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Analysis of rRNA gene methylation in *Arabidopsis thaliana* by CHEF-Conventional 2D gel electrophoresis

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Summary

Contour-clamped homogenous electric field (CHEF) gel electrophoresis, a variant of Pulsed-field gel electrophoresis (PFGE), is a powerful technique for resolving large fragments of DNA (10 kb to 9 Mb). CHEF has many applications including the physical mapping of chromosomes, artificial chromosomes and sub-chromosomal DNA fragments, etc. Here we describe the use of CHEF and two-dimensional gel electrophoresis to analyze rRNA gene methylation patterns within the two ~ 4 million base pair nucleolus organizer regions (NORs) of *Arabidopsis thaliana*. The method involves CHEF gel electrophoresis of agarose-embedded DNA following restriction endonuclease digestion to cut the NORs into large but resolvable segments, followed by digestion with methylation-sensitive restriction endonucleases and conventional (or CHEF) gel electrophoresis, in a second dimension. Resulting products are then detected by Southern blotting or PCR analyses capable of discriminating rRNA gene subtypes.

Keywords

Arabidopsis; 45S rRNA gene; rDNA; Nucleolus organizer region (NOR); DNA methylation; Protoplast; Pulsed-Field Gel Electrophoresis (PFGE); Contour-clamped Homogenous Electric Field (CHEF); two-dimensional gel analysis; Southern blotting

1. Introduction

Pulsed-field gel electrophoresis (PFGE) is an invaluable technique to resolve large fragments of DNA (10 kb to 9 Mb) that cannot be resolved by conventional gel electrophoresis [1,2]. Contour-clamped homogenous electric field (CHEF) gel electrophoresis is a variation of PFGE that enables excellent resolution of long DNA molecules [3]. The CHEF procedure involves the application of an electric field that alternates between two orientations at a predetermined angle [3,4]. For the analyses of chromosomes and subchromosomal fragments whole cells are first embedded in an agarose gel matrix and digested with

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¹Although MS plates can be prepared in advance and stored at 4°C, we recommend preparing them fresh before use. A drop of 1 M KOH solution is enough to bring the pH of MS solution to 5.7. While pouring and further handling MS plates take care not to contaminate the media! For small plates (100 mm × 20 mm) pour ~ 25 mL of media and for large plates (150 mm × 25 mm), pour ~ 120 mL of media. Partially open the lids and allow the plates to dry for about 45 min in a laminar flow hood.

proteases and one or more restriction endonucleases that cut the chromosomes within the agarose matrix, whose dimensions match the wells of an agarose gel [5,6]. The agarose “plugs” containing the cut DNA are then inserted into the wells and electrophoresis is conducted.

CHEF gel electrophoresis conducted either in one or two dimensions, combined with the Southern blotting technique [7–10], has been useful for multiple applications including, Restriction Fragment Length Polymorphism (RFLP) analysis, physical mapping of gene order, sizing and isolation of intact yeast chromosomes or Bacterial Artificial Chromosomes (BACs) or Yeast Artificial Chromosomes (YACs), analysis of genomic polymorphism in bacteria called epidemiological typing, etc. [5,11,12,1,13,4].

45S ribosomal RNA (rRNA) genes in eukaryotes are tandemly repeated at chromosomal loci known as nucleolus organizer regions (NORs) [14–16]. In *Arabidopsis thaliana*, rRNA genes are organized into two NORs; NOR2 and NOR4 on chromosomes 2 and 4, respectively, with each NOR consisting of 350–400 gene copies [17,11]. About half of the rRNA genes are silenced during development by repressive chromatin modifications, including DNA hypermethylation [18–21]. Recent evidence has indicated that rRNA gene silencing in *Arabidopsis* takes place at a subchromosomal level, affecting NOR2. A prediction is that DNA fragments derived from NOR2 are expected to be hypermethylated relative to NOR4. This hypothesis can be tested by analyzing rRNA gene methylation within large DNA fragments derived from the two NORs. We describe here a method to perform such studies using a two-dimensional gel electrophoresis approach. The method involves CHEF gel electrophoresis of agarose-embedded DNA following restriction endonuclease digestion with an enzyme that cuts infrequently within one or both NORs, followed by CHEF gel electrophoresis to resolve the fragments in a first dimension. The products resolved in the first dimension are then subjected to in-gel restriction digestion using a methylation-sensitive endonuclease, followed by a conventional (or CHEF) gel electrophoresis in a second dimension. Southern blotting, probing and detection of resulting products provide insight into the methylation status of large DNA fragments derived from the different NORs.

2. Materials

All solutions should be made using ultra pure water with 18.2 MΩ•cm (25°C) resistivity (e.g. Milli-Q water, type 1) and analytical/molecular biology grade reagents. Filter-sterilize the solutions using a 0.22 or 0.45 μm vacuum filtration unit and store them at room temperature (unless indicated otherwise). Follow recommended safety procedures for handling and disposing of chemicals.

2.1 Seed sterilization and plant growth

1. Seed Sterilization Solution: 76% ethanol, 20% commercial bleach (*see* Note 1), 4% water.

¹Bleach made by the Clorox company works fine but bleach from some of the companies can be harsh on seeds and could severely affect *Arabidopsis* seed germination rate. Ensure the safety of your Seed Sterilization Solution before performing any experiment.

2. Murashige & Skoog (MS) plates (0.5× strength): For 1 L of MS media dissolve 2.2 g of MS salts (with vitamins) in water and add sucrose to a final concentration of 1% (10 g/L). Adjust pH of the solution to 5.7 with potassium hydroxide (KOH). Add 5 g of phytoagar, autoclave for 20 min and then pour MS media into sterile petri dishes (100 mm × 20 mm) under sterile conditions in a laminar flow hood (*see* Note 2).

2.2 Materials needed to generate protoplasts embedded in agarose plugs

1. Protoplasting Solution (prepare fresh prior to use): 0.4 M mannitol, 0.33% cellulase (10 ku/g), 0.17% macerozyme R-10 (*see* Note 3), 3 mM MES Buffer, pH 7.5 (adjust pH with KOH), 7 mM CaCl₂, 5 mM glutathione.
2. Filtering devices with the following pore sizes: 1.13 mm, 230 μm, 38 μm (*see* Note 4).
3. Protoplast Resuspension Solution: 0.34 M mannitol, 7 mM CaCl₂.
4. 50 mM TBE: 50 mM Tris, 50 mM Borate, 1 mM EDTA.
5. Reusable agarose plug molds (Bio-Rad Cat#1703622).
6. NDS solution: 100 mM EDTA (*see* Note 5), 1% N-laurylsarcosine, 1 mM Tris. For 1 L, dissolve EDTA and Tris in 750 mL of water. To help dissolve EDTA, add solid sodium hydroxide to reach a pH between 8.0–9.0. In a separate vessel, dissolve the N-laurylsarcosine in 100 mL of water (incubation in a 65°C water bath for few minutes might be necessary for complete solubilization). Add the sarcosine solution to the EDTA solution, adjust pH to 8.5 with sodium hydroxide or hydrochloric acid and adjust the final volume with water.

2.3 Components for restriction digestion of agarose-embedded DNA and CHEF gel analysis

1. T₁₀E₁₀ buffer: 10 mM Tris, 10 mM EDTA, pH 8.0.
2. Stop Solution: 20 mM Tris, 50 mM EDTA, pH 8.0.
3. Certified megabase agarose (Bio-Rad) and low-melting temperature agarose.
4. CHEF gel apparatus.

2.4 Southern blotting components

1. Ethidium Bromide Solution: Prepare ethidium bromide solution (1.5 μg/mL) in 50 mM TBE.
2. Depurination Solution: 0.25 M HCl.

²Cellulase and macerozyme from different companies may not work equally well. We obtain these enzymes from Biophoretics.

³Our filter units are constructed by gluing stainless steel mesh disks (cut from sheets supplied by the manufacturer) to the bottoms of 50 mL plastic conical tubes with their bottom cones cut off.

⁴Some protocols recommend 500 mM EDTA in NDS solution but we find that 100 mM EDTA is sufficient.

⁵Do not plan to analyze more than 4 genotypes at a time because isolation of protoplasts and their embedding into agarose plugs is a tedious procedure. Plants from two MS plates (150 mm × 25 mm) are enough to generate ten agarose plugs at a concentration of 2×10^7 protoplasts/mL.

3. Denaturation Solution: 3 M NaCl, 0.4 M NaOH.
4. Transfer Buffer: 3 M NaCl, 8 mM NaOH.
5. *GeneScreen Plus* hybridization transfer membrane (Perkin Elmer).
6. Neutralization Buffer: 50 mM sodium phosphate, pH 6.5.
7. UV Crosslinker apparatus (e.g. GS UV crosslinker, Bio-Rad)
8. Southern transfer apparatus.
9. Reaction components to generate radioactive random-primed probe: Gel-purified 45S rRNA gene DNA template, random hexamers, New England Biolabs (NEB) Buffer 2, dATP, dTTP, dGTP, [α - 32 P]-labeled dCTP, Klenow fragment (3'→5' exo-).
10. Church's Hybridization Buffer: 1% (w/v) BSA, 1 mM EDTA, 0.5 M sodium phosphate, pH 7.2, 7% (w/v) SDS.
11. Non-stringent Wash Buffer: 0.2× SSC, 0.1% SDS (20× SSC buffer contains 3 M NaCl, 0.3 M sodium citrate, pH 7.0).
12. Stringent Wash Buffer: 0.1× SSC, 0.1% SDS.
13. Phosphorimaging screen and cassette.

3. Methods

Protoplasts can be generated from plants grown on soil or on MS plates. However, the quality of DNA appears to be better using cells of plants grown on MS plates. Therefore, the method below involves growing plants on MS media for protoplast generation. To develop this method we have incorporated previously described procedures with some modifications [5,22–26].

3.1 Seed Sterilization

1. Add a desired amount of Arabidopsis seeds (~25 μ L/plate) to a 1.7 mL microcentrifuge tube and add 1 mL of Seed Sterilization Solution. Mix and incubate the tube at room temperature for 15 min using a rotating mixer (*see* Note 6).
2. Spin down the seeds in a microcentrifuge at 13,000 g for 1 min and discard the solution. Wash the seeds twice with 1 mL of 100% ethanol.
3. Add 1 mL of 100% ethanol to the seeds and spread them using a pipettor on an empty sterile petri dish containing a piece of sterile filter paper, in a laminar flow hood. Allow the seeds to dry.

⁶Take all possible precautions and care to avoid contamination of MS plates. Use only sterile equipment/solutions to spread seeds. There are several ways to place seeds onto MS plates. One of the methods is: place sterilized seeds on a sterile piece of paper (autoclaved). Fold the paper and gently tap it to drop seeds on to MS plates. Alternatively, you can also resuspend the sterilized seeds in a sterile 0.08% agarose solution and drop the seeds onto MS plates using a pipettor and a tip.

4. Spread the seeds onto freshly prepared MS plates under sterile conditions (*see* Note 7).
5. Seal the plates with Micropore paper tape to prevent desiccation while allowing aeration, and place them at 4°C for three days before transferring them to a growth chamber set for short day growth conditions (8 h light, 24°C: 16 h dark, 23°C, 60% relative humidity). Place the plates on a shelf covered with an aluminum foil, surface-sterilized with 70% ethanol. Within the growth chamber, the plates are kept about 40 cm away from light source consisting of fluorescent and incandescent bulbs. Let the plants grow for 2 weeks (*see* Note 8).

3.2 Generation of protoplasts

1. After two weeks take the MS plates to a hood and gently harvest the plants either by cutting them with sterilized surgical scissors or pulling them out of the media with forceps, without collecting any chunks of solid media (*see* Note 9).
2. Combine plant material harvested from 2 plates on the lid of a sterile petri dish and lightly mince the plants into small pieces using sterile surgical scissors or a razor blade.
3. After mincing, place the plant material in a sterile petri dish (100 mm × 20 mm) and add enough 0.5 M mannitol to cover the plant material. Allow the cells to plasmolyze for 30–60 min.
4. After plasmolysis, remove the mannitol using a pipette (or by gently pouring into a waste container) and add 15 mL of freshly-made Protoplasting Solution. Gently mix the plant material with the solution, seal the petri dish with a parafilm tape and cover with aluminum foil. Place the plates on a rotary shaker with gentle agitation (50 rpm) and allow cell wall digestion to proceed overnight (*see* Note 10).

(From this stage onwards be very gentle with the protoplasts. It is critical to minimize physical trauma to the protoplasts, therefore avoid pipetting until the very last step).
5. Separate liberated protoplasts from the rest of the digested plant material by gently pouring the digestion mixture, successively, through three different filtering devices with pore sizes of 1.13 mm, 230 µm, and 38 µm (*see* Note 11).

⁷Alternatively you can store sterilized *Arabidopsis* seeds in a sterile container at 4°C for 3 days and then place them on freshly-made MS plates. In this case, MS plates will be immediately placed (after sealing with a Micropore tape) in a growth chamber. Do not grow plants for more than 14 days. Plan your experiments accordingly.

⁸Discard contaminated plates and use only healthy plants for protoplast generation. It might be a good idea to grow an extra plate of plants (if there is no limitation of seeds) to compensate for the loss of plants, if any, due to contamination.

⁹Digestion for longer time (>18 h) may not be desirable and therefore plan the timings for this procedure accordingly.

¹⁰To avoid physical trauma to the protoplasts, slowly pour the protoplast-containing solution along the walls of a filtering device using a clean funnel. The filtrate should be collected into a clean 50 mL beaker placed on ice. While collecting the filtrate, make sure that the filter is as close to the bottom of the beaker as possible. The filters will get clogged frequently by plant debris and therefore, it will be convenient to have multiple clean filtering devices of each pore size to avoid washing and reusing of the filtering devices while performing the protocol.

¹¹Filtered protoplast solutions will be light green in color and the protoplasts will form a distinct dark green pellet if they are healthy and intact.

When using the filtering device with the pore size of 38 μm it will help to pre-wet the filter from below by placing it onto a drop of water placed on a sterile surface. This overcomes surface tension and removes trapped air bubbles so that the protoplasts in solution can flow through.

6. Gently transfer the final filtrate to a clean 50 mL conical tube placed on ice and centrifuge at $30 \times g$ for 10 min in a swinging bucket rotor at room temperature. Remove the supernatant (usually very green) using a sterile transfer pipette without touching the protoplast pellet. Add 13 mL of Protoplast Resuspension Solution and resuspend protoplasts by gently inverting the tube several times. After the resuspension of the protoplasts is complete, gently transfer the solution to a 15 mL conical tube placed on ice.
7. Centrifuge the protoplasts again at $30 \times g$ for 10 min, remove the supernatant and resuspend the protoplasts as before but in 10 mL of Protoplast Resuspension Solution and store them on ice (*see* Note 12).
8. Take 10 μL of resuspended protoplasts and dilute them in 90 μL of Protoplast Resuspension Solution and count the protoplasts using a hemocytometer. Adjust the volume to give 2×10^7 protoplasts/mL, a concentration sufficient for analyzing high copy 45S rRNA genes (*see* Note 13).

3.3 Preparation of agarose-embedded DNA

1. We use reusable plug molds (Bio-Rad) to make agarose plugs. Keep clean, assembled plug molds ready on ice. After resuspending protoplasts to the required concentration, prepare an equal volume of 2% low melting agarose solution in 50 mM TBE and heat in a boiling water bath until the agarose melts (*see* Note 14).
2. Equilibrate the temperatures of both the protoplasts and the 2% agarose solution by incubating them in a 37°C water bath for 5–10 min. Then mix the agarose and the protoplast solutions together by gentle but thorough pipetting and immediately pipette the mixture into the plug molds kept on ice (*see* Note 15). Allow about 15–20 min for the agarose plugs to solidify.

¹²Most likely the resuspended protoplast concentration will be less than $2 \times 10^7/\text{mL}$. If this is the case, then centrifuge the protoplasts again at $30 \times g$ and remove supernatant to the required volume. For example, if the protoplast concentration in the 10 mL solution is $4 \times 10^6/\text{mL}$, then remove 8 mL of the supernatant after pelleting the protoplasts. This leads to a fivefold increase in the protoplast concentration to $2 \times 10^7/\text{mL}$.

¹³It is important to maintain the concentration of the agarose solution at 2%. Lower concentration will increase the fragility of the agarose plugs and higher concentration will decrease the porosity of the agarose plugs, which in turn might affect all the downstream steps such as protease digestion, restriction digestion and release of large DNA fragments from the agarose plugs into the CHEF gel. Alternatively, you can buy 2% agarose solution from Bio-Rad (CleanCut Agarose #1703594). To make agarose plugs you may use disposable plug molds (Bio-Rad, Cat#1703713) in place of reusable molds.

¹⁴Because the protoplast and the agarose solutions will be viscous, use 1 mL wide orifice tips to mix them. If you don't have the wide orifice tips, cut about 2 mm off of the tips of 1 mL pipette tips, with scissors or a razor blade, before use.

¹⁵While displacing the agarose plugs into NDS solution stay as close to the tube as possible to minimize the impact of dropping. About 10 agarose plugs can be placed in 40 mL of NDS per 50 mL conical tube. Do not use dry bead baths in place of water baths. At least in our hands, bead baths did not work well. In a 50°C bead bath, the agarose plugs melted due to higher temperatures at the bottom of the bead bath. We use protease from *Streptomyces griseus* (Sigma Cat#5147) which is cheaper than Proteinase K. NDS with protease sufficient for two successive incubations can be prepared and stored at 4°C.

3. Once the agarose plugs are solidified, gently disassemble the plug molds. Holding the plug-retaining side of the plug mold upside down, gently displace the protoplast-containing agarose plugs (using a small spatula) into a 50 mL conical tube containing 40 mL of NDS with 1 mg/mL protease (source: *Streptomyces griseus*, Sigma, Cat#5147). Seal the tubes with parafilm tape and incubate them overnight in a 50°C water bath (see Note 16).
4. Replace the NDS/protease with fresh solution and let it incubate overnight again at 50°C (see Note 17).
5. Replace the NDS/protease with fresh NDS but without protease and incubate at 50°C overnight again.
6. The following day replace the NDS with fresh NDS and store at 4°C (see Note 18).

3.4 Restriction digestion of agarose-embedded DNA for first dimension CHEF gel electrophoresis

To obtain large rDNA fragments, choose a restriction enzyme which is an infrequent cutter of the 45S rRNA genes and provides a distinct banding pattern. For example, only some rRNA gene subtypes in *A. thaliana* ecotype Col-0 have a *HindIII* site [5].

1. Take two pieces of an agarose plug (one for first dimension analysis and the other for second dimension analysis) slightly smaller than the well of a CHEF gel and incubate in a 15 mL conical tube containing 10 mL of T₁₀E₁₀ buffer with 2 mM PMSF for 1 h or more at 4°C (see Note 19).
2. Replace the T₁₀E₁₀ buffer with fresh 10 mL solution without PMSF and equilibrate for 30 min at room temperature. Repeat this step three more times.
3. Place the sample in a flat-bottom 2 mL centrifuge tube containing 1 mL of 1× restriction enzyme buffer and equilibrate for 1 h at room temperature (see Note 20). Repeat this step one more time by replacing the buffer.

¹⁶While discarding NDS solution be gentle and use a spatula to prevent agarose plugs from falling out of the tube. Alternatively, you can use screened plug caps from Bio-Rad (Cat#170-3711), or make your own, to discard the solution safely without losing the agarose plugs. While adding fresh NDS solution, take care to ensure the safety of the agarose plugs.

During incubation, gently invert the tubes once or twice to avoid tight stacking of the agarose plugs, which could hamper the protease digestion of the embedded protoplasts.

¹⁷After protease digestion, agarose plugs should have lost all green color. However some plugs may still have a greenish tinge, which is fine. DNA should now be free from all histones and other proteins due to the protease digestion. A high concentration of EDTA (100 mM) will prevent contaminating DNase activity, if any. Agarose plugs can be stored at 4°C for several months. Because agarose plugs are fragile, repeatedly opening and closing the storage tube might physically damage the plugs. To avoid this, and for convenience of handling, aliquot plugs into flat 6 well culture plates for storage.

¹⁸The well size of a CHEF gel varies depending on your choice of comb. Measure the size of a well from the comb you intend to use and cut the agarose plug accordingly to fit the well.

The first equilibration period in T₁₀E₁₀ buffer can be extended to overnight, if needed. It is a bit hard to see the plug clearly in the solution because it is transparent, therefore take care that you don't lose the plug while discarding the buffer during T₁₀E₁₀ washes.

¹⁹We buy most restriction enzymes from NEB. All NEB restriction enzyme buffer compositions are available at the NEB website.

²⁰The amount of enzyme and incubation time needed may have to be empirically defined.

Some restriction enzymes require incubation at higher temperatures. Because the DNA is embedded in low-melting temperature agarose, the incubation temperatures should not exceed 55°C. For restriction enzymes that require higher temperature (e.g. *BstEII* requires incubation at 60°C) NEB sells high fidelity versions of the same enzyme that sometimes require lower incubation temperature (*BstEII*-HF requires incubation at 37°C).

4. Remove the buffer and then add about 200–250 μ L (enough to cover the agarose plug) of 1 $\times$ restriction enzyme buffer containing 50 units of restriction enzyme and incubate at the appropriate temperature (e.g. 37°C for *Hind*III digestion) for few hours to overnight (*see* Note 21).
5. At the end of the reaction, remove the reaction solution and incubate the plug in 500 μ L of Stop Solution for 5–10 min. Then proceed to CHEF gel electrophoresis (*see* Note 22).

3.5 CHEF gel electrophoresis (first dimension)

1. About 45 min before the restriction digestion ends, cast a 1% agarose gel using Certified Megabase Agarose (Bio-Rad) (or agarose of equivalent quality) in 50 mM TBE. A 1% gel is ideal to resolve 10 kb – 1.5 Mb DNA. Use the comb type/size depending on your choice of gel type. You can run either a wide gel or a long gel (Fig. 1). The CHEF gel running conditions given below are for a wide gel. After melting the agarose in TBE it helps to slightly seal the edges and corners (but not the round holes!) of the gel casting tray with the melted agarose solution before pouring the gel. Once cooled sufficiently, pour the melted agarose solution on to the gel casting tray without introducing air bubbles. Let it cool and solidify for about 40 min. During this time prepare 1% low melting temperature agarose solution (about 4–5 mL) and incubate at 65°C until it is needed (*see* Note 23).
2. Once the gel is dried, carefully remove the comb. Take the digested plugs from the reaction tube and gently insert them into the wells using a small spatula. Two sets of plug samples should be separated on the gel to allow cutting the gel into two halves with each half having one set of samples. In one of the wells, insert a piece of agarose plug containing CHEF gel markers (e.g. Yeast Chromosome PFG marker from NEB which has size range of 225 kb to 1900 kb; product # N0345S). Gently press the plugs against the front wall of the wells to remove air bubbles. Seal the wells with the molten 1% low-melting temperature agarose solution without introducing air bubbles and let it solidify for about 10 min (*see* Note 24).
3. After the gel is cast, lift the casting tray holding the gel and place it inside the gel-holding square positioned in a CHEF gel apparatus (Fig. 1) containing 2 L of

²¹If you intend to run the CHEF gel a day later, remove the Stop Solution and store the digested agarose plug at 4°C. If you store the plug along with the solution, shorter DNA molecules may diffuse from the plug into the solution.

²²Don't add ethidium bromide while casting the gel. We use a Bio-Rad wide-long gel casting stand (Cat#170-3704). For 1.5 – 3.0 Mb separations, use 0.8% gel.

²³Pieces of agarose plugs often stick to the walls of microcentrifuge tubes. Using a spatula to draw the sample out is not recommended as this will likely damage the plug. Rather, gently invert the tube along with the buffer few times until the plug is suspended in the buffer. Then open the cap of the tube to pour the buffer along with the plug onto a lid of a clean petri dish (use the inner surface). Take care not to damage plugs while inserting into the wells. CHEF gel markers of different sizes are available from NEB and Bio-Rad.

²⁴Under 4°C cold room conditions, the mean gel running temperature is ~14°C. If you don't have cold room facilities to run CHEF gels at 4°C, use a chiller system with the cooling module in conjunction with CHEF gel apparatus to maintain the gel running temperature of 14°C.

cold 50 mM TBE. We run CHEF gels in a cold room at 4°C without further cooling (*see* Note 25).

4. Load any additional DNA markers as needed (e.g. Kb-plus DNA marker) and run the CHEF gel with the following parameters (*see* Note 26). The parameters mentioned in this protocol are for running gels using a CHEF DRII pulsed-field gel electrophoresis system from Bio-Rad. Some modifications in the parameters may be required if you use other systems (e.g. CHEF DRIII or CHEF Mapper system).

Stage I:

Initial switch time: 60 sec

Final switch time: 60 sec

Run time: 13 h

Voltage 200 V

Stage II:

Initial switch time: 90 sec

Final switch time: 90 sec

Run time: 7 h

Voltage: 200 V

3.6 Southern analysis of CHEF gels (first dimension)

Procedures described for Southern transfer of DNA from CHEF gels onto a membrane are similar to those described for conventional gels except for the requirement of pretreatment of the gel with UV irradiation (*see* Note 27).

1. After gel running is complete, carefully transfer the gel to a container and cut into half. Use one half for one dimensional analysis and save the other half for two-dimensional analysis (*see* Note 28).

²⁵While running a CHEF gel, the TBE buffer is constantly circulated using a pump. Make sure that the speed setting is not too high as this may result in damage and/or displacement of the agarose gel. We use a speed setting of 35 with the Variable Speed Pump from Bio-Rad.

If you need to vary the CHEF gel running parameters, first try varying the gel running times and if results are not still satisfactory, try varying switch times.

²⁶For efficient blot transfer of DNA fragments larger than 20 kb, the CHEF gel is treated with UV irradiation, which results in cleavage of DNA molecules. Depurination of the gel with acid treatment also results in DNA cleavage. Although either of these gel pretreatments is enough for DNA cleavage, we use both. If you intend to use only one of these two, then go for UV irradiation.

²⁷Remember that restriction digestion has been performed on two identical sets of agarose plugs (one set for one dimensional analysis and the other set for two-dimensional analysis). Therefore, it is recommended to use one whole agarose plug (cut into required pieces) for both one dimensional and two-dimensional analysis for consistent results.

It is a good idea to first complete one dimensional analysis before starting two-dimensional analysis. Half of the gel required for two-dimensional analysis can be saved for few days at 4°C in a clean container with a lid. Cover the gel with a couple of clean paper towels, soaked with 50 mM TBE, to prevent desiccation.

²⁸Ethidium bromide staining of DNA is necessary for DNA nicking by UV light and therefore, a fresh staining solution is ideal.

2. Place one half of the gel to be used for one dimensional analysis in a plastic box containing fresh Ethidium Bromide Solution and stain for about 40 min by gently agitating the gel on a rotary shaker (*see* Note 29).
3. Take a picture of the ethidium bromide-stained gel after UV exposure and proceed to pretreatment (*see* Note 30).
4. Irradiate the gel with 60 mJoule of 254 nm UV light. We use a Bio-Rad GS Gene Linker UV chamber on setting 'nic'.
5. Treat the gel with the Depurination Solution for no longer than 6–8 min.
6. Wash the gel twice for 30 min each with the Denaturation Solution.
7. Equilibrate the gel with the Transfer Buffer for 15 min.
8. Equilibrate a GeneScreen Plus (Perkin-Elmer) membrane (cut to the shape of the gel) with the Transfer Buffer at least for 5 min.
9. Transfer DNA from the agarose gel to the membrane for about 24 h by assembling the Southern transfer apparatus (*see* Note 31).
10. After the DNA transfer, mark the DNA side of the membrane with a pencil and neutralize the membrane twice, 5 min each, using the Neutralization Solution.
11. Briefly air-dry the membrane and UV cross-link with 150 mJoules of 254 nm UV light. We use the C3 setting on a Bio-Rad GS UV Crosslinker for this purpose.
12. Place the membrane in a hybridization bottle containing about 15–25 mL of Church's Hybridization Buffer. Prehybridize the membrane for 1 h or longer at 60°C (*see* Note 32).
13. While the prehybridization of the membrane is occurring, prepare the probe. First, mix the following components and incubate them at 95°C for 5 min to denature the template DNA.
 - 1 μ L (~50 ng) gel-purified 45S rRNA gene DNA template (*see* Note 33)
 - 1 μ L random hexamers (250 ng/ μ L)
 - 8 μ L water

²⁹To see if the ethidium bromide staining is sufficient or not, check the DNA marker staining. Digestion products from the agarose plugs will likely form a smear.

³⁰We use a downward transfer apparatus (Schleicher and Schuell) for DNA transfer to blots. You may use upward transfer, if preferred.

To make sure that DNA transfer is complete, stain the gel after transfer and take a picture after UV exposure, using the same exposure settings used prior to DNA transfer. Check the DNA markers on the gel to determine the efficiency of DNA transfer. If need be, the duration of the DNA transfer step can be extended up to 48 h.

³¹Alternatively, the blot membrane can be wrapped in a plastic wrap and stored between –20 to 4°C for later use.

³²We digest a 45S rDNA-containing BAC clone (e.g. F1J10) [27] with *I-PpoI* endonuclease (which cuts once per gene) and gel-purify the 10.5 kb rDNA fragment (corresponding to a single copy of 45S rRNA gene) to be used as DNA template for making probes.

Alternatively, you can use gel-purified PCR amplicons of 45S rDNA.

³³Klenow fragment (3'→5' exo-) has DNA polymerase activity but not 3'→5' exonuclease activity.

Add the following components, mix and incubate for 45–60 min at 37°C in a radioactive area

2 µL NEB Buffer 2

3 µL dNTP solution (1 mM each of dATP, dTTP and dGTP)

4 µL α -³²P-dCTP (40 µCi)

1 µL Klenow fragment (3'→5' exo-) (*see* Note 34)

20 µL total reaction volume

14. Bring the volume up to 100 µL with water and run the sample through a PERFORMA spin column (Edge Bio) to remove the unincorporated radioactive nucleotide. Monitor the flow through with a Geiger counter to make sure that it is “hot”.
15. Boil the microcentrifuge tube with the probe for 5 min and then add the denatured probe to the bottle containing the prehybridized membrane and the hybridization buffer (*see* Note 35). Allow probe hybridization to occur overnight at 60°C.
16. Rinse the membrane twice with Non-stringent Wash Buffer at room temperature and then wash with the same buffer for 10 min at 65°C.
17. Wash twice with the Stringent Wash Buffer for 15 min each at 65°C (*see* Note 36).
18. Briefly dry the radioactive membrane, wrap in plastic wrap and expose to a phosphorimager screen for 6 h to overnight. Process the screen using a Phosphorimaging scanner to obtain an image of the gel (*see* Note 37).

An image of the membrane obtained for agarose-embedded DNA of Col-0, subjected to *Hind*III digestion and CHEF gel electrophoresis, is given in Figure 2. After one dimensional CHEF gel electrophoresis, gel elutions corresponding to each band on the gel image can be obtained from gel lanes carrying the same sample but not subjected to Southern blot analysis (*see* Note 38). Similar images of one dimensional analysis for other ecotypes of Arabidopsis have been published earlier [5,11,12].

³⁴It is important to remove some hybridization buffer just before adding the probe. The amount of hybridization buffer in the bottle should be just enough to soak the membrane. Too much hybridization buffer will dilute the probe and reduce hybridization efficiency.

³⁵The amount of wash buffer should be just enough to immerse the membrane. Excess wash buffer and longer wash times may result in weaker signals during detection.

³⁶Monitor the membrane with a Geiger counter before and after each wash step to check the levels of radiation. The membrane should remain fairly “hot” even after all the washes but not nearly as “hot” as after hybridization.

³⁷For eluting the bands from a CHEF gel, first measure the distance of each band from the well on the gel image obtained after Southern blot analysis. According to these measurements cut the bands from a gel lane containing the same sample, but not subjected to Southern blot analysis. If you plan to use a kit to extract DNA from gel slices, choose a kit designed to extract longer than 10 kb DNA fragments (e.g. QIAEX II from Qiagen, Cat#20021).

³⁸Once you standardize this protocol in your hands, you can straightaway proceed to the second dimension analysis after the first dimension analysis without storing the CHEF gel at 4°C. In this case, however, you will not be able to see the results of the first dimension analysis before proceeding to the second dimension analysis.

3.7 In-gel restriction digestion of resolved CHEF gel samples (for second dimension gel electrophoresis)

For the second dimension electrophoretic step you can perform either CHEF gel or conventional gel electrophoresis depending on the size of DNA fragments to be resolved. We describe below the use of a conventional gel for the second dimension. For methylation analysis, choose a methylation-sensitive endonuclease (e.g. *HpaII*).

1. Use the half of the CHEF gel stored at 4°C for second dimension gel electrophoresis. Slice an individual gel lane containing the sample lengthwise, into two halves. Use one half of the lane for restriction digestion with a methylation-sensitive enzyme (e.g. *HpaII*) and keep the other half for a control (no enzyme) (*see* Note 39).
2. Place the gel slice in a plastic container with 100 mL of 1× restriction enzyme buffer and incubate at room temperature for 2 h.
3. Repeat Step 2 and then place the gel slice in a sealable bag containing 10 mL of 1× restriction enzyme buffer and 1000 units of restriction enzyme (or no enzyme for the control sample).
4. Seal the bag, avoiding bubbles, and incubate overnight at the appropriate temperature (e.g. 37°C for *HpaII*) (*see* Note 40). For efficient digestion, hang the bag to a wall of a plastic container using glue tape and then place the container in an incubator maintaining the required temperature (Fig. 3).
5. The following morning add 1000 units of restriction enzyme again (except for the control sample) and incubate for an additional 6 h (*see* Note 41).
6. 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 h.
7. Repeat Step 6. Just prior to the end of the incubation, prepare 1% melted agarose solution in 50 mM TBE. After the wash, lay the gel strip at the top of a gel casting tray perpendicular to the direction of electrophoresis and pour the gel as shown in Figure 4. Optionally, a small gel comb can be held (with the help of a clamping system) next to the gel slice to create few lanes for loading markers (Fig. 4) (*see* Note 42).

³⁹You can use zip lock bags for incubating sliced gel lanes. We use bags with a dimension of 6 in × 2 in. Using bigger bags will increase the amount of buffer and restriction enzyme required for efficient digestion.

⁴⁰You can vary the amount of restriction enzyme and incubation time as needed. To further increase the efficiency of digestion you can also incubate the bag (secured to a container (Fig. 3) in a 37°C shaker set for gentle shaking (not more than 25 rpm!). Overshaking may result in the loss of some small DNA fragments from the gel slice into the buffer solution.

⁴¹The width of the gel casting tray should allow for a few lanes for loading markers. The length of the gel can be varied to obtain a desirable resolution. We use a large gel casting tray (10 in × 9 in) and a wall comb (a flat comb with no wells) to make gels of desirable size. If you make a gel bigger than you need you can cut the unwanted portion using a razor blade either before or after gel electrophoresis.

Instead of using a comb held by a clamping system, you can also gently cut out wells in an area next to the CHEF gel slice to load markers.

⁴²The duration of electrophoresis will vary depending on the gel electrophoresis system and the dimension of the gel. Tracking the loading dye front relative to markers will help you decide when to stop the electrophoresis.

8. After the gel is hardened, load the appropriate markers and perform standard electrophoresis in 1× TBE at 160 V for 3–4 h (see Note 43).
9. Perform Southern blotting, probing and detection as described above.

A gel image obtained after *Hind*III digestion and CHEF gel electrophoresis in the first dimension and *Hpa*II digestion and conventional gel electrophoresis in the second dimension is shown in Figure 5. See also [11]. Differential *Hpa*II sensitivities of fragments resolved in the first dimension is apparent in the second dimension (Fig. 5). Note that after two-dimensional gel electrophoresis, DNA fragments can be eluted from different positions of a gel for PCR or other analyses, rather than Southern blot analysis.

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⁴³To detect bands of interest, long exposures to a phosphorimager screen may be needed (up to 5 days). If sensitivity is an issue, you can increase the concentration of protoplasts in the agarose plugs. Additionally, you can also try generating higher specific activity radioactive probes.

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Figure 1. CHEF gel orientations: Wide gel (on the left side) allows more space for samples with less resolving distance where in long gel (on the right side) provides less sample space with longer resolving distance.

CHEF gel electrophoresis (first dimension)

Mohannath, G. unpublished data

Figure 2.

CHEF gel electrophoresis of *Hind*III-digested agarose-embedded DNA of *Arabidopsis thaliana* ecotype Col-0 (first dimension). On the left side is an ethidium bromide-stained gel image. On the right side is the image obtained after southern blotting and probing with rRNA gene radioactive probe.

M1=GeneRuler 1kb Plus DNA ladder; M2=Yeast Chromosome PFG marker (NEB: N0345S). The topmost band in the M2 lane corresponds to yeast (*Saccharomyces cerevisiae*) chromosome 12, which contains ribosomal genes [28] and cross-reacts with the probe. Also, some fragments in M1 cross-react with the probe.

Figure 3.
Setting up a CHEF gel slice for in-gel restriction digestion.

Figure 4.
Casting a new agarose gel with a CHEF gel slice at the top, for the second dimension gel electrophoresis

Mohannath, G. unpublished data

Figure 5.

Two-dimensional gel electrophoresis of agarose-embedded DNA of *Arabidopsis thaliana* ecotype Col-0. CHEF gel electrophoresis of *Hind*III-digested products in the first dimension and conventional gel electrophoresis of non-digested (image at the top) or *Hpa*II (methylation-sensitive endonuclease)-digested products (image at the bottom) in the second dimension.

The DNA fragments marked with dotted circles are hypomethylated compared to the DNA fragments marked with undotted circles.

M= *Hpa*II-digested products of 4 Arabidopsis rRNA gene-containing BAC clones [27].

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