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Investigation of Transposon DNA Methylation and Copy Number Variation in Plants Using Southern Hybridisation

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Abstract

Plant genomes are pronouncedly enriched in repeat elements such as transposons. These repeats are epigenetically regulated by DNA methylation. Whole genome high-depth sequencing after bisulfite treatment remains an expensive and laborious method to reliably profile the DNA methylome, especially when considering large genomes such as in crops. Here, we present a simple reproducible Southern hybridisation–based assay to obtain incontrovertible methylation patterns from targeted regions in the rice genome. By employing minor but key modifications, we reliably detected transposon copy number variations over multiple generations. This method can be regarded as a gold standard for validation of epigenetic variations at target loci, and the consequent proliferation of transposons, or segregation in several plant replicates and genotypes.

Keywords: Epigenetics, DNA methylation, Repeats and transposons, Copy number variation, Plants

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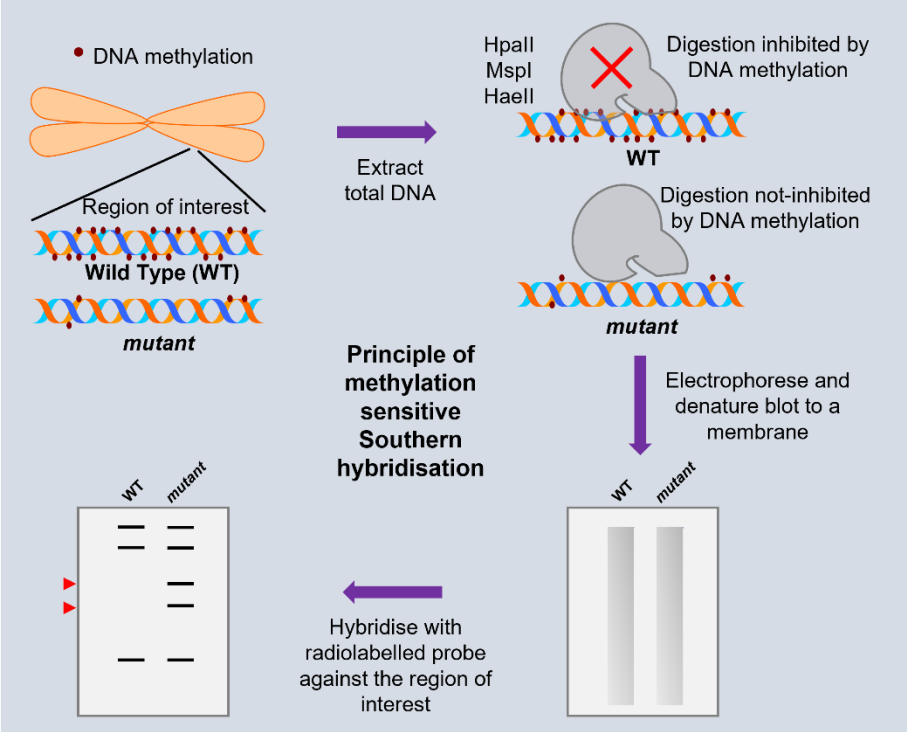
Graphical abstract:



Background

Epigenetics mediated by DNA methylation plays an important role in plant development, regulation of repeats and transposons, immunity, stress tolerance, and transgenerational memory (Zhang *et al.*, 2018). Profiling methylation at targeted loci provides the primary data in delineating the causes and consequences of DNA methylation across genotypes or treatments. This can be investigated by methylation-sensitive enzyme digested DNA PCR (ChoP-PCR), or bisulfite amplicon sequencing (BSAS) (Zhang *et al.*, 2014; Masser *et al.*, 2015). However, these methods have the inherent bias in PCR amplification, and require the usage of primers with very low GC content, which tend to amplify non-specific regions. Hence, additional validation by high throughput methods is necessary for conclusiveness.

Southern hybridisation, on the other hand, involves restriction of the genomic DNA using specific DNA methylation responsive restriction enzyme(s), followed by electrophoresis, blotting, and hybridisation, using targeted regions of interest. This offers several advantages for targeted methylation profiling, a few of which are enumerated below:

1. Unbiased detection of all the copies of transposons, irrespective of methylation status, truncation, or sequence variants.
2. DNA methylation among eukaryotes can be distinguished into CG, CHG, and CHH contexts, based on the adjacent nucleotides to cytosines in the genome. Methylation in these contexts is distinctly modulated, and interpreted by different cellular processes. This method offers the ability to distinguish between these different contexts of DNA methylation, by employing restriction enzymes specific to each category.
3. The hybridised membrane can be stripped and re-probed with multiple probes, which may serve as control regions.
4. Regions such as rDNA repeats and others, which are recalcitrant for amplification due to presence of a tandem array of repeats, can be profiled for DNA methylation in a reproducible manner.
5. DNA methylation differences might occur only in a few of the available copies of the specific class of transposons. Such differences that occur in only a few of the identical copies cannot be detected by PCR–based

assays. The method described here can reliably detect such variations, as banding patterns differ for every copy of the transposon.
6. This method can be used to unequivocally prove the proliferation of transposons, upon silencing perturbation, or stress-induced transposon activation. If such an event occurs only in specific tissues or a sub-population of cells, this method can detect such copy number variation.
7. Since this method characterises the variation in restriction fragments in the genome, the possibility of probing transgene copy variations is an added advantage.
8. This method can be effectively adapted for any eukaryote. Once considerable quantity and quality of DNA is obtained, the universality of the methylation sensitivity, DNA fragment electrophoresis, and transfer enables probing a region of interest from any source.

Materials and Reagents

1. Whatman sheets (GE Healthcare, catalog number: 3030-931)
2. 10 mL sterile serological pipette (Biofil, catalog number: GSP211010)
3. 1.5 mL microcentrifuge tubes
4. Purified total genomic DNA (~5 µg) from each sample
5. DNA probes designed for the regions of interest
6. Restriction Enzymes
   a. HpaII (New England Biolabs, catalog number: R0171S)
   b. MspI (New England Biolabs, catalog number: R0106S)
   c. HaeII (New England Biolabs, catalog number: R0107S)
   d. PstI-HF (New England Biolabs, catalog number: R3140S)
   e. EcoRV-HF (New England Biolabs, catalog number: R3195S)
   f. 10× CutSmart Buffer (New England Biolabs, catalog number: B7204S)
7. Ultra-pure water (Invitrogen, catalog number: 10977-023)
8. Amersham Rediprime II DNA Labeling System (GE Healthcare, catalog number: RPN1633)
9. Illustra MicroSpin G-50 Columns (GE Healthcare, catalog number: 27533001)
10. Zeta-Probe® Blotting Membranes (Bio-Rad, catalog number: 162-0165)
11. UltraPure™ Agarose (Thermo Fisher Scientific, Invitrogen, catalog number: 16500-100)
12. 1 kb DNA ladder (New England Biolabs, catalog number: N3232S)
13. Sodium hydroxide (Fisher Scientific, catalog number: 27815S)
14. Sodium chloride (HiMedia, catalog number: MB023-1Kg)
15. Tris-base (Fisher Scientific, catalog number: 15965-500G)
16. Hydrochloric acid (Fisher Scientific, catalog number: 29505)
17. Tri-sodium citrate (Fisher Scientific, catalog number: 14005)
18. Boric acid (Fisher Scientific, catalog number: 12005)
19. Ficoll-400 (Sigma, catalog number: F2637)
20. Bromophenol blue sodium salt (Sigma, catalog number: B5525)
21. Sodium phosphate dibasic, Molecular weight 141.96 g/mol (Sigma, catalog number: 71642)
22. Double distilled autoclaved water (called “water” unless mentioned otherwise)
23. Ethidium Bromide (Himedia, catalog number: MB071)
24. Sodium Dodecyl Sulphate (SDS) (Himedia, catalog number: GRM205)
25. Ethylene diamine tetra-acetic acid disodium salt (EDTA), Molecular weight 372.24 g/mol (Fisher Scientific, catalog number: 12635)
26. Ortho-phosphoric acid (Fisher Scientific, catalog number: 29245)
27. dCTP [α-^{32}P], 10 mCi/mL (Board of Radiation and Isotope Technology, India, catalog number: PLC102)
28. 20× Saline-Sodium citrate (SSC) (see Recipes)
29. Depurination solution (see Recipes)
30. Denaturation solution (see Recipes)
31. Neutralisation solution (see Recipes)
32. Hybridisation buffer (see Recipes)

33. Ethidium Bromide staining solution (see Recipes)
34. 2 M sodium phosphate buffer pH 7.2 (see Recipes)
35. 10% SDS (see Recipes)
36. 0.5 M EDTA pH 8.0 (see Recipes)
37. 1 M Tris buffer pH 8.0 (see Recipes)
38. 10× Tris-Borate-EDTA (TBE) buffer (see Recipes)
39. 1× Tris-EDTA (TE) buffer (see Recipes)
40. 8× DNA loading buffer (see Recipes)
41. Wash buffer 1 (see Recipes)
42. Wash buffer 2 (see Recipes)
43. Wash buffer 3 (see Recipes)

Equipment

1. Water bath (Neolab Instruments, India, model: WB-343)
2. Microcentrifuge (Eppendorf, model: 5415R)
3. Thermomixer (Eppendorf, model: Thermomixer comfort)
4. Chemical fume hood
5. DNA gel electrophoresis apparatus with accessories (Bio-Rad, Sub-Cell GT Cell)
6. Hybridisation oven (Thermo Fisher Scientific, Hybaid oven)
7. Hybridisation cylinders (WHEATON® Safety Coated, Hybridisation bottle, DWK life sciences, catalog number: 805027)
8. Typhoon Phosphor-Imager scanner system (Amersham Biosciences, GE Healthcare, model: Typhoon TRIO)
9. Phosphor screen (GE Healthcare, 20×24 cm)
10. Phosphor exposure cassette (GE Healthcare, 20×24 cm)
11. Electrophoresis power supply unit (Bio-Rad, PowerPac HC)
12. Glass trays
13. Microwave oven
14. Gel rocker (Bionova India, model: SLMGR100)
15. Bubble level
16. UV-gel imaging system (Vilber Gel documentation system)
17. UV crosslinker (UVP, model: CL1000)
18. PCR thermal cycler (Bio-Rad, model: S1000)
19. Radioactive material safe work space with radioactive disposal facility

Software

1. ImageJ
2. Typhoon TRIO image acquisition system

Procedure

A. Genomic DNA digestion

1. Place approximately 3–4 µg of DNA (estimate using fluorimetry, spectrophotometrically, or via gel electrophoresis) from each sample in a 1.5-mL microcentrifuge tube, and make up to 40 µL with ultra-pure water.
2. Add 5 µL of 10× CutSmart buffer, and 40 units of the corresponding enzyme of interest. Make up the total reaction volume to 50 µL with ultrapure water.

3. Incubate the sealed tubes in a water bath set to the optimal temperature for the enzyme being used for 12 to 16 h, to facilitate complete digestion. In case the water bath does not contain a flow agitator to mix the samples, manually mix by tapping at 3 h intervals.
4. Post-incubation, add 8 µL of 8× DNA loading buffer to the reaction mix. Gently tap and spin the tube to collect the contents, and proceed for gel electrophoresis.
   Note: If required, digested DNA with loading buffer can be stored at -20°C indefinitely.

Inference

LINE1 is a conserved transposon that is regulated *via* DNA methylation in plants (Cui *et al*., 2013). In this example, DNA methylation and transposon copy number status were compared at the LINE1 locus between a wild type (WT) rice variety - Pusa Basmati1 (*Oryza sativa Indica* spp.), and a knockdown mutant of a plant specific RNA silencing mutant (for details, please refer to Sundar *et al*., 2021). Figure 1A demonstrates DNA hypomethylation in the mutant genotype, as additional lower size bands were observed in specific plant samples (HpaII lanes). Genomic DNA subjected to test was extracted from leaf samples of the equally-grown 40 days old WT and mutant rice plants. When the DNA is digested with methylation sensitive enzymes, portions that are hypomethylated are prone to enzyme activity, hence, accumulating shorter DNA fragments compared to their methylated counterparts. When hybridised to a complementary labelled strand, we observed additional lower sized fragments reflecting their hypomethylated states. Similar hypomethylation was observed in the MspI and HaeII lanes, for the corresponding sample. Taken together, these differential banding patterns corroborate that specific lines of mutant genotypes show DNA hypomethylation in the LINE1 locus.

Figure 1B shows the transposon display map (transposon copy number profile) of the LINE1 transposon in mutant lines. Additional bands (red arrow) in the mutants show that the LINE1 element has proliferated in the genome, upon loss of DNA methylation in the mutant genotype. When the transposon proliferates, the new integration sites in the genome will have a different pattern of enzyme recognition sites adjacent to the location of integration, compared to the original copies of the transposon. When digested with different enzymes, these additional copies tend to create a hybridisation profile that exhibits these new digested fragments.

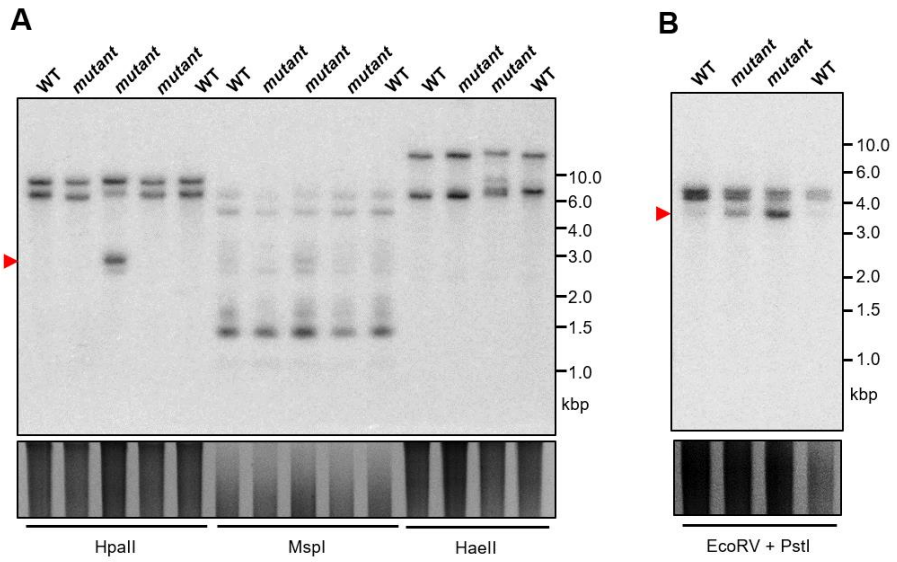


Figure 1. Southern blots for DNA methylation and copy number investigation of LINE1 transposon in different genotypes of rice.

A. Profiles of LINE1-probe–hybridised fragments from different genotypes of rice. DNA digested with methylation-sensitive enzymes (HpaII, MspI, and HaeII) was electrophoresed, blotted, and hybridised with the probe amplified from the LINE1 fragment in rice genome, using the oligos 5'-tctctggacgagcctgttccaa-3' and 5'-ggctaagtcgtcagttgaatgc-3'. The additional bands (red arrow) denote that the particular mutant genotypes have a differential methylation pattern when compared to the WT samples. B. Transposon copy number blot

showing additional transposition events of LINE1 transposon in the mutant lines (red arrow).

B. Gel electrophoresis

1. Setup a clean gel casting apparatus with a suitable comb (each well should be able to hold approximately 75 µL). Use a bubble-level to make sure that the gel tray is assembled on a straight surface without inclination.
2. Weigh the amount of UltraPure™ agarose required for casting a 0.8% (w/v) gel, and melt this in a microwave oven, in an appropriate volume of 1× TBE buffer inside a glass bottle. Avoid boiling of the gel mix, as this may change the concentration of the buffer. Measure the molten gel mix again, and make up for the evaporation loss with sterile water. Allow the mixture to cool down. Once the temperature is approximately 65°C, pour the gel mix into the gel casting tray, and let it set for 45 min.
   Note: For a standard 15 × 10 cm gel tray, around 160 mL of 0.8% 1× TBE gel is needed for a ~1.5 cm-thick gel, accommodating ~75 µL-well volume.
3. Fill the gel electrophoresis tank with the required volume of freshly diluted 1× TBE buffer, and place the gel in the apparatus until it is completely immersed.
4. Load the digested DNA in the wells and include a 1-kb DNA ladder (~1 µg). Electrophorese at 80 V (~8 V/cm of gel length) until the bromophenol blue tracking dye reaches the bottom of the gel (~2.5 h).

C. Ethidium Bromide staining and visualisation

1. Transfer the gel to a clean glass tray (holding capacity of approximately 1 L) containing enough volume of ethidium bromide staining solution (~350 mL). The staining solution contains 0.5 µg/mL of ethidium bromide in water.
2. Shake gently on a gel rocker for 15–20 min, and image the gel in a UV gel imaging system. This image acts both as loading control, and to examine the extent of digestion.

D. Depurination, denaturation, and neutralisation

1. To aid in the efficient transfer of higher molecular weight DNA fragments, rock the gel gently in an immersing amount of depurination solution for 20–30 min, until the bromophenol blue dye changes to an orange colour.
   Note: In case the expected fragment sizes are less than 5 kb, the depurination step can be avoided.
2. Discard the depurination solution, replace with copious amounts of denaturation solution, and gently shake in a rocker for 1 h.
3. Discard the denaturation solution, and wash the gel with copious amounts of water (~20 gel volumes). Repeat four times to eliminate any residual buffer.
4. Pour 20 gel volumes (~350 mL) of neutralisation solution, and continue gentle rocking for 1 h. Using a sharp scalpel, trim the wells of the gel retaining only the electrophoresed gel portion.

E. Capillary transfer to nylon membrane (Figure 2A)

1. In a large trough, place an inverted gel tray (acting as a plank). Cut a Whatman sheet into a long rectangular wick, whose width is equal to the width of the trimmed gel. The length of the wick should be long enough to fold within the tray bottom.
2. Pour the trough with sufficient 20× SSC buffer, to completely wet the wick. Carefully invert the gel (with the loading side facing the bottom plank), and place it on the wet wick. Cut the Zeta Probe nylon membrane to the exact trimmed gel dimensions, and dip it in water for 3 s in a small tray first, and then immerse it in 20× SSC in another tray.
3. Thoroughly pour the gel with the 20× SSC buffer, and carefully place the membrane on the gel, avoiding air bubbles.
   Note: Once placed on the gel, the membrane must not be moved. The gel contact-side of the membrane shall be labelled with pencil on the corner, as required for identification of the blot. Once the gel is placed,

if air bubbles are to be removed, a sterile serological pipette must be used to gently roll over the membrane, eliminating air pockets.

4. Cut three Whatman sheets of membrane dimensions, and carefully place sequentially over the membrane. Dip the first two sheets in 20× SSC buffer and place on the gel, while the third sheet is placed on the wet Whatman sheets, without pre-wetting. Use a serological pipette to gently roll over the sheets, to eliminate gaps and air pockets.
5. Vertically stack up the setup with 10–12 cm of Whatman blotting sheets cut to the dimension of the membrane. Place a plank over the sheets, and top this up with a ~200 g weight (like a glass bottle). Allow the capillary transfer to proceed for 14–16 h post-setup at room temperature.
   Note: Make sure the trough has sufficient amount of 20× SSC buffer at the bottom for the entire duration of capillary transfer.

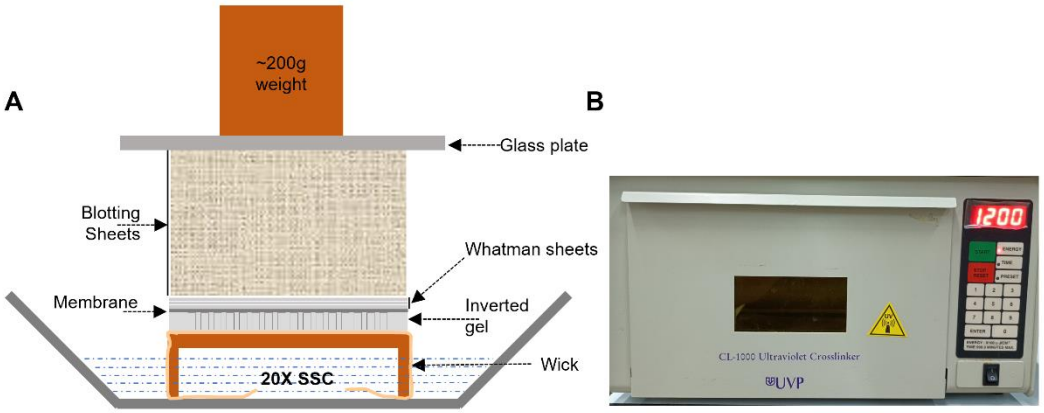


Figure 2. Southern capillary transfer and crosslinking.
A. Graphical representation of the Southern capillary blotting setup. B. Photograph of the UV crosslinker that generates continuous UV-C beam at the required dosage.

F. UV-crosslinking the membrane

1. Carefully disassemble the setup, and keep a tray of freshly prepared 2× SSC buffer.
2. Using forceps, carefully withdraw the membrane from the gel, and immerse it in a tray of 2× SSC for 15–20 s. This step removes salt precipitates deposited on the membrane.
3. Immediately place the membrane on a dry Whatman paper, with the transferred side facing up, and proceed to the UV-crosslinker.
4. Crosslink the DNA with the membrane using UV-C irradiation of 1200 Joules of continuous pulse. The UV irradiator provides a continuous beam of UV light, until 1200 joules of energy is irradiated uniformly over the surface of the membrane (Figure 2B).
5. Place the membrane in a sealed plastic cover, and store at 4°C until hybridisation.
   Note: The post-transfer gel can be re-stained with ethidium bromide to check for residual DNA, and hence the transfer efficiency. It is normal to observe partial retention of high molecular weight DNA fragments (~above 25 kb).

G. Radioactive labelling of the probe

1. Place approximately 25 ng of purified homogeneous DNA of the probe region of interest in a volume of 45 µL topped up with 1× TE buffer, in a 0.2-mL PCR tube.
   Note: The desired probe should be gel eluted and purified post PCR amplification or plasmid digestion, so that there is no other contaminating dsDNA molecule. The probe must be eluted in 1× TE buffer. See notes for characteristics of an ideal probe.
2. Heat the probe solution in the thermal cycler at 99°C for 6–7 min, and immediately place on ice without delay, to retain the probe in a denatured state.

3. Add the probe mix from ice to the vial of Rediprime labelling kit. To this vial, add 5 µL of [α-^{32}P] dCTP, and gently mix 10 times using a pipette, to dissolve the lyophilised reaction mix without bubbles. Incubate in a thermomixer at 37°C for 30 min.
4. Add 2 µL of 0.5 M EDTA to stop the reaction. Purify the reaction mix to remove unlabelled [α-^{32}P] dCTP and other reaction components, using a G-50 column, as per the manufacturer's instructions. Briefly, pinch off the plastic seal of the G-50 column, and spin at 800 × *g* for 1 min to remove the preservative buffer. Place the tube on the fresh 1.5-mL collection tube, and add the labelled reaction mix carefully over the column bed. Spin again at 800 × *g* to collect the purified probe.

H. Hybridisation, stringency washes, and exposure to imaging screen

1. Heat the hybridisation buffer to 65°C in an oven. Place the rolled membrane in a hybridisation bottle, and add 50 mL of pre-heated (to 65°C) hybridisation buffer. Place the bottle in a pre-heated hybridisation oven. Allow this pre-hybridisation step at 65°C to proceed for 2 h with slow rotation (around 12–15 rpm) of the hybridisation bottles. (This is to facilitate uniform coverage of the membrane with hybridisation buffer, and to block the regions without DNA).
2. Remove the hybridisation buffer, and replace with 10–12 mL of the fresh pre-heated hybridisation buffer. Allow rotation in the oven for 10–15 min.
3. Denature the purified radio-labelled probe in a thermomixer at 99°C for 5 min. Immediately place on ice.
4. Add the denatured probe into the hybridisation bottle at 65°C as quickly as possible. Allow the hybridisation to proceed with slow rotation at 65°C for 16–20 h.
5. Discard the hybridisation buffer into the radioactive waste, and add 30 mL of wash buffer 1 pre-heated to 65°C. Shake and rinse the blot vigorously, and replace with 50 mL of fresh wash buffer 1. Proceed to rotation in an oven for 30 min.
   Note: All the wash buffers must be pre-heated to 65°C, and the washing steps occur at the same temperature with gentle rotation.
6. Discard wash buffer 1, and replace with 40 mL of wash buffer 2. Incubate in an oven for 30 min. Replace wash buffer 2 with wash buffer 3, and again proceed for 30 min.
7. After the washes, rinse the blot with 60 mL of 2× SSC buffer (at room temperature). Seal the blot in a wet state in a plastic cover, and fix the sealed blot in an exposure cassette with the DNA side facing up. Place the freshly erased phosphor imaging screen on the blot, and close the exposure cassette. Ideally, 2–3 days of exposure time at room temperature is sufficient.

I. Imaging the screen, and stripping the blot

1. Image the screen on a molecular imager, with a suitable wavelength of laser excitation and maximum photo multiplier tube (PMT) excitation voltage. The image can be acquired with the software provided with the molecular imager (.gel format), and analysed using ImageJ software package.
2. The blot is now ready for stripping and re-probing.
   Note: Ascertain that the blot is always kept wet with a layer of 2× SSC buffer, if stripping and re-probing is to be performed. Dry blots are hard to strip.
3. Pre-heat the oven and wash buffer 3 to 92°C. Add the hot wash buffer 3 to a hybridisation bottle containing the blot, and rotate at the same temperature for 15 min. Discard, replace with fresh wash buffer 3, and continue for 15 more minutes.
4. Wash the blot extensively with 2× SSC buffer at room temperature, and store it at 4°C in a sealed cover until a further round of hybridisation with a new probe.

Notes

1. Ideally, choose a probe between 600 bp to 2,000 bp long that is unique to a transposon or repeat of interest. Avoid regions of long terminal repeats (LTRs), as there might be conservation of stretches between different

LTR transposons. Choose regions such as the ones coding for transposase, or other transposon-associated coding regions of the genome.
2. Choose restriction enzymes that are proven to show reduced star activity and increased stability over prolonged incubation. Avoid adding more than 50 units of restriction enzyme, as this might cause over digestion or star activity.
3. It is normal to observe reduced quality of Southern images upon repeated stripping and re-probing. To avoid loss in quality, scale up the reaction volume with an increased DNA amount and post-digestion concentrate DNA by vacuum concentration or ethanol precipitation.
4. Should the protocol be adopted for other eukaryotic organisms, please scale the DNA quantity up/down according to the genome size, while all the other steps remain unchanged. Do not exceed 10 µg of genomic DNA for any source.

Recipes

1. **20× Saline-Sodium citrate (SSC) [3 M Sodium chloride, 0.3 M tri-sodium citrate, pH 7.0] buffer**
   Weigh 175.3 g of Sodium chloride and 88.2 g of tri-sodium citrate and dissolve them in 800 mL of water. Adjust the pH using hydrochloric acid (HCl) to 7.0, and make up the volume to 1,000 mL. Autoclave the solution, and store at room temperature (RT).

2. **Depurination solution [0.15 N HCl]**
   Prepare fresh depurination solution (0.15 N HCl) by adding 4 mL of concentrated HCl to 280 mL of water and making the volume up to 300 mL.

3. **Denaturation solution [1 M Sodium chloride, 0.5 M Sodium hydroxide]**
   Dissolve 20 g NaOH and 58.44 g Sodium chloride in 800 mL of water, and make the volume up to 1,000 mL. Autoclave, and store at RT.

4. **Neutralisation solution [1.5 M Sodium chloride, 0.5 M Tris-Cl]**
   Dissolve 87.66 g NaCl and 60.55 g of Tris base in 800 mL of water. Adjust pH to 7.0 using concentrated HCl, and make the volume up to 1,000 mL. Autoclave, and store at RT.

5. **2 M Sodium phosphate buffer pH 7.2**
   Weigh 56.8 g of Sodium phosphate dibasic salt, and dissolve in 140 mL of water pre-heated to ~70°C. Set pH to 7.2 using ortho-phosphoric acid. Make up the volume to 200 mL, autoclave, and store at RT. In case this precipitates, re-heat the buffer to dissolve the components.

6. **10% SDS (w/v)**
   Dissolve 50 g of SDS in 450 mL of water. Make up the volume to 500 mL, and store at RT.

7. **0.5 M EDTA pH 8.0**
   Dissolve 18.6 g of EDTA in 60 mL of double distilled water. Keep adding NaOH pellets slowly, until the EDTA is completely dissolved. Adjust the pH to 8.0 using 10 N NaOH. Make the volume up to 100 mL, autoclave, and store at RT.

8. **Hybridisation buffer [0.5 M Sodium phosphate buffer pH 7.2, 7% SDS, 1 mM EDTA]**
   Pre-heat 10% SDS and 2 M Sodium phosphate buffer pH 7.2 solutions to 65°C. Mix 70 mL of 10% SDS, 25 mL of 2 M Sodium phosphate buffer, 4.8 mL of water, and 0.2 mL of 0.5 M EDTA pH 8.0, and heat the solution to 65°C in a water bath.

9. **10× Tris borate EDTA (TBE) buffer [0.89 M Tris-Cl, 0.89 M Boric acid, 20 mM EDTA]**

Weigh 108 g of Tris base, 55 g of boric acid, and 7.5 g of EDTA. Dissolve them in 800 mL water. The pH should self-adjust to ~8.2. Make the volume up to 1,000 mL, autoclave, and store at RT.

10. **1 M Tris buffer pH 8.0**
    Dissolve 121.14 g of Tris base in 800 mL of water. Adjust pH using concentrated HCl to 8.0. Make the volume up to 1,000 mL, autoclave, and store at RT.

11. **1× Tris-EDTA (TE) buffer [10 mM Tris-Cl pH 8.0, 1 mM EDTA]**
    To 8 mL of ultra-pure water, add 100 µL of 1 M Tris buffer pH 8.0, and 20 µL of 0.5 M EDTA pH 8.0. Make the volume up to 10 mL. Store at RT.

12. **8× DNA loading buffer [20% Ficoll-400, 88 mM EDTA, 26.4 mM Tris-Cl pH 8.0, 0.12% Bromophenol blue disodium salt]**
    For 10 mL of loading buffer, dissolve 2 g of Ficoll-400 in 8 mL of ultra-pure water. Add 1.76 mL of 0.5 M EDTA pH 8.0, and 0.264 mL of 1 M Tris buffer pH 8.0. Add 12 mg of Bromophenol blue sodium salt, mix, and dissolve. Make the volume up to 10 mL with ultra-pure water. Store at 4°C.

13. **Wash buffer 1 [3× SSC and 0.1% SDS]**
    To 80 mL of water, add 15 mL of 20× SSC buffer, and 1 mL of 10% SDS. Make the volume up to 100 mL. Prepare fresh.

14. **Wash buffer 2 [0.5× SSC and 0.1% SDS]**
    To 80 mL of water, add 2.5 mL of 20× SSC buffer, and 1 mL of 10% SDS. Make the volume up to 100 mL. Prepare fresh.

15. **Wash buffer 3 [0.1× SSC and 0.1% SDS]**
    To 80 mL of water, add 0.5 mL of 20× SSC buffer, and 1 mL of 10% SDS. Make the volume up to 100 mL. Prepare fresh.

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Competing interests

The authors declare that they have no competing interests.

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