

Bisulfite Genomic DNA Sequencing

-for high-resolution mapping of methylated cytosines in a DNA region of interest-

by Rick Lawrence and Craig Pikaard, Washington University in St. Louis.

This protocol is adapted from a protocol from Steve Jacobsen's lab.

Conversion:

1. Digest ~4ug of genomic DNA with enzymes that cut outside region of interest (but as close as possible) in a total volume 100 ul, using 10-20 Units of each enzyme. The purpose of digestion is to cut the DNA into linear fragments that are more readily denatured for bisulfite conversion than total genomic DNA.
2. Run restriction digestion products on 1% low melt agarose gel (in TBE buffer) and purify DNA size range of interest using a Gene Clean Turbo Kit.
3. Make fresh bisulfite solution: dissolve 40.5 g of sodium bisulfite in 80 ml of water with slow stirring to avoid aeration. Adjust pH to 5.1 with 10M NaOH (this stock solution is 8g NaOH/20ml water; freshly prepared). Add 3.3 ml of 20 mM hydroquinone (this stock solution is 1.1g hydroquinone/500mls water). Adjust volume to 100 ml and protect from light.
4. Take 40ul of purified DNA and add to 0.5ml tube. Heat at 97°C for 5 min in a thermocycler to denature the DNA. As quickly as possible, put the reaction tubes in an ice-water bath and cool at 0°C for 1 min. Quick chilling minimizes duplex formation, keeping individual DNA strands as denatured as possible.
5. Add 2ul of 6.3M NaOH (5.04g/20ml freshly prepared). Incubate at 39°C for 30min. We use a thermocycler for this incubation step.
6. Prewarm bisulfite solution to 55°C and add 416 ul of bisulfite solution to the tube containing denatured DNA (while tube is still in the thermocycler to maintain temperature). Overlay the reaction mixture with mineral oil to prevent evaporation of the aqueous phase.
7. Program the Thermocycler such that reactions are incubated at 55°C for a total duration of 16 hours, punctuated every three hours by a 5 minute denaturation step at 95°C.

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 denaturation steps done carefully. Make sure there is no salt in the DNA for the 96C denaturation step.

8. Desalt samples using the Wizard DNA Clean-up System (Promega Corporation).
9. Measure the exact volume of DNA recovered (in TE buffer) and add 6.3 M NaOH to a final concentration of 0.3M. Incubate at 37°C for 15min.
10. Add 10M NH₄OAC pH 7.0 (ammonium acetate) to a final concentration of 3M, add 2 ul of 20 ug/ul tRNA or glycogen (to act as carrier for high efficiency precipitation), and 3

volumes of 100% EtOH. Incubate at -20°C for 30min. Spin 15min. at top speed (14,000 x g) in microcentrifuge. Wash the DNA pellet with 70% EtOH and resuspend in 100ul TE. Use 2ul per PCR reaction.

NOTES: Primer design in our hands does not seem to be critical for amplifying bisulfite converted rRNA gene sequences. However, it is important to use only freshly converted DNA (no more than a few days old) in the PCR reactions to obtain clean results.

Cloning and Sequencing:

PCR the region of interest in triplicate, pool the PCR products, and clone fresh PCR products into plasmids for sequencing (we use the TOPO TA Cloning kit from Invitrogen). Be sure to spread enough plates to be sure you will get enough clones for your analyses (we typically obtain ~20-30 colonies per plate). Sequence each individual clone (we use ABI cycle sequencing reagents and automated sequencing with Big Dye chemistry). Compare sequences of bisulfite-converted DNA to mock-treated controls. Unmethylated cytosines are converted to uracils by bisulfite treatment, thus the DNA sequence will appear to be altered relative to controls at unmethylated cytosine positions.