Bisulfite Protocol

Adapted from a protocol by Mike Huang, Daniel Zilberman, modified by Simon Chan 6/15/04

**DAY 1**

1)  Digest 250ng–2 μg of genomic DNA with enzymes that cut just outside of the region of interest, total volume of 100 μl, about 20 units of each enzyme, 2 hours – overnight.

•  DNeasy works well for maize endosperm, CTAB extraction works well for maize and soybean leaf.

2) Add 100 μ L phenol:chloroform (pH 8.0) to the solution in step 1, mix, spin for 5 minutes at 12,000 rpm, then transfer 90 μ l of aqueous phase to each fresh tube. Add 1-2 μ l (20 μ g/ μ l) tRNA or glycogen and 9 μ l 4M NaOAC and 350 μ l EtOH, mix well. Spin 10 minutes at 12,000 rpm, 70% EtOH wash very carefully and remove all liquid. Repeat this wash again. Dry pellet completely.

3) Resuspend in 20 μl water. <Pause point, can store digested DNA at -20 ° C>

4) Prepare fresh bisulfite. Dissolve 8.1g of sodium bisulfite (Fisher S654-500) in 16mL of water with slow stirring to avoid aeration. Adjust pH to 5.1 with 10M NaOH (takes about 0.4mL, 8g/ 20mls,**freshly prepared**). Add 0.66mL of 20mM hydroquinone (0.11g/ 50mls water; Sigma H-9003). Adjust volume to 20mL with water.

•  It takes at least 10 minutes for the sodium bisulfite to dissolve, start preparing sodium bisulfite solution while pellet in step 2 is drying

5) Heat digested DNA at 97 ° C 1 minutes in PCR machine. Quench in ice water for 1 minute. Spin down for 2-3 seconds.

6) Add 1 μ l of 6.3M NaOH ( **freshly prepared NaOH from above**). Incubate 39 ° C for 30 minutes.

•  Add 208 μ l of the bisulfite solution to each denatured DNA. Do this in the PCR machine, so that the denatured DNA is still at 39 ° C. Incubate in PCR machine at 55 ° C for 16 hours, with a jolt to 95 ° C for 5 min every three hours. Store at 4 ° C until ready to proceed the next day.

**DAY 2**

8) Desalt samples with QIAGEN PCR purification columns. Elute in 100µL EB.

9) Add 6.3M NaOH (from day 1) to a final concentration of 0.3M (5µL), mix well, incubate at 37 ° C for 15 min.

10) Add 33µL 10M NH 4 OAC pH 7.0 to a final concentration of 3M, add 1-2 μ l of (20 μ g/ μ l) tRNA or glycogen, and 342µL (=3 volumes) 100% EtOH . Spin 15 min for 13,000 rpm, 70% wash and dry pellet. Resuspend in 100 μ l of QIAGEN EB or TE/H20. Use 2 μ l for each PCR reaction.

**Note:**The most critical aspect of the protocol is complete denaturation of the genomic DNA so that the bisulphite conversion is complete. This means that the enzyme digestion should be complete, and the denatuation steps done carefully. Make sure there is no salt in the DNA for the 97 ° C denaturation step.

**PCR**based on Brain Research Protocols 2000 5:167-171 with changes.

**FOLLOW EXACTLY!!!!**

Use Takara ExTaq.

2 µl bisulfite treated DNA

4 µl dNTPs (2.5mM each)

5 µl 10X ExTaq buffer

1 µl reverse primer

38 µl H20

1) 95 ° C 5min

add 1µl ExTaq (5U)

2) 95 ° C 20secs

3) 60 ° C 3min

4) 72 ° C 3min

5) run 5 cycles

Add 1µl forward primer

6) 95 ° C 20secs

7) 60 ° C 1.5 min

8) 72 ° C 2min

9) run 10 cycles

10) 95 ° C 20secs

11) 50 ° C 1.5 min

12) 72 ° C 2 min

13) run 30 cycles

14) 72 ° C 5min

15) 4 ° C forever

Do not run PCR for more cycles than indicated, as this leads to sibling clone problems.

Gel purify PCR product and clone with Invitrogen TOPO TA kit (or your cloning kit for sequencing of choice). Sequence – aim for about 20 for each biological sample to get reasonable values for methylation.

Bisulfite primer design

I start out by differentially highlighting the C’s in my region of interest (for example, make all CNN, make all CG and all CNG). This makes it easier to visually pick out regions that might be good for primer design. Design primers according to the rules below. Try to keep product to <500bp (smaller PCR products generally amplify better than large ones). Remember, after bisulfate conversion, the two strands of DNA will no longer be complementary (converted Cs, now Ts, will no longer be able to base pair with the G on the other strand). You have to treat each strand on its own for PCR primer design.

**• Forward (top strand) primer**

1) Criteria for good primer:

– Find G-rich region.

– Minimal number of CGs or CNGs to reduce degeneracy in the primer.

– Look for two asymmetrics Cs very close to the 3′ end (at least 2 in the last five nucleotides) and make one of these the last nucleotide in your primer; this will select for converted DNAs. Ideally, your primer will have two asymmetric Cs right at the end.

– If possible, avoid polyT and A runs near the 3′ end i.e. include some G. This will prevent amplification of simple sequences (and primer dimer with similar sequences in bottom strand primer).

2) Convert Cs to Ts. Convert Cs in CG or CNG context to Ys (i.e. C or T). Try to avoid having more than 3 degeneracies per primer. Fewer is better.

3) Adjust the length so the Tm is above 65 degrees. If it’s impossible to meet the rules about CGs, asymmetrics at the end, and some Gs near the end, look for another region for your primer.

**Reverse (for the top strand) primer (use first in bisulfite PCR reaction)**

4) Criteria for good primer:

– Same criteria as above, pick a suitable region and reverse complement it to make your reverse primer

– If you must include a degeneracy in your primer, the base complimentary to the C in the CG or CNG context becomes an “R” in your revere complemented primer. See example below