Benchmarks

Methylation Analysis Using Bisulfite Genomic Sequencing: Application to Small Numbers of Intact Cells

BioTechniques 22:272-274 (February 1997)

This report describes the application of bisulfite genomic sequencing to samples consisting of between 50 and 200 intact cells. Bisulfite genomic sequencing, originally described by Frommer et al. (5), allows the determination of methylation status of each cytosine residue in a defined sequence for individual strands of genomic DNA. This technique relies on the ability of bisulfite to specifically convert unmethylated cytosine residues, but not 5-methylcytosine, to uracil by deamination (6,7,10,12). Bisulfite-modified DNA is then polymerase chain reaction (PCR)-amplified to yield products in which uracil residues have been amplified as thymine and 5-methylcytosine residues only have been amplified as cytosine. The methylation pattern of a target sequence is revealed when PCR products are sequenced directly, to reveal an average methylation pattern for the population of DNA molecules, or cloned and sequenced to reveal the methylation pattern of an individual allele. Published variations of the bisulfite genomic sequencing protocol require microgram amounts of restriction enzyme-digested genomic DNA as starting material (2,4,5,9) or dilutions to picogram amounts of previously digested genomic DNA (2).

In this report, we describe a method that allows the bisulfite genomic sequencing technique to be used for the methylation analysis of genes when starting material consists of between 50 and 200 intact cells. This method will allow researchers to investigate the role of methylation in the control of gene expression in germ cells, embryogenesis, inherited diseases and cancers, when samples are limiting. However, since it may be possible to amplify from a single allele, minimizing cell numbers too drastically will give methylation profiles that are not repre-

sentative of the population of cells. This is especially the case since no method of DNA purification, restriction enzyme digestion or PCR amplification can be 100% efficient. Thus, overall experimental design should allow for this by including as many cells per sample as possible, and multiple individual samples must be assayed.

We have used this method in our laboratory to generate comprehensive methylation profiles of the *Xist* gene in mouse germ cells and during pre-implantation development in order to study the role of methylation in the genomic imprinting of this gene. F1 generation embryos derived from evolutionary distinct strains of mice were used in these experiments so that polymorphisms could be used to identify the parental origin of individual alleles.

In the first step of the procedure, genomic DNA is prepared from cells treated with a lysis buffer containing proteinase K and guanidine hydrochloride (8). Genomic DNA is then recovered from the lysate by ethanol precipitation in the presence of glycogen, which acts as a carrier for low concentrations of DNA. Bisulfite modification of isolated genomic DNA is then performed essentially as described by Clark et al. (2) with variations introduced to increase the sensitivity of the procedure for small amounts of starting material. As bisulfite induced deamination of cytosine residues is only efficient for single-stranded DNA (6,11), the genomic DNA is first denatured by

restriction enzyme digestion followed by alkali denaturation. The efficiency of the procedure was increased by including plasmid DNA in the restriction enzyme digest and by increasing the stringency of denaturation by raising the reaction temperature from 37°C to 75°C. Raizis et al. (9) report that shearing genomic DNA before alkali denaturation is an unnecessary step; however, in our experience this step was found to be essential (data not shown). Prolonged bisulfite treatment causes degradation of DNA as a result of depurination (9). For this reason, the kinetics of the bisulfite modification reaction were maximized by using 4.8 M bisulfite, which allowed reaction time to be reduced to 4 h. For PCR amplification, we recommend using touchdown PCR (3) for amplifying bisulfite-modified DNA in picogram amounts. There are at least two advantages of using touchdown PCR over the traditional PCR approach. First, it can avoid the amplification of nonspecific products, which is a possibility with the T-rich forward primers or A-rich reverse primers used to amplify bisulfite-modified DNA. Second, it can help overcome the problem of arriving at a suitable empirically derived annealing temperature for these primers without time-consuming optimization procedures.

The optimized protocol for bisulfite genomic sequencing starting with small samples of intact cells is as follows: Cells in a volume of phosphate-buffered saline (PBS) + 0.1% bovine

Figure 1. Amplification of a 569-bp fragment from *Xist* using bisulfite-modified genomic DNA derived from mouse pre-implantation embryos. DNA was extracted, bisulfite-treated and PCR-amplified as described in the text. The 569-bp product was amplified from a region of *Xist* located approximately 1 kb into the coding sequence using asymmetric nested primers (forward primer, 2M2: TGAGTTATTGTATTATTTGGAATATTTATGTT; and reverse primers, 2M1: TAACTCAAACAT-AAACTAATATAACAC and 2M3: TACTACACACCAAAACAAAAATTAACTAT). Lane 1, size standards (1-kb ladder; Life Technologies, Gaithersburg, MD, USA). Lanes 2–5, PCR products generated using bisulfite-modified DNA derived from 50 4-cell, 10 8-cell, 11 morula and 4 blastocyst stage pre-implantation embryos. Lane 6, PCR products generated using bisulfite-modified DNA derived from 9 isolated inner cell masses. Additional bands at the bottom edge of the gel represent excess primer bands.

serum albumin (BSA) or M2 embryo culture medium (Sigma-Aldrich, Castle Hill, NSW, Australia) not exceeding 20 μ L were transferred directly into 165 μ L of freshly prepared proteinase K-guanidine hydrochloride lysis buffer (8) in a sterile 1.5 mL Eppendorf tube. Samples were then incubated at 60°C for 2 h. Genomic DNA was ethanol-precipitated by adding 1.0 μ g of glycogen (Boehringer Mannheim, Castle Hill, NSW, Australia) as carrier and 3 volumes of 95% (vol/vol) ethanol. Glycogen was used as a carrier in this protocol as an alternative to yeast tRNA (2). The sample was stored overnight at -20°C, and the DNA was pelleted by centrifugation, washed twice with 70% ethanol and then resuspended in 10 μ L of water. Restriction enzyme digestion was performed in a total volume of 20 μ L in the presence of 100 ng of pGEM®-5Zf DNA (Promega, Annandale, NSW, Australia) using an enzyme that does not cut within the sequence of interest. Denaturation of DNA was achieved by adding 2 μ L of 3 M NaOH directly to the digest and incubating at 75°C for 15 min. Bisulfite modification was initiated by adding 250 μ L of freshly made 4.8 M bisulfite, pH 5.0, and 14 μ L of 10 mM hydroquinone. The bisulfite solution was prepared by dissolving 9.1 g of Na₂S₂O₅ (BDH, Kilsyth, VIC, Australia) in 19 mL of deionized water, adjusting the pH to 5.0 with 10 M NaOH, then bringing the final volume to 20 mL with water. The reaction mixture was covered with paraffin oil, and incubated at 55°C for 4 h in the dark. The sample was removed from under the paraffin oil and the DNA was recovered using the Prep-A-Gene® DNA purification kit (Bio-Rad, Regents Park, NSW, Australia). DNA was allowed to bind to 5 μ L of Prep-A-Gene matrix for 1 h at room temperature with gentle shaking. Following standard washing procedures, the DNA was eluted from the matrix twice with 20- μ L aliquots of water at 37°C for 30 min. The longer than recommended binding and elution times were to enhance recovery of dilute single-stranded DNA. We found that the Prep-A-Gene DNA purification kit was simple, efficient and consistent for desalting bisulfite-modified DNA. However, other DNA purification kits may be as

effective. Desulfonation was performed by adding 4.5 μ L of 3 M NaOH and incubating at 37°C for 15 min. The solution was then neutralized by adding 28 μ L of 5 M ammonium acetate, pH 7.0, and the DNA was precipitated at -20°C overnight by adding 1.0 μ g of glycogen as carrier and 3 volumes of ethanol. Precipitated DNA was resuspended in 20 μ L water from which 4–5 μ L were used for PCR amplification. PCR amplification was performed with nested primers in two rounds of touchdown amplification (3) using the hot-start approach (1) for initiating the reaction.

In our laboratory, this method has been used to analyze the methylation profile of two regions of the mouse *Xist* gene, thought to play a role in genomic imprinting. Figure 1 shows PCR products amplified from a region located approximately 1 kb into the coding sequence of *Xist* using asymmetric nested primers. These PCR products were generated from bisulfite modification reactions consisting of between 4 and 50 mouse pre-implantation embryos (80 to 200 cells in total) at different stages of development. PCR products were similarly amplified from the minimal promoter region of *Xist* (data not shown). For methylation analysis, PCR products were purified on agarose gel, blunt-end cloned into the *EcoRV* site of pGEM-5Zf and sequenced. This method resulted in complete conversion of cytosine residues for all samples analyzed.

REFERENCES

1. Chou, Q., M. Russel, D.E. Birch, J. Raymond and W. Bloch. 1992. Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications. *Nucleic Acids Res.* 20:1717-1723.
2. Clark, S.J., J. Harrison, C.L. Paul and M. Frommer. 1994. High sensitivity mapping of methylated cytosines. *Nucleic Acids Res.* 22:2990-2997.
3. Don, R.H., P.T. Cox, B.T. Wainwright, K. Baker and J.S. Mattick. 1991. "Touchdown" PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res.* 19:4008.
4. Feil, R., J. Charlton, A.P. Bird, J. Walter and W. Reik. 1994. Methylation analysis on individual chromosomes: improved protocol for bisulfite genomic sequencing. *Nucleic Acids Res.* 22:695-696.
5. Frommer, M., L.E. McDonald, D.S. Millar, C.M. Collis, F. Watt, G.W. Grigg, P.L. Molloy and C.L. Paul. 1992. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc. Natl. Acad. Sci. USA* 89:1827-1831.
6. Hayatsu, H. 1976. Bisulfite modification of nucleic acids and their constituents. *Prog. Nucleic Acid Res. Mol. Biol.* 16:75-124.
7. Hayatsu, H., Y. Wataya, K. Kai and S. Iida. 1970. Reaction of sodium bisulfite with uracil, cytosine, and their derivatives. *Biochemistry* 9:2858-2866.
8. Jeanpierre, M. 1987. A rapid method for the purification of DNA from blood. *Nucleic Acids Res.* 15:9611.
9. Raizis, A.M., F. Schmitt and J. Jost. 1995. A bisulfite method of 5-methylcytosine mapping that minimizes template degradation. *Anal. Biochem.* 226:161-166.
10. Shapiro, R., V. Difate and M. Welcher. 1974. Deamination of cytosine by bisulfite. Mechanism of the reaction. *J. Am. Chem. Soc.* 96:906-912.
11. Shortle, D. and D. Nathans. 1978. Local mutagenesis: a method for generating viral mutants with base substitutions in preselected regions of the viral genome. *Proc. Natl. Acad. Sci. USA* 75:2170-2174.
12. Wang, R.Y.-H., C.W. Gehrke and M. Ehrlich. 1980. Comparison of bisulfite modification of 5-methyldeoxycytidine and deoxycytidine residues. *Nucleic Acids Res.* 8:4777-4790.

We thank Dr. Susan Clark for useful discussions on bisulfite genomic sequencing before publication of this manuscript. Address correspondence to Graham F. Kay, Queensland Institute of Medical Research, Post Office Royal Brisbane Hospital, Queensland, 4029, Australia. Internet: grahamk@qimr.edu.au

Received 14 March 1996; accepted 11 July 1996.

**Louise E. McDonald and
Graham F. Kay**
*Queensland Institute
of Medical Research
Post Office Royal
Brisbane Hospital
Queensland, Australia*