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A modified and improved method for bisulphite based cytosine methylation analysis

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ABSTRACT

Sequencing of bisulphite modified genomic DNA is the most powerful method to determine methylation patterns in chromosomal DNA. In many experimental systems, the amount of material available for analysis is very small which makes it necessary to perform experiments at extreme levels of sensitivity and reproducibility. In this communication, we present an improved modification of the bisulphite based sequencing method. Our strategy is to perform the bisulphite treatment and subsequent PCR steps on material embedded into agarose beads. This prevents loss of DNA during the experimental procedure and ensures an optimal bisulphite reactivity by maintaining the DNA in the single stranded form. The modification improves previously published protocols in that it facilitates the handling of probes and reproducibly reaches a very high level of sensitivity.

Various approaches can be used to analyse the positions of C5-methylated cytosines in genomic DNA. The development of bisulphite based genomic sequencing has been a major experimental innovation allowing a direct and positive determination of methylation patterns of individual DNA strands. The method is based on a chemical modification of cytosine residues in the presence of sodium bisulphite (1). In the first step of the bisulphite reaction, cytosines are sulfonated and deaminated converting them to uracil sulphonate. A subsequent desulphonation at a basic pH completes the conversion from cytosines to uracils. C5-methylcytosine is not modified under the conditions used. After bisulphite treatment the chromosomal region of interest is PCR-amplified and the PCR products sequenced. Only methylcytosines are detected as cytosines in this sequencing reaction, whereas all unmethylated cytosines appear as thymidines.

In this communication we introduce technical and experimental modifications which improve previously described methods (2–4) in several respects: an easier and safer handling of samples, a shortening of time required for the experimental work and an easier reproducibility of experimental results reaching a very high standard of sensitivity. In addition, the procedure facilitates bisulphite methylation analyses of very small amounts of tissues or cells; this is often desirable when studying the fate of methylation patterns in specific tissues/cells during development and differentiation. The simple rationale behind our approach is to use low melting point (LMP) agarose into which either the denatured DNA or cells, of which DNA is to be analysed, are embedded. The embedding prevents denatured DNA strands from renaturating during the bisulphite treatment thereby optimising the reactivity of the chemical. Embedding also minimises the loss of material because all precipitation and purification procedures are substituted for short steps of equilibrations.

A protocol for our method is outlined in the following examples in which intact mammalian cells (I) or isolated chromosomal DNA (II) were used as starting materials.

I. Preparation of cells for the bisulphite treatment. Trypsinized cells (in this case of the murine myoblastic cell line C2) were recovered in 1× PBS. For the purpose of testing the sensitivity of our method the number of cells in suspension was determined and appropriate dilutions of this cell suspension were mixed (at 37°C) with molten LMP agarose (SeaPlaque, FMC) prepared in 1× PBS. The final concentration of the agarose in this agarose/cell mixture was 1.6%. A 10 µl drop of this mixture containing a defined number of cells (aliquots were counted in a hematocytometer) was then pipetted into 300 µl cold mineral oil (Sigma) laid over 800 µl of lysis solution (10 mM Tris–HCl, 10 mM EDTA, 1% SDS, 20 µg/ml Proteinase K). The agarose/cell drop immediately solidified in the oil and the agarose beads were mechanically pushed into the lysis solution. After overnight incubation at 50°C the Proteinase K was inactivated by PMSF (40 µg/ml, pH 7 in 1× TE, 2 × 45 min) followed by several equilibrations (each 15 min) with 1 ml of 1× TE (pH 9). Wash/equilibration solutions were simply exchanged using a pasteur pipette or 1 ml Gilson pipette. The beads were then equilibrated against the appropriate restriction enzyme buffer (2 × 100 µl) and digested for at least 5 h with 10 U of the *Eco*RI restriction enzyme. This procedure was followed by an equilibration against 0.3 M NaOH (500 µl) for 2 × 15 min at room temperature to achieve effective denaturation of the DNA strands. Since a later molten agarose bead would not resolidify in 0.3 M NaOH, a third dialysis against 0.1 M NaOH for 5 min was required. The individual beads were then overlaid with mineral oil and heated to 80°C for 15 min in order to melt the agarose and fully separate individual DNA strands in the liquid agarose. The DNA–agarose solutions were re-solidified to agarose beads by chilling the reaction tube on ice.

II. Preparation of agarose beads containing isolated chromosomal DNA. Isolated chromosomal DNA of C2 cells was digested with *Eco*RI, then boiled for 5 min, quickly chilled on ice and subsequently incubated in 0.3 M NaOH for 15′ at 50°C. Dilutions

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of this material were mixed with 2 vol of 2% LMP agarose dissolved in H_2O. Agarose/DNA mixtures containing 50 pg–200 ng DNA were directly pipetted into chilled mineral oil to form agarose beads.

Bisulphite treatment of agarose beads

Aliquots of 100 µl of a 5 M bisulphite solution (2.5 M sodium metabisulphite, Merck; 125 mM hydroquinone, Sigma; pH 5.0) were added to each reaction tube containing a single bead. The beads were brought into the aqueous phase by gently inverting the reaction tube. The reaction mixtures were then incubated for 4 h at 50°C under exclusion of light.

Treatments were stopped by equilibrations against 1 ml of 1× TE (6 × 15 min) followed by desulphonation in 500 µl of 0.2 M NaOH (2 × 15 min). The reactions were neutralised with 1/5 vol of 1 M hydrochloric acid. Finally, beads were washed with 1 ml 1× TE followed by equilibrations against 1 ml of ddH_2O (2 × 15 min). The beads were used directly for PCRs. We have also kept beads at 4°C for several weeks and never detected any loss of quality (data not shown).

PCR amplifications of bisulphite treated DNA

To assess the quality and sensitivity of the bisulphite treatments described above, PCR amplifications were performed with individual beads containing increasing amounts of cells (10–2000) or isolated chromosomal DNAs (50 pg–200 ng). The primers used were specific for a 616 bp region of the mouse Igf2 gene known to exhibit variable methylation (5). To compare the efficiency of our method with 'conventional' bisulphite procedures we performed parallel amplifications with chromosomal DNAs treated according to the most sensitive published method (2), (conditions are outlined in the legend of Fig. 1). All reactions were subjected to two rounds of amplifications using a nested primer approach and products were examined on agarose gels. The results obtained after first round amplifications (35 cycles; Fig. 1A–C) showed that reactions performed with embedded cells appeared to be most efficient. Visible products were detected in amplifications containing as little as 100 cells (~500 pg) of starting material (Fig. 1B). The minimum amount of DNA required to obtain amplification products from beads containing isolated chromosomal DNA was 10 ng (Fig. 1C, lane 4). No amplification products were detectable in the samples treated according to the conventional method (Fig. 1A). Aliquots of 1 µl of PCR reactions (Fig. 1A–C) were subjected to second round amplifications (21 cycles) using a pair of nested primers. Correct products of 583 bp (Fig. 1D–F) were detected for all three treatments, however at somewhat variable efficiencies. For example, weak bands could even be observed in lanes 1 of (D) (conventional procedure) and (F) (agarose imbedded DNA) containing only 50 pg of chromosomal DNA, but in total the results obtained with agarose embedded material appeared to be more reproducible and efficient.

In order to check the completeness of cytosine conversion in our agarose treatments PCR products (Fig. 1B, lane 4) were gel purified (JetSorb, Genomed), ligated into a TA cloning vector (InvitroGen) and individual clones were sequenced. In all sequences cytosines were only detected within a CpG dinucleotide context (see also legend to Fig. 2). The CpG methylation patterns, however, varied between individual clones showing that the cloned PCR products were derived from different chromosomes.

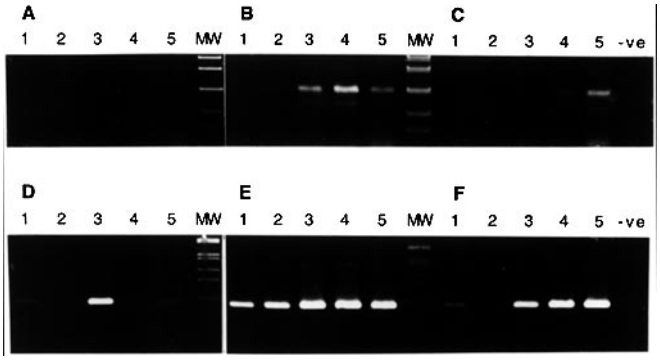

Figure 1. Agarose gel analysis of PCR products. (**A**) and (**D**) PCR products obtained from chromosomal DNA treated according to the method published by Clarke *et al.* (2). Five individual bisulphite modifications were performed containing increasing amounts of chromosomal DNA (50 pg–100 ng). Their protocol was followed strictly with only one minor modification. After bisulphite treatment the chromosomal DNAs were spiked with 1 µg of carrier DNA (pBluescript KS+) and extracted using a JetSorb (Genomed) kit since we found this technique to recover DNA to be most efficient in our hands. The recovered and desulphonated DNA was dissolved in 10 µl ddH_2O and directly used for PCR (2). (**B**) and (**E**) PCR products with agarose beads from treatments of whole cells; (**C**) and (**F**) PCR products with agarose bead treatment of isolated chromosomal DNA. MW DNA standard VII (Boehringer, Mannheim); –ve shows the control PCR lanes without DNA template. The PCR primers were designed to be fully complementary to the deaminated DNA strand and do not include CG dinucleotides: Primer #1: 5′-AACTAAAATTATCTATC-CTATAAAAC-3′; Primer #2: 5′-TTGATGGATTTATATTGTAGAATTAT-3′; Primer #3: 5′-GGAATTCCCTATAAAACTTCCAAACAAACCTTCAAA-3′; Primer #4: 5′-GGAATTCCTGATTTATTGATGGTTGTTGGATATTTT-3′. First round amplifications shown in (A), (B) and (C) were performed in 100 µl reactions containing individual 10 µl beads (B) and (C) or 10 µl of isolated chromosomal DNA (A), 200 µM dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl pH 8.3, 0.5 µl (2.5 U) *Taq* polymerase (Boehringer, Mannheim), in a Biometra Trioblock-Thermocycler under the program conditions 94°C/5 min followed by 35 cycles of 94°C/30 s, 54.5°C/90 s, 72°C/60 s, and one extension step of 5 min at 72°C. DNA concentrations used for amplifications analysed in lanes 1–5 of (A) and (C) are 50 pg, 500 pg, 2.5 ng, 10 ng and 100 ng respectively. Concentration of agarose embedded cells used for amplifications shown in lanes 1–5 of (B) are 10, 100, 500, 2000, 20000 cells. The DNA content of a single mouse cell is ~5 pg. Amplifications using nested primers #3 and #4 (D), (E) and (F) were performed using 1 µl of the primary PCR products [shown in lanes 1–5; (A), (B) and (C) respectively]. PCR conditions were the following: 21 cycles at 94°C/30 s, 58°C/1 min, 72°C/1 min. All first and second round PCR reactions were performed twice with identical results.

We never observed any indication of incomplete bisulphite reactivity. Figure 2 shows an example of a sequence obtained from a treatment performed with intact cells. We would like to note that control sequences obtained from agarose bead treated plasmid DNA containing the cloned *Igf2* region showed complete conversion of all cytosines to thymidines (data not shown).

ADVANTAGES OF OUR METHOD

Before introducing the agarose embedding in our laboratory, we have gained several years of experience working with modified conventional bisulphite modification methods (2,3,5). However, we found that the simple trick of agarose embedding represents a significant technical and experimental improvement. This does not only concern a simplified practical design of the procedure (no DNA isolation or precipitation required) but also the reproducibility of optimal, i.e. complete treatment (due to the

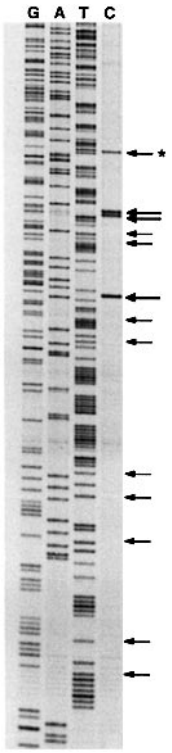

Figure 2. Sequence of a cloned PCR product. A sequence obtained from cloned fragment of amplified bisulphite treated C2 myoblast cells in agarose beads. Thin arrows indicate CpG cytosine positions which have been converted to uracil during the treatment. The bold arrows mark cytosines in CpGs that have not been converted, indicating the presence of 5-mC in the original DNA strand. In the sequence of this particular clone one cytosine marked by an asterisk is located outside of the CpG context. Since the original sequence contains a thymidine at this position this cytosine has probably to be attributed to a polymerase error during PCR.

physical separation of DNA strands) and high experimental sensitivity (mainly by minimising loss of DNA).

The method is easy to use and extreme levels of sensitivity can be reached. In our example we have amplified sequences of a single copy gene from as little as 50 pg of bisulphite treated chromosomal DNA (~10 individual cells). This level of sensitivity gives us the opportunity to determine methylation patterns in very small tissue samples. We are aware of the fact that successful DNA amplification after bisulphite treatment strongly depends on the choice of suitable primer pairs and optimised PCR conditions. Our method, however, has successfully been applied by other laboratories (H. Tamaru, A. Codon and T. Fornet, personal communication) who encountered problems with 'conventional' protocols both with respect to sensitivity and completeness of the treatment.

We have performed a large number of experiments with the agarose bisulphite method and never observed incomplete conversion of the DNA (A. Olek and J. Oswald, unpublished results). The elimination of such problems most probably results from the immobilisation of single strands in the agarose which enhances complete reactivity. Even very CG-rich regions including repeated structures, which we have previously found to be relatively resistant to bisulphite treatment, could be completely deaminated by our procedure.

We are currently performing methylation analyses of larger chromosomal regions and succeeded in amplifying fragments of up to 3 kb. All our previous attempts to amplify fragment >1 kb using conventional published protocols (2–4) had been unsuccessful.

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