optimized all conditions for *Arabidopsis* and for the BioRad CHEF DRII system.

### **Materials and Resources**

**Germination Media (GM) plates:** 0.43% MS salts + Gamborg vitamins (Sigma), 2.0% sucrose, pH to 5.7 with KOH, 0.8% Agar (Sigma)

Autoclave 20 minutes with no more than 500 ml/ liter bottle, and pour 25 ml/petri dish under sterile conditions in the laminar flow hood.

**Protoplasting solution:** 0.4M mannitol, 0.33% cellulysin (Calbiochem), 0.17% macerozyme R-10, 3 mM MES pH 7.5 (pH with KOH), 7 mM CaCl<sub>2</sub>

Optionally, Glutathione (Sigma) may be added to a concentration of 5 mM (23 mg/15 ml) as a free radical scavenger.

**NDS:** 500 mM EDTA, 1% N-laurylsarcosine, 1 mM Tris

For 1 L dissolve the EDTA and Tris in 700 ml of ddH<sub>2</sub>O by adding solid NaOH to a pH between 8.0 - 9.0. In a separate vessel dissolve the N-laurylsarcosine in 100 ml ddH<sub>2</sub>O (helps to use 65°C bath). Add the sarcosine solution to the EDTA solution and pH to 9.5, and add ddH<sub>2</sub>O to volume.

**T<sub>10</sub>E<sub>10</sub>:** 10 mM Tris pH 8.0, 10 mM EDTA pH 8.0

**Restriction Enzyme Reaction Solution:** 1X restriction enzyme buffer, 8 mM spermidine\*, 0.1 mg/ml BSA, 50 units restriction enzyme

\* Spermidine (Sigma) should only be added to those reactions that have at least 50 mM salt (spermidine will inhibit endonuclease activity in low salt).

**STOP:** 0.5X TAE (Tris Acetate EDTA gel buffer), 10 mM EDTA (pH 8.0), 1% Fidy (20 % Ficol 400, 100 mM EDTA (pH 8.0), 1% SDS, 0.1% Orange G)

**Gel Denaturing Solution:** 400 mM NaOH, 600 mM NaCl

**Gel Neutralizing Solution:** 1.5 M NaCl, 500 mM Tris pH 7.5

**20X SSC:** 3 M NaCl, 0.3 M sodium citrate  
adjust pH to 7.0

**Prehybridization Solution:** 6X SSC, 0.5% SDS, 5X Denhardt's solution, 100 µg/ml denatured, sheared, salmon sperm DNA or yeast tRNA

Prehybridization solution should be made fresh and added at a volume equal to 0.2 ml/cm<sup>2</sup> of filter membrane. Also, salmon sperm DNA (Sigma) is a good inexpensive blocking agent but causes reduced signal and high background with some probes (such as telomere probes). In such cases we substitute yeast tRNA (Boehringer Mannheim) which is considerably more expensive.

**50X Denhardt's:** 1% Ficol 400, 1% polyvinylpyrrolidone, 1% BSA (fraction V)

**Hexamer labeling reaction**

4X reaction stock*	15 ul
BSA (10 mg/ml)	2.4 ul
Klenow fragment of DNA polymerase I(NEB)	1.0 ul
alpha- <sup>32</sup> P-dCTP (3000 Ci/mM)	6.0 ul
alpha- <sup>32</sup> P-dATP (3000 Ci/mM)	6.0 ul

\***4X reaction stock:** 200 mM Tris-Cl pH 8.0, 20 mM MgCl<sub>2</sub>, 20 mM DTT, 80 uM dNTP, 800 mM HEPES (not pH'd), 27 units Hexamer (Pharmacia)

To make the HEPES stock dissolve 4.766 g of the free acid (Sigma) in 10 ml of ddH<sub>2</sub>O and store as 1 ml aliquots at -20 C.

To make the hexamer stock dissolve 50 A260 units of pd(N)<sub>6</sub> (Pharmacia) in 556 ul ddH<sub>2</sub>O and store at -20 C.

To make the 1 mM dNTP stock mix equal volumes of 1 mM dGTP and TTP (or which ever two you haven't used as hot nucleotides).

**Hexamer Labeling Stop Solution:** 200 mM EDTA pH 7.2, 1% SDS, 40 mg/ml Blue Dextran, 0.1 mg/ml Bromphenol Blue

**Sephadex:** Add 100g of dry sephadex G-50 powder to 1 liter of G-50 buffer(10 mM Tris-Cl pH 7.5, 2 mM NaCl, 0.5 mM EDTA, 0.1% SDS). Swirl, cover and let stand for an hour. Pour off excess buffer and replace with fresh buffer, mix and let settle again. Repeat the wash and autoclave.

**Hybridization Mix:** 6X SSC, 10 mM EDTA, probe DNA, 5X Denhardt's solution, 0.5% SDS, 100 µg/ml salmon sperm DNA

Use 0.05 ml/square cm of membrane. See notes on salmon sperm DNA under pre hybridization.

**The Nottingham Arabidopsis Stock Centre**

Dr. Mary Anderson, NASC, Dept. of Life Science, University of Nottingham, University Park, Nottingham NG7 2RD, U.K.; Tel +44 602 791216; email PL2MLH@UK.AC.Nottingham.ccc.vax

**Arabidopsis Biological Resource Center**

The Ohio State University, 1735 Neil Avenue, 309 Botany & Zoology Bldg, Columbus Ohio 43210, USA; Tel 614-292-9371; FAX 614-292-0603; email arabidopsis+@osu.edu

**Lehle Seeds**

P.O. Box 2366, Round Rock TX, 7680-2366, USA. Tel: 1-800-881-3945, FAX: 1-572-388-3974, World Wide Web site = <http://www.arabidopsis.com>

**Small Parts Inc.**

13980 N.W. 58th Court, P.O. Box 4650, Miami Lakes Fl 33014-0650; Tel 305-557-8222; FAX 1-800-423-9009

**Grace-Sierra Horticultural Products Company**

1001 Yosemite Dr., Milpitas, CA 95035

## I. Growing plant material

Whenever possible choose a reliable source for seed and document strain and batch numbers. Often samples which are called by similar names will be different genetically. For instance, there are at least 5 different strains of *Arabidopsis thaliana* commonly called Landsberg and each one gives distinctive rDNA polymorphisms (unpublished data). It is therefore imperative to document the history of the strains you use. Our lab uses The Nottingham Arabidopsis Stock Centre and The Ohio State University Arabidopsis Biological Resource Center. Both these resources provide material (seeds, DNA stocks etc...) free of charge to academic researchers. Lehle Seeds is a good commercial supplier.

Optimally plants should be grown in sterile conditions [6, 7]. Sterile growing conditions will prevent errors due to contamination by bacterial, fungal and insect DNA. To grow sterile plants, add the desired amount of seed (~10 uL of seeds/plate to be planted) to an eppendorf tube and fill with 95% ethanol. Incubate at room temp for 2-3 minutes. Remove the ethanol and add 700 uL of ddH<sub>2</sub>O and 700 uL of commercial bleach (Chlorox) to the tube. Adding 0.01% Triton X-100 makes the seeds much easier to handle but it is not necessary for sterilization. Incubate at room temperature for 20-30 minutes with occasional mixing. From this point on all work should be done in a laminar flow hood. In the hood remove the chlorox solution and rinse the seeds four times with 1 ml of sterile water, leaving the final ml in the tube with the seeds. Using a wide bore pipette tip (these can be made manually by cutting the top off a 200 uL tip or they can be purchased) take up 25 uL of seeds and sow the seeds onto GM plates at a density of about 1-2 seeds/0.5 cm<sup>2</sup> or 150 seeds/plate; do this by touching the tip of the pipette to the media repeatedly. Generally, it is a good idea to sow approximately 5 plates per stain/line to be examined. This will give enough agarose-embedded DNA for more than 100 restriction digests. If seeds are limiting one can make a single agarose plug from as few as six two-week old plants. Also, don't try to sow seeds of too many strains/lines in one day as it is difficult to process large numbers later in the procedure. We usually plant 1-4 lines in a day. Wrap the plates of sown seeds with parafilm and poke holes in the parafilm with a needle to allow gas exchange. Incubate the plates in a growth chamber (16 hr 24 C days 8 hr 21 C nights) for 2 weeks. Any plates with contaminating fungus or bacteria should be discarded. If contamination is a recurring problem, seeds can be sown on GM plates containing carbenicillin. However this could conceivably change your experimental results.

It is possible to harvest DNA from individual plants when the experiment demands, for example when sib analysis is necessary. In this case plant seeds, sterilized as described above, in 3 inch pots which contain autoclaved potting mixture (rediearth:vermiculite, 60:40) that has been saturated with sterile water. Place these pots in a plastic flat and cover the flat with a clear plastic dome lid. Place the flat in a short day (8 hr. light, 24 C) growth chamber. Keep the flat covered until the plants germinate and then crack the lid for 2-3 days before removing it to acclimate the plants to a lower humidity. The soil should be allowed to dry (but not too dry!) before watering. Also the plants tend to grow better if they are given 15:16:17 Peters fertilizer (Grace-Sierra Horticulture Products Co.) at 125-150 ppm nitrogen once a week or so. Once the rosettes are large and bushy (6-9 weeks depending on the ecotype) harvest the leaves giving preference to the smallest and youngest which will yield better protoplasts. This method will yield enough protoplasts to make 1-2 agarose plugs. However, the quality of the DNA is somewhat poorer than that derived from young tissue grown on GM plates such that the background following Southern blotting and hybridization is higher.

## II. Harvesting plant material

Generation of plant protoplasts is achieved by the method of Doelling and Pikaard with slight modification [6, 7]. After the plants have grown for two weeks take them to the hood and pull them from the agar with forceps. Combine the plants from 2 plates in the lid of one of the petri dishes and lightly mince the plants into small pieces using surgical scissors. Immediately following mincing place the plant material in a clean petri dish and add 15 ml (or enough to cover the plant material) 0.5M mannitol (in water) to plasmolyze the cells for 30 min. - 1 hr. After plasmolysis remove the mannitol using a pipette and add 15 ml of Protoplasting Solution. Protoplasting solution should be made immediately prior to use and filter sterilized using a 0.22 or 0.45  $\mu$ m vacuum filtration unit. Wrap the petri dish with parafilm, cover with aluminum foil, and place on a rotary shaker with gentle agitation ( 48 rpm on our Bellco orbital shaker). Allow the digestion to proceed overnight.

Separate liberated protoplasts from unwanted plant material by pouring the digestion mixture through successively finer stainless steel wire mesh. We use 1.13 mm, 230  $\mu$ m, and 38  $\mu$ m mesh steps (mesh was purchased from Small Parts Inc.). Our filter units were constructed by gluing mesh disks (cut from sheets supplied by the manufacturer) to the bottoms of 50 ml plastic conical tubes with the bottom cone cut off. Minimizing physical trauma to the protoplasts is critical, therefore avoid pipetting whenever possible. When using the smallest mesh it is necessary

to pre-wet the filter from below by placing the filter onto a drop of solution. This overcomes surface tension and removes trapped air bubbles so that the protoplasts in solution can flow through. Transfer the final filtrate to a clean 50 ml conical tube and centrifuge at 26 X g for 10 minutes in a swinging bucket rotor at room temperature. Remove the supernatant (usually very green) and resuspend the pellet containing the protoplasts in 14 ml of 0.34 M mannitol, 0.07 M CaCl<sub>2</sub> and transfer to a 15 ml conical tube. Centrifuge the protoplasts again at 26 X g for 10 minutes, remove the supernatant and resuspend the protoplasts as before. Count the protoplasts on a hemacytometer and adjust the volume to give the desired protoplast/ml. For our studies of the chromosomal arrangement of rRNA genes, a high copy gene, we use  $1.8 \times 10^7$  protoplasts/ml. Anand and Southern [8] recommend a concentration of  $1 \times 10^7$  protoplasts/ml for high copy number genes and  $3 \times 10^7$  for low or single copy number genes. Concentrations as high as  $5 \times 10^7$  can be used but band sharpness and resolution suffer. As a general rule lower protoplast concentrations yield better resolution but the lower the DNA content, the harder it is to detect rare sequences and autoradiography time increases. Once you have determined your desired protoplast concentration adjust the volume of your protoplast suspension to accommodate this concentration. To increase concentration spin the protoplast suspension at 25 X g for 10 minutes and remove the appropriate amount of supernatant and resuspend the protoplasts in the remaining supernatant. To decrease concentration add the appropriate amount of 0.34 M mannitol, 0.07 CaCl<sub>2</sub>.

### III. Preparing agarose embedded DNA

Agarose embedded DNA is prepared according to Anand and Southern's protocol for cultured animal cells with minor modifications [8]. Following resuspension of the protoplasts at the correct concentration, prepare an equal volume of 2% lowmelt agarose solution (we use Fischer DNA grade low-melt agarose) in 50 mM TBE (50 mM Tris, 50 mM Borate, 1 mM EDTA, 0.5X TBE is 45 mM with respect to the Tris and Borate) and heat in a boiling water bath until the agarose is molten. Equilibrate the temperature of both the protoplasts and the agarose to 37 C in a water bath (about 5-10 minutes) then mix the agarose and the protoplast solution together. Immediately pipette the mixture into plug molds. We use BioRad plug molds with dimensions of 0.9 cm (width) X 2 cm (height) X 0.1 cm (thickness). The width and thickness of the plugs should match the width and thickness of the comb used to form the wells of the CHEF gel. Place the filled plug molds on ice to quickly harden the agarose before the protoplasts can begin to settle towards the bottom of

the molds. Once hardened (5-10 minutes) removed the agarose plugs from the molds with a small spatula into a 50 ml conical tube containing NDS with 1 mg/ml pronase (pronase is a much cheaper substitute for proteinase K; we buy ours from Sigma). We usually put 10-40 plugs into 40 ml of NDS per 50 ml conical tube. Place the tube containing the plugs in a 55 C water bath and let it incubate overnight. Replace the NDS/pronase with fresh solution and let it incubate overnight again. Replace the NDS/pronase with fresh NDS WITHOUT pronase and incubate at 55 C overnight again. Replace the NDS with fresh NDS and store at 4 C. At this point the plugs should have lost all green color. Plugs made from young tissue grown on GM media should be a translucent white/tan color whereas plugs made from older soil-grown tissues are often a more opaque white/tan color. These plugs in NDS are stable for years. We have used 3 year old plugs to resolve pieces of DNA as large as 3 Mb with no appreciable differences in DNA quality as compared to fresh plugs.

#### IV. Restriction digests of agarose embedded DNA

Agarose-embedded DNA is digested with restriction endonucleases according to the methods of Van Daelen and Zable [9]. Place a piece of plug slightly smaller than the well of a CHEF gel (we use plugs cut into thirds with final dimensions of ~ 0.9 cm X 0.6 cm X 0.1 cm) in a 15 ml conical tube containing 10 ml of T<sub>10</sub>E<sub>10</sub> with 2 mM PMSF (Sigma) and allow the plug to equilibrate 1 hour (to overnight) at 4 C (this equilibration is done at 4 C because PMSF is fairly unstable at room temperature in aqueous solution). Replace the solution with fresh T<sub>10</sub>E<sub>10</sub> WITHOUT PMSF and equilibrate for 30 min. at room temperature. Repeat the T<sub>10</sub>E<sub>10</sub> washes 3 times. Place the sample in a microcentrifuge tube containing 1 ml of the desired 1X restriction enzyme buffer without the DTT and BSA and let it equilibrate for 1 hour. We find that using 2 ml flat bottomed microcentrifuge tubes reduces breakage of the agarose plug. Repeat the buffer wash once more. Remove the buffer wash and add an appropriate volume of reaction solution. We use 250 uL of reaction solution for our sample size (larger sample may require more solution to cover the plugs). Incubate the agarose embedded DNA with the restriction enzyme at the appropriate temperature for 8 hours (or overnight). Keep in mind that when using restriction enzymes that require high temperatures, such as BstE II, the samples are embedded in low-melt agarose. Therefore, reactions should not exceed 55 C. After the initial incubation, add 50 additional units of restriction enzyme and incubate for an additional 6 hours. This results in 100 units of enzyme used per reaction which, based on our experience performing deliberate partial digestion series, should be a vast excess of what is necessary to

achieve complete digestion[10]. It should also be noted that when choosing a restriction enzyme, close attention should be paid to its sensitivity to methylation, particularly CpG and CpNpG methylation [11].

## V. Running the CHEF gel

Preparation and running of the CHEF gel is based on the manufacturer's recommendations [12-15]. To pour the gels, make a fresh agarose solution for each gel. This avoids changes in agarose concentration due to multiple heatings and subsequent buffer evaporation. We use a 13 cm X 14 cm casting tray and we pour 110 ml gels. The casting tray is leveled each time with a bubble level to assure constant gel thickness. The well combs are placed at a distance of 0.8 cm from the top edge of the gel, with a 0.1 cm gap between the bottom of the comb and the casting tray. We use 1% pulsed-field certified DNA grade agarose (BioRad) for 10 kbp - 1.5 Mbp separations and 0.8% chromosomal grade agarose (BioRad) for 1.5 - 3.0 Mbp separations. All gels are made in 50 mM TBE. The agarose solution is heated in a microwave on medium power with frequent mixing, to avoid boiling over, and allowed to cool until it is comfortable to handle with bare hands. Once cooled sufficiently, the agarose is poured into the casting tray. The gel is allowed to harden completely before loading.

After the samples are digested with the appropriate restriction enzyme(s) remove the reaction solution and replace it with 500 uL STOP solution and allow the samples to equilibrate for 5 - 10 minutes. Pour the sample into a plastic petri dish, and using a plastic micro-spatula, scoop the plug up and place it into a well in the gel (using a 1 ml pipette tip to guide the plug helps). Useful DNA size standards include *Saccharomyces cerevisiae* chromosomes (NEB) for 100 kbp - 1.5 Mbp separations and *Hansenula wingei* chromosomes (BioRad) for 1 - 3 Mbp separations. Once the sample and controls are loaded, seal each well with molten low melt agarose of the same concentration as the gel using a syringe fitted with a medium gauge needle. Be careful to avoid introducing air bubbles. Allow the seals to harden for 5 minutes.

We run our CHEF apparatus in a 4 degree C cold room with no further cooling. The buffer (2 liters of 50 mM TBE) is pre-equilibrated to cold-room temperature and is circulated in the running tank at a rate of 1 liter per minute. Under these conditions the mean temperature of the gel is ~14 degrees C. The parameters for switch time, voltage, and run length will, of course, vary with the size of DNA fragments being separated. We find that a good starting program for 10 kbp - 1.5 Mbp separation is as follows:

**Stage I:**

initial switch time	60 seconds
final switch time	60 seconds
run time	14 hours
voltage	200 V

**Stage II:**

Initial switch time	90 seconds
final switch time	90 seconds
run time	8 hours
voltage	200 V

For 1.5 - 3.0 Mbp separations a good starting program is:

**Stage I:**

initial switch time	1200 seconds
final switch time	1800 seconds
run time	30 hours
voltage	67 V

**Stage II:**

Initial switch time	90 seconds
final switch time	180 seconds
run time	11 hours
voltage	200 V

## **VI. Southern analysis of CHEF gels**

After the CHEF gel run is complete, remove the gel from the running tank and place it in a deep plastic tray containing 500 ml 50 mM TBE containing 1.5 ug/ml ethidium bromide (this solution can be reused several times). Incubate the gel in the staining solution for 30 minutes at room temperature while gently agitating on a rotary shaker. Following staining, visualize the DNA in the gel using a short wavelength UV transilluminator. Record a photographic image of the gel for your records. Often for gels resolving large pieces of DNA, bands will not be visible with EtBr.

Because of the large size of the DNA fragments, it is necessary to nick them to achieve efficient transfer [16]. We use a BioRad GS Gene Linker UV Chamber on setting 'nic', which delivers 60 mJoule of 254 nm UV light. After nicking, denature the gel in 500 ml of fresh Denaturing Solution for 30 minutes at room temperature with gentle agitation. Following denaturation, neutralize the gel in 500 ml of Neutralizing Solution for 30 minutes at room temperature with gentle agitation (the gel has a tendency to float in this high salt solution so agitation should be made sufficient to assure submersion). Following neutralization, proceed

with standard Southern transfer using 10X SSC as a transfer buffer. We use Nitrobind nitrocellulose as a transfer membrane (MSI) which we prewet with 6X SSC before blotting. Following Southern transfer, rinse the membrane briefly with 6X SSC to remove any agarose and crosslink the DNA to the membrane using UV light. Again, we use the BioRad Gene Linker on program 'C3' which delivers 150 mJoule of 254 nm UV light. At this point the blot can be stored in 6X SSC in a sealed plastic bag or it can be probed immediately. We never allow our blots to dry.

To probe the blot, place it in a heat-sealable plastic bag containing Prehybridization Solution (avoiding bubbles) and incubate in a 65 degree C water bath overnight (incubation times as short as 4 hours are sufficient). While the gel is pre-hybridizing label the probe to be used. We use the hexamer labeling protocol of Feinberg and Vogelstein [17] but other methods could be used. For hexamer labeling dilute the template (probe) DNA to 100 ng/30 ul. For template DNA we use plasmid fragments of the appropriate sequence that have been isolated on agarose gels and purified using the Gene Clean kit (BIO101). Also, template DNA should be at least 400 bp for hexamer labeling. Boil 30 ul of the template DNA for 5 minutes then immediately cool the sample on ice. Once the sample has cooled add the remaining Hexamer Labeling Reaction ingredients. Mix the reaction components well and spin briefly in a microcentrifuge to remove drops from the side of the tube, and incubate overnight. Note that both the membrane prehyb and the hexamer labeling reaction are incubating during the same night. The following day add 66 ul of Hexamer Stop Solution to the labeling reaction. Plug a 5 inch glass Pasteur pipette with glass wool at the point where the aperture begins to narrow. Fill the pipette with Sephadex G-50 in G-50 buffer until the bed of resin reaches 1 cm below the top of the column. When the meniscus of the G-50 buffer reaches the bed of resin carefully pipette the stopped reaction onto the column. Once the sample has entered the column add more G-50 buffer. Collect the fractions that contain the Blue Dextran (faster migrating of the two dyes in the STOP solution). These fractions will contain the labeled DNA. The unincorporated nucleotides will migrate more slowly, usually with the Bromphenol blue (slower) dye. Weigh a microcentrifuge tube, transfer the labeled probe to the tube and weigh it again to estimate the volume. Remove 5 ul of the probe and count it in a scintillation counter to determine the specific activity of the probe. Heat the remaining probe in a boiling water bath to denature the DNA (be careful to secure the top of the tube with a tube lock or use a screw cap tube) for 5 minutes. Immediately chill the probe on ice for 10 min. Add the cooled probe to the Hybridization Mix. Remove the prehybridization solution from the blot, replace with Hybridization Mix and probe, and reseal the sealable bag (being careful to avoid bubbles). Place the bag in a shaking water

bath, secure it below the surface of the water, and incubate with gentle agitation at 67 degrees C overnight.

Following hybridization, move the blot from the bag to a plastic container with a sealable lid or to a fresh sealable bag. Wash the blot once with 300 ml 2X SSC, 0.5% SDS at room temperature for 15 minutes, once with 300 ml 2X SSC, 0.1% SDS at room temperature for 15 minutes, and once with 300 ml 0.1X SSC, 0.5% SDS at 68 degrees C for 2 hours. Rinse the blot briefly with 50 ml of 0.1X SSC, 0.5% SDS and wrap the damp blot in plastic wrap. Tape the edges of the plastic wrap to prevent any leakage and visualize the blot by autoradiography or phosphorimaging.

Stripping and reusing of CHEF gel blots has proved difficult and inconsistent in our hands so we don't do it.

## **VII. Two Dimensional CHEF analysis**

For 2D analysis, run the first dimension as described above but before staining the gel with EtBr use a straight edge and a scalpel to slice half (lengthwise) of the lane of interest out of the gel. Using only half of the first dimension lane for the second dimension serves two purposes. First, it leaves half of the lane on the original gel which can then be processed and used as a control for the second dimension. Second, it makes the bands (or spots) in the second dimension much tighter and easier to differentiate. Place the first dimension gel slice in a small container with 100 ml of the 1X restriction enzyme buffer to be used in the second dimension, amended to contain 2 mM spermidine, 1 mM DTT, and 2 ug/ml BSA, and incubate at room temperature for 2 hours [18, 19]. Repeat the wash once more for 2 hours and then place the gel slice in a sealable bag containing 10 ml of the appropriate 1X restriction enzyme buffer amended to contain 2 mM spermidine, 1 mM DTT, 2 µg/ml BSA, and 1000 units of restriction enzyme. Seal the bag, avoiding bubbles, and incubate at the appropriate temperature 8 hours to overnight. Following the initial incubation add 1000 additional units of restriction enzyme and incubate for an additional 6 hours. After digestion remove the gel slice from the bag and place it in a tray with 200 ml of 50 mM TBE and incubate at room temperature with gentle agitation for 1 hour. Repeat the wash once more for 1 hour. Following the final wash, lay the gel strip in the casting tray perpendicular to the direction of electrophoresis and pour the gel as before. It is useful to use the well combs to hold the gel slice in place while initially pouring the gel but be sure to remove them before the gel sets. Once the gel has hardened use the appropriate electrophoresis regime as described above. It is also sometimes useful to run a CHEF gel in the first dimension and a conventional gel in the second dimension if products resulting from the

second digestion are small. To see real-life examples of gels resulting from these protocols, see the recent papers by Gregory Copenhaver [3-5].

## References

1. Schwartz, D.C., Saffran, W. Welsh, J., Haas, R., Goldenberg, M., and Cantor, C.R. (1982) Cold Spring Harbor Symp. Quant. Biol. 47, 198-195
2. Chu, G., Vollrath, D., and Davis, R.W. (1986) Separation of large DNA molecules by contour-clamped homogeneous electric fields. *Science*, 234, 1582-1585
3. Copenhaver, Gregory P., Jed H. Doelling, J. Scott Gens and Craig S. Pikaard (1995). Use of RFLPs larger than 100 kbp to map the position and internal organization of the nucleolus organizer region on chromosome 2 in *Arabidopsis thaliana*. *The Plant Journal* 7: 273-286.
4. Copenhaver, Gregory P. and Craig S. Pikaard. RFLP and physical mapping with an rDNA-specific endonuclease reveals that nucleolus organizer regions of *Arabidopsis thaliana* adjoin the telomeres on chromosomes 2 and 4. *The Plant Journal* 9, 259-272
5. Copenhaver, Gregory P. and Craig S. Pikaard (1996). Two-dimensional RFLP analyses reveal megabase-sized clusters of rRNA gene variants in *Arabidopsis thaliana*, suggesting local spreading of variants as the mode for homogenization during concerted evolution. *The Plant Journal* 9, 273-282
6. Doelling, J.H. and Pikaard, C.S. (1993). Transient expression in *Arabidopsis thaliana* protoplasts derived from rapidly established cell suspension cultures. *Plant Cell Rep.* 12, 241-244.
7. Doelling, J.H., Gaudino, R.J. and Pikaard, C.S. (1993). Functional analysis of *Arabidopsis* rRNA gene and spacer promoters *in vivo* and by transient expression. *Proc. Natl. Acad. Sci. USA*, 90, 7528-7532.
8. Anand, R. and Southern, E. (1990) Pulsed field gel electrophoresis. In *Gel Electrophoresis of Nucleic Acids a Practical Approach, 2nd Edition*, (Rickwood, D. and Hames, B.D., ed.) IRL press, Oxford p. 101-123
9. Van Daelen, R., and Zabel, P. (1992) Pulsed-Field Gel Electrophoresis, in *Plant Genomes: Methods for Genetic and Physical Mapping*, (Beckmann, J.S., and Osborn, T.C., ed.) Dordrecht: Kluwer Academic Publishers

10. New England Biolabs Corporation Bulletin: *The NEB Transcript*.  
Volume 3. No. 1 1991

111. McClelland, M., Nelson, M., Raschke, E. (1994) Effect of site-specific modification on restriction endonucleases and DNA modification methyltransferases. *Nucleic Acid Res.* 22, (No. 17) 3640-3659

12. *CHEF-DR II Pulsed Field Electrophoresis System Instruction Manual and Application Guide*. (1988) BioRad Corporation

13. *Applications in Pulsed Field Electrophoresis*. BioRad Bulletin 1593

14. *Addendum to Pulse Controller Manual*. BioRad Bulletin 1631 A

15. Birren, B. (1990) *Preparation and Separation of Mammalian DNA by Pulsed Field Gel Electrophoresis*. BioRad Bulletin 1589

16. *UV Nicking of DNA in Agarose Gels for Enhanced Transfer and Detection of Megabase Size DNAs*. (1991) BioRad Bulletin 1672

17. Feinberg, A.P. and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. BioChem.* 132, 6-13.

18. Warburton, P.E., Wevrick, R., Mahtani, M.M. and Willard, H.F. (1992). Pulsed-field and two dimensional gel electrophoresis of long arrays of tandemly repeated DNA. In *Methods in Molecular Biology, Vol 12: Pulsed-field Gel Electrophoresis* (Burmeister, M. and Ulanovsky, L. ed.) The Humana Press Inc. Totowa, NJ p. 299-317

19. Bautsch, W. (1992) Bacterial Genome Mapping by Two-Dimensional Pulsed-Field Gel Electrophoresis (2D-PFGE), in *Methods in Molecular Biology, Vol. 12: Pulsed-Field Gel Electrophoresis*, (Burmeister, M., Ulanovsky, L., ed.) The Humana Press Inc. Totowa, NJ p. 185-201