

Plant DNA methylation analysis by "bisulfite genomic sequencing" (PROT14)



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Introduction

The following "bisulfite genomic sequencing" protocol has been optimized for the determination of DNA cytosine methylation at transgene loci in plants. The conditions used are based on the method given by Pelissier and Wassenegger (2000).

Procedure

Preparation of Plant Genomic DNA

Prepare plant genomic DNA using a Qiagen DNeasy Plant Maxi or Mini kit (see [comment 1](#)).

Prior to the bisulfite-treatment, the plant genomic DNA is cleaved at sites outside of region intended for amplification to resolve structures like direct or inverted repeats that could interfere with PCR (see [comment 2](#)).

To a 1.5ml reaction tube,

1. Add genomic DNA dissolved in bidist. water (e.g. 2.5µg for *Arabidopsis* or 25µg for tobacco depending on the genome size of the respective plant species; less DNA (>0.5µg) may be used without "downscaling" the protocol, but downstream PCR might then require more cycles and/or a larger volume of bisulfite-treated target DNA solution);
2. Fill up with bidist. water to a final volume of 270µl;
3. Add 30µl 10X reaction buffer (appropriate for restriction enzyme);
4. Add restriction enzyme, ~10U/µg of genomic DNA;
5. Incubate over night (~14 hours) at temperature appropriate for used restriction enzyme;
6. Add 0.5ml Tris-buffer-saturated phenol-chloroform, vortex;
7. Centrifuge with Eppendorf 5414 or equivalent at 10000 x g for 2 minutes;
8. Transfer the upper aqueous phase to clean 1.5ml reaction tube;
9. Add 0.5ml chloroform, vortex;
10. Centrifuge as in step 7;
11. Transfer the upper aqueous phase to clean 1.5ml reaction tube;
12. Add 35µl 3M NaOAc/HOAc pH 5.0; 850µl abs. ethanol;
13. Incubate >1 hour at -20°C (see [comment 3](#));
14. Centrifuge Sigma 1-15K (or equivalent) >15 minutes at 4°C 12000rpm (12000 X g);
15. Discard supernatant, add 1.0ml 70% ethanol (room temperature), vortex;
16. Centrifuge Sigma 1-15K (or equivalent) >15 minutes at 4°C 12000rpm (12000 X g);
17. Discard supernatant, vacuum dry for 10 minutes at room temperature;
18. Dissolve cut DNA in 10µl bidist water (0.25 or 2.5 µg/µl, respectively), store at -20°C;

Bisulfite treatment

The [bisulfite solution](#)

should always be made fresh the same day as it will be used. One should get started early to make it, as it will usually take some hours for the sodium bisulfite and urea to dissolve completely. It is essential to adjust the pH of the solution to the required 5.0 at room temperature. Otherwise, incomplete conversion of unmethylated cytosines could be the result. 10M NaOH should always be prepared freshly as well.

The genomic DNA is first denatured by alkaline treatment and then incubated with bisulfite in the presence of urea. To support complete conversion, repeated heat denaturation steps are performed during the incubation. After the reaction, the DNA is cleaned up and desulfonated for PCR amplification.

First day:

1. To a 0.5ml thin walled PCR reaction tube (Eppendorf 0030 124.502); add 4.0µl (1µg) restriction-cut genomic DNA (see above), add 6.0µl bidist. water, add 0.35µl 10M NaOH;
2. Incubate 20 minutes at room temperature;
3. Add 68µl [bisulfite solution](#) freshly made as indicated above;
4. Overlay 3 drops (~100µl) mineral oil;
5. Incubate in a PCR cyclor over night: 2 minutes at 95°C 1 time, 2 hours at 75°C followed by 1 minute at 95°C 9 times, hold at 75°C. Some precipitate will form, but this will not interfere with later steps; proceed to the DNA cleanup immediately after completion of the bisulfite reaction.

Second day:

1. Transfer the reaction solution (including the oil, but without the precipitate) to a 1.5ml reaction tube;
2. Add 1.0ml Wizard DNA clean up resin (Promega kit #A7280), vortex (see [comment 4](#));
3. Load resin on provided column, apply vacuum (vacuum flask, membrane pump);
4. Add 2.0ml 80% (v/v) 2-propanol on column, apply vacuum (vacuum flask, membrane pump);
5. Transfer column with resin to 1.5ml reaction tube, add 50µl bidist. water preheated to 64°C for DNA elution;
6. Incubate 1 minute at room temperature;
7. Centrifuge Eppendorf 5414 2 minutes;
8. To DNA solution (~50µl), add 40µl bidist. water, add 3.0µl 10M NaOH (prepared the day before);
9. Incubate 20 minutes 37°C (incubator);
10. Add 200µl 4.5M ammoniumacetate (Sigma A-1542, 17.34g dissolved in bidist. water to a final volume of 50ml) (see [comment 5](#));
11. Add 750µl absolute ethanol (see [comment 6](#));
12. Incubate >1 hour at -20°C;
13. Centrifuge Sigma 1-15K (or equivalent) >15 minutes at 4°C 12000rpm (12000 X g);
14. Discard supernatant, add 1.0ml 70% ethanol (room temperature), vortex;
15. Centrifuge Sigma 1-15K (or equivalent) >15 minutes at 4°C 12000rpm (12000 X g);
16. Discard supernatant, vacuumdry for 10 minutes at room temperature;
17. Dissolve DNA in 50µl bidist. water (20ng/µl or 200ng/µl, respectively), store at -20°C;

PCR

See [comment 7](#). The upper limit for the length of PCR products to be amplified from bisulfite-treated DNA should be in the range of some 400 to 500 nucleotides. Primers for PCR amplification are designed to contain degenerate nucleotides (G/A for the 1st primer, C/T for the second primer) to allow for alternative C or "T" (actually dU) nucleotides in the bisulfite-treated template DNA. Primer length should be approximately 21 to 24 nucleotides; the T_m should be in the range of 50-60°C. Preferably, primers should not contain more than 3 degenerate bases, but more (up to 5?) can be tolerated for one of the two primers. In this case, it can be favourable to replace the G/A (R) or C/T (Y) degenerate nucleotides by inosine (I), which is able to basepair with any of the nucleotides present in DNA. Some examples for primer pairs that were used successfully (Aufsatz *et al.* 2002, Kanno *et al.* 2004) are:

NOSpro-NPTII top strand (optimal T_{amp} for PCR: 55.0°C)

NOSpro-top-2 5' YAT GAG YGG AGA ATT AAG GGA GT 3'

nptII-top-2 5' CCR AAT ARC CTC TCC ACC CAA 3'

NOSpro-NPTII bottom strand (optimal T_{amp} for PCR: 50.0°C):

NOSpro-bot-2 5' CAC RTT ATR ACC CCC RCC 3'

nptII-bot-3 5' AGI AGI IGA TTG TIT GTT GTG 3'

alpha'pro-GFP top strand (optimal T_{amp} for PCR: 52.3°C):

T2f 5' AYG YGA TAG AAA AYA AAA TAT AG 3'

T1r 5' CCT TTA CTC ATT RTT ATA TCT CC 3'

Primers were ordered from Metabion "OPC-purified" as stock solutions of 100pmol/µl.

For one 50µl reaction using AmpliTaq Gold DNA pol.(Applied Biosystems #4311814), combine in a Eppendorf 0.5ml thin

walled PCR reaction tube:

- bidist. water
- 10X reaction buffer (no MgCl₂)
- 4.0μl (final conc.: 2mM)
- 25mM dNTP mix
- 100 pmol/μl 1st primer
- 100 pmol/μl 2nd primer
- 5U/μl AmpliTaq Gold DNA pol
- 20ng/μl template DNA 37.35μl(28,35μl)
- 5.0μl
- 25mM MgCl₂
- 0.4μl
- 1.0μl
- 1.0μl
- 0.25μl
- 1.0μl (10μl for diluted DNA)

Overlay with ~50μl mineral oil.

PCR reaction: 10 minute at 94°C 1 time; 30 seconds at 94°C, 30 seconds Temp., 45 seconds at 72°C 40 times (up to 45) (Temp. to be optimized for each primer pair, see above, ~50-60°C), 10 minutes at 72°C, hold at 8°C (see [comment 8](#)).

10μl of the PCR product solution are tested by electrophoresis on a standard 2.5% agarose gel in 1 X TBE followed by ethidiumbromide-staining. The remaining 40μl of the PCR solution can be stored for up to several weeks at 4°C prior to cloning (see [comment 9](#)).

Cloning and Sequencing of PCR Products

If a clear defined band is visible on the ethidiumbromide-stained agarose gel, the PCR products are cloned and sequenced. An economic and reliable method is given below, but of course, there are many alternative cloning procedures applying more sophisticated vector systems that will do as well. PCR products are purified using the QIAquick PCR Purification Kit (Quiagen Cat. No. 28104) and cloned using the pGEM-T Easy Vector System I (Promega Cat.# A1360).

PCR product purification:

1. Add 200μl "binding buffer" PB (equals 5 volumes) to the remaining 40μl PCR product solution in the original reaction tube still containing the mineral oil, vortex;
2. Transfer the solution including the mineral oil to a QIAquick spin column sitting in the provided 2ml collection tube
3. Centrifuge in a table-top centrifuge at full speed at room temperature for 1 minute;
4. Discard flow-through, place the QIAquick column back in the same collection tube;
5. Add 750μl "washing buffer" PE to the QIAquick column;
6. Centrifuge Eppendorf 5414 at room temperature for 1 minute;
7. Discard flow-through, place the QIAquick column back in the same collection tube;
8. Centrifuge Eppendorf 5414 at room temperature for 2 minutes to dry the column;
9. Place the QIAquick spin column in a clean 1.5ml reaction tube;
10. Add 40μl bidist. water directly onto the membrane of the QIAquick spin column;
11. Incubate for 1 minute at room temperature;
12. Centrifuge Eppendorf 5414 at room temperature for 2 minutes;
13. Discard QIAquick spin column, store eluate at 4°C.

pGEM-T Easy Vector (Promega Cat.# A1360) - PCR product ligation

In a 1.5ml reaction tube sitting on ice, combine:

- 3.0μl - purified PCR product solution (see above)
- 5.0μl - 2X rapid ligation buffer (provided in the kit)
- 1.0μl - 50ng/μl pGEM-T Easy Vector (provided in the kit)
- 1.0μl - 3U/μl T4 DNA ligase (provided in the kit)

Briefly mix, spin down, incubate over night at 4-8°C in a refrigerator.

Transformation of *E. coli* DH5alpha (Invitrogen)

Competent *E. coli* DH5alpha:

1. Grow *E. coli* DH5alpha over night on a [L-Agar](#) plate to single colonies at 37°C;
2. Inoculate 1.5ml [LB](#) in a baked glass culture tube from a single colony;
3. Shake 4 hours at 37°C;
4. Use this preculture to inoculate 40ml [LB](#) in a baked wide-neck 500ml Erlenmeyer flask;
5. Shake 2 hours at 37°C;
6. Determine OD600 (1cm path) for the culture; allow further growth while shaking at 37°C until the OD600 value will be in the range of 0.50-0.55 (it should never exceed 0.60);
7. Split the culture in 2 times 20ml into 50ml conical tubes (Greiner Nr. 227261) sitting on ice;
8. Centrifuge Heraeus Megafuge 1.0R 3000rpm 4°C 5 minutes;
9. Discard supernatant, add 2 times 10ml ice-cold [100mM CaCl2](#), resuspend the bacteria by "punching" the tubes against one another;
10. Incubate 20 minutes on ice;
11. Centrifuge Heraeus Megafuge 1.0R 3000rpm 4°C 5 minutes;
12. Discard supernatant, add 2 times 1.0ml ice-cold [100mM CaCl2](#), resuspend the bacteria by "punching" the tubes against one another;
13. Store "competent" *E. coli* DH5alpha on ice for up to 24 hours.

Transformation

1. Transfer 5µl of ligation solution into a sterile culture tube sitting on ice;
2. Add 150µl "competent" *E. coli* DH5alpha prepared as described above;
3. Incubate 45 minutes on ice;
4. Incubate 2 minutes 42°C in a water bath;
5. Immediately add 1.0ml [LB](#), mix gently by rotating the culture tube;
6. Incubate 1 hour at 37°C, the "transformation culture" can then be stored at 4°C for up to several weeks;
7. Prepare [L-Amp50-agar-XGal-IPTG](#) plates freshly;
8. Spread up to 200µl of "transformation culture" per plate;
9. Incubate over night at 37°C until clearly visible colonies will have formed;
10. Incubate several hours at 4°C to allow the "development" of optimal blue/white color contrast;
11. Pick clearly white colonies to inoculate 2ml [LB](#) in a sterile culture tube;
12. Shake over night at 37°C;
13. Perform plasmid DNA minipreparations using a QIAprep Spin Miniprep Kit.

Plasmid preparations can be checked by EcoRI digestion and agarosegel electrophoresis for the presence inserts of the size expected for the PCR product.

Sequencing is done by standard methods. But some systems may have problems to produce sequencing runs of sufficient length and quality from the A/T rich sequencing templates that can result if there is little or no cytosine methylation in the genomic plant DNA of interest. In our hands, the polyacrylamidegel-based LI-COR 4300 DNA Analysis System turned out to be most useful for this purpose (see [comment 10](#)).

Materials & Reagents

Bisulfite treatment	10M NaOH: dissolve 10.0g NaOH in bidist. water to a final volume of 25ml (Greiner tube).
bisulfite solution	38.0g - 2M "sodium disulfite" (Merck 1.06528.1000) 36.0g - 6M urea (BioRad 161-0731) 6.6mg - 0.60mM 1,4 benzenediol (Sigma H9003) Bake a 100ml wide-neck Erlenmeyer flask containing a stirring bar 4 hours at 150°C; Collect solids as indicated above into the flask; Add 60ml bidist. water (will give a final volume of 100ml); Stir at room temperature until most solids will be dissolved (can take several hours); Add 400µl freshly prepared 10M NaOH (remaining solids should now dissolve); Check pH, adjust pH to 5.0 by slowly adding 10µl-aliquots of 10M NaOH.
LB	10g - Bacto Tryptone (Nr. 211707) 5.0g - Difco Yeast Extract (Nr. 212750) 10.0g - NaCl Dissolve in 1.0 liter deionized water; Set pH to 7.0 using 1M NaOH;

	Autoclave 20 minutes 141°C in 0.5 liter bottles.
L-Agar	Add 7.5g Select Agar (Invitrogen Cat No.30391-023) per 500ml LB in a 0.5 liter bottle prior to autoclaving; Mix by shaking; Autoclave 20 minutes 141°C; Mix again; Set bottle in a waterbath at 65°C for > 2 hours; Per bottle, pour 15 plates in 94mm/15mm dishes (Greiner Nr. 632190) in a clean bench; Allow to solidify over night; store upside down at 4°C.
L-Amp50-agar	Stock solution for ampicillin: 50mg/ml. Dissolve 500mg ampicillin (Sigma A-9518) in 10.0ml bidist. water; sterilize by filtration; store at -20°C; Prepare L-Agar as above; set temperature to 65°C in the waterbath; Add 500µl sterile 50mg/ml ampicillin stock solution immediately before pouring plates; proceed as described above.
L-Amp50-agar-XGal-IPTG	On L-Amp50-agar plates prepared as above, spread immediately before plating bacteria: 70µl 20mg/ml XGal (AppliChem A1007 in NN-dimethylformamide Sigma D-4254); 70µl 20mg/ml IPTG (AppliChem A1008 in bidist. water).
100mM CaCl2	Dissolve 2.94g calcium chloride dihydrate (Merck 2382) in bidist. water to a final volume of 200ml; autoclave 20min 141°C; store 4°C.

Reviewer Comments

Reviewed by: [Ales Kovarik](#) and coworker, Institute of Biophysics, Brno

1. General comments: We recommend setting up a control of bisulfite conversion every time when methylation of a new locus is to be investigated. This is because bisulfite reaction is known to be often incomplete due to renaturation of DNA strands. Since renaturation rate of different DNA molecules might vary (depending on GC content and other physical-chemical properties of the target template) we recommend using a plasmid DNA containing the cloned target gene. Plasmid DNA is usually methylation free with exception of rare sites modified by *E. coli* methylation systems. Ideally on sequencing electropherograms the bisulfite-treated plasmid DNA should appear as a C-free track.

The controls reactions are especially important when analysing plant DNA since plant genomes contain high level of cytosine methylation, and methylated Cs can be located at any sequence context. Consequently, it is sometimes difficult discriminate between high density of genomic methylation and incomplete bisulfite conversion of molecules. The reliability of bisulfite sequencing should be therefore verified by an independent method e.g. methylation-sensitive enzymes in combination with Southern blot hybridization using gene specific probes or RFLP-PCR. In an ideal world the degree of methylation of a restriction site determined by bisulfite seq. and restriction enzymes-based methods should be comparable.

2. Such structure may also stimulate undesirable of DNA renaturation.
3. Ethanol precipitation at -20°C for > 1 hour is usually unnecessary for DNA concentration >20ng/ml. We follow the protocol of Sambrook and Russel (Molecular Cloning manual, 3rd ed., A8.14, 2001) allowing 15 minutes precipitation at 4°C. The shortened incubation intervals will speed up the whole procedure.
4. The Qiaex II kit from Qiagen works equally well. In general, any kit allowing purification of single stranded DNA can be used.
5. For neutralization add ammonium acetate to a final concentration of 3M. For ~40 µl of DNA we add 20µl of 10M ammonium acetate - it is useful to keep the volumes low because of low concentration of bisulfite-treated DNA. There are reports that sodium bisulfite might cause significant degradation of DNA (Nucl Acids Res 29 (13) e65, 2001). Consequently the DNA concentrations might significantly differ before and after the [Bisulfite treatment](#). For ammonium acetate, please give pH - we use 7.0.
6. Better: Add 2.5 volume of ethanol at -20°C.
7. The primer design is critical. We think that this paragraph deserves some more attention.
 - The DNA strands are analyzed separately since they are no longer complementary. For a primer design, try to find regions which are relatively rich in G's. The PCR is initiated with the 3' primer that should be C-rich. The 5' primer should

- be G-rich. (the 5' primer is homologous to the sequenced strand, the 3' side is complementary to the sequenced strand);
- The best primers should contain 6-8 G/C's;
 - Often, especially when dealing with large genomes e.g. tobacco it is necessary to use nested (4 primers) or semi-nested PCR (3 primers) for a sufficient yield of the PCR product.
8. The 3 minutes of initial denaturation step is enough - shortened denaturation intervals will better preserve enzyme activity.
 9. We use 1-1.5% agarose, which works well and saves some money!
 10. Cloning and sequencing of PCR products. In the protocol I miss the description of sequencing data processing. This can be done manually or with an aid of appropriate software. Some hints are mentioned below:
 - Manual comparison of sequences. Write down the sequences in an order: the native strand should be written in the top line; in the modified sequences below. A mutation of C into T in bisulfite-treated clones indicates absence of methylation. Preservation of C's (no mutation) indicates the presence of methylated cytosine in genomic DNA.
 - The sequences can be aligned by a number of programs e.g. Pile UP (implemented in the Wisconsin GCG package software) and Clustal W (freely accessible on web).
 - Use a toolbox to visualize and analyze DNA methylation data. The web server at Methtools offers a number of good programs that might be downloaded. A direct analysis of aligned sequences via www is also possible. (There is an interface on the Epigenome NoE site under the Tools & Resources button)
 - The bisulfite sequencing generates a large number of data that might not always be simple to interpret. To reveal biological meaning of methylation patterns the data should be evaluated with respect to methylation density and sequence context of methylated residues.

References

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