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Urea improves efficiency of bisulphite-mediated sequencing of 5′-methylcytosine in genomic DNA

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ABSTRACT

The detection of 5′-methylcytosine by the bisulphite-mediated genomic sequencing method has considerably aided study of the role of methylation in areas such as X chromosome inactivation, genomic imprinting and cancer research. However on occasion difficulty has been experienced in obtaining complete conversion of cytosine to uracil in regions of the target DNA. We report here a simple improvement to the method involving addition of urea to the bisulphite reaction, a step which greatly improves the reaction efficiency, presumably by maintaining the target DNA in single stranded form, thereby allowing complete and reliable conversion.

The bisulphite-mediated genomic DNA sequencing method (1,2) for positive detection of methylated cytosines in genomic DNA involves PCR amplification of chemically modified DNA in which unmethylated cytosine residues have been converted to uracil by hydrolytic deamination, but methylated cytosine residues remain unconverted. Deamination is performed on single stranded DNA by treatment with a high concentration of sodium bisulphite at pH 5.0. The bisulphite sequencing method has several advantages over other methods (discussed in 3) which has resulted in increasing numbers of laboratories using it. However, although several improvements to the original method (1) have been reported (2,4–6), problems still persist in obtaining consistent conversion with certain DNA sequences. These problems seem to be associated with retention of double stranded structures in the DNA, since the rate of bisulphite deamination of cytosine residues in double stranded DNA is negligible (7).

Whilst we have found the standard technique (2) to be efficacious for many sequences, we occasionally have difficulty in conversion of some samples, due either to their sequence characteristics or to sample impurity. Reported improvements to the protocol include reducing DNA size before conversion by digestion with endonucleases closely flanking the PCR primers (4), introducing multiple heat denaturation steps during conversion (4) and performing conversions at 0°C (5). However in our hands, application of these methods alone did not prove consistently successful for difficult samples. Therefore we have developed a simple procedure, based on an earlier idea (6) that overcomes this problem and should be of general applicability. This consists of adding urea to the bisulphite reaction, a procedure that we have found not to affect the methylated cytosine residues in DNA, or the normal reaction of bisulphite with cytosine residues.

The new protocol is as follows: genomic DNA (typically 2 µg) is digested with flanking restriction endonucleases, then denatured by the addition of 1/9 vol of freshly prepared 3 M sodium hydroxide and incubation for 15 min at 37°C. A 6.24 M urea/2 M sodium metabisulphite (4 M bisulphite) solution is made by dissolving 7.5 g of urea in 10 ml of sterile distilled water, adding 7.6 g of sodium metabisulphite, adjusting the pH to 5 with 10 M sodium hydroxide and adding sterile water to a final volume of 20 ml. Note that the above reagents should be added in the order indicated, otherwise difficulty may be experienced in dissolving the salts. The urea/bisulphite solution and hydroquinone are then added to the denatured DNA to final concentrations of 5.36 M, 3.44 M and 0.5 mM respectively. Typical reaction mixes contain: 22.2 µl of denatured DNA, 208 µl of urea/bisulphite solution and 12 µl of 10 mM (freshly prepared) hydroquinone. The reaction is performed in a 0.5 ml PCR tube overlaid with 100 µl of mineral oil and, in the experiments described here, subjected to 20 cycles of 55°C for 15 min followed by denaturation at 95°C for 30 s in a PCR machine (Corbett 3200) essentially as described by Rein *et al.* (4). In subsequent experiments we have found that urea/bisulphite conversion at 55°C for 15 h, as in the standard method, works equivalently well for our samples. The bisulphite treated DNA is further processed as described in (2) except that a Bresaclean DNA purification (silica) step is used to desalt the DNA.

The DNA sequence used to illustrate this method comprises a 1.2 kb region containing 66 CpG dinucleotides in the promoter region of the human multidrug resistance gene *MDR1*. Primers used to amplify the converted DNA are as described in (8). Figure 1 shows PCR products obtained upon conversion of DNA isolated from an *MDR1* expressing human leukemia cell line, with and without urea in the conversion reaction. PCR products in lanes 2 and 3 were amplified from *Eco*RI digested DNA and those in lanes 4 and 5 from DNA digested with *Rsa*I, which cuts closer to the region to be amplified. As can be seen, the largest amount of product was obtained using urea and *Rsa*I (lane 5). In other experiments using the same DNA preparation we performed the conversion at 0°C as suggested in (5) but obtained no PCR product (results not shown).

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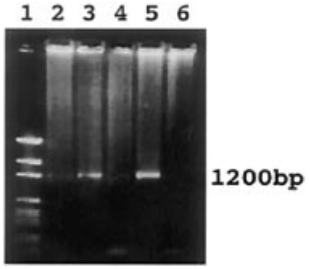

Figure 1. PCR products obtained after bisulphite conversion of the human *MDR1* gene. Lane 1, pGEM markers; lane 2, *Eco*RI digested DNA converted using standard bisulphite method; lane 3, *Eco*RI digested DNA converted using urea/bisulphite protocol; lane 4, standard bisulphite conversion of *Rsa*I digested DNA; lane 5, *Rsa*I digested DNA converted using urea/bisulphite; lane 6, negative control.

In previous experiments with PCR products obtained using the standard technique, we sometimes found upon sequencing that the region between the converted strand specific primers was not fully converted. That is, whilst the primer proximal sequence was fully converted, patches of highly GC rich sequence within the region remained unconverted. However, upon subcloning and sequencing of the PCR products in lanes 3 and 5, all non-CpG cytosines were found to be converted. In contrast, multiple unreacted non-CpG cytosines were seen in clones derived from the PCR product in lane 2, obtained upon conversion in the absence of urea. Figure 2A shows typical sequence of clones obtained from conversion with and without urea. Similar complete conversion of non-CpG cytosines was obtained in 20 independent clones sequenced from the urea treated samples.

To confirm that addition of urea to the conversion reaction did not cause deamination of methylated CpGs, we used the urea/bisulphite method to convert and amplify DNA from a cell line (HL60) that we had previously shown by bisulphite mediated sequencing to be extensively hypermethylated in the region under examination (8). The results were entirely analogous to those previously obtained using the standard bisulphite method. Figure 2B shows typical sequence obtained for *Eco*RI digested HL60 DNA, converted in the presence of urea. Three methylated CpG sites are shown here, site 1 was previously found to be methylated in 18 out of 50 HL60 PCR subclones examined, site 2 was methylated in 25 of 50 and site 3 in all of 50 DNA molecules sequenced (8). The observation that each of these three CpG cytosines remained unconverted in the urea conversion protocol, whilst close non-CpG cytosines were converted indicates that incorporation of urea into the protocol does not result in deamination of methylated cytosines. Similar results were obtained for other subclones examined. In summary, addition of urea to the bisulphite conversion method aids the conversion of difficult samples without causing deamination of methylated sites. It is our experience that this improved method can be used on genomic DNA that has been prepared more crudely due to the increased denaturation capability of urea in the reaction. We anticipate that it will also enable the use of smaller cell sample sizes.

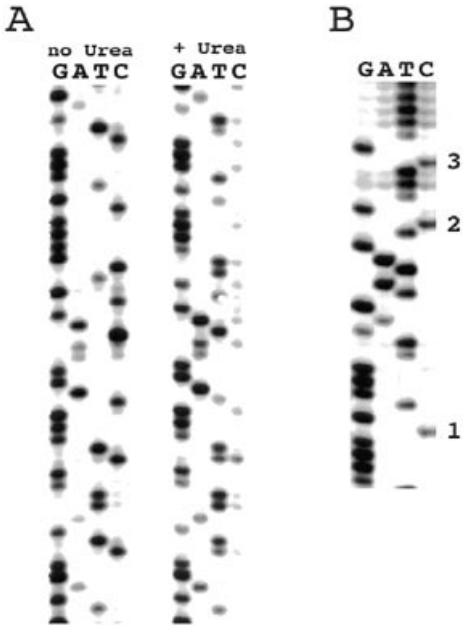

Figure 2. (**A**) *Eco*RI digested DNA from an *MDR1* expressing cell line was bisulphite converted with and without urea. PCR products were subcloned (pGEM-T vector) and subclones sequenced (Sequitherm cycle sequencing kit) on a Li-Cor automated sequencer. The unconverted native sequence of the region shown is GTGAGGCTGATTGGCTGGGCAGGAACAGCGCCGGG-GCGCGGGCTGAG. (**B**) *Eco*RI digested DNA from the HL60 cell line was converted using the urea/bisulphite method and PCR products subcloned and sequenced. Methylated CpG dinucleotides are indicated on the figure by the numbers 1, 2 and 3. The unconverted native sequence of the region shown is GGGGCGTGGGCTGAGCACAGCCGCTTCGCTCTC.

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