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Benchmark

Methylation Analysis of Formalin-Fixed, Paraffin-Embedded Sections Using a Nontoxic DNA Extraction Protocol

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DNA methylation is an epigenetic mechanism responsible for gene silencing (4). The methylation status of a gene can be assessed by a variety of methodologies (1,7). These commonly involve sodium bisulfite treatment of genomic DNA that converts unmethylated, but not methylated, cytosine residues to uracils (2), followed by PCR amplification. We have adapted the basic bisulfite modification protocol specifically to examine methylation using DNA from formalin-fixed, paraffin-embedded tissue sections.

Formalin-fixed, paraffin-embedded sections have been extensively used as a source of genomic DNA for PCR-based analysis. A significant advantage associated with the use of paraffin sections is that they can be microdissected to enrich for subpopulations such as tumor cells, thus reducing the confounding effect of contaminating normal tissue that can complicate data interpretation. This necessitates efficient methodologies for isolating DNA from the very small numbers of cells present in these sections. The ability to extract DNA from paraffin sections allows the use of material for retrospective studies and understanding tumor evolution. In the first part of our methodology, we have developed an efficient, nontoxic protocol to

prepare PCR-quality DNA from formalin-fixed, paraffin-embedded tumor sections. This protocol uses Histoclear™ (National Diagnostics, Atlanta, GA, USA) instead of xylene or octane for deparaffinizing the sections.

Ten-micron sections were prepared from paraffin-embedded blocks of formalin-fixed breast tumor biopsies. A hematoxylin and eosin-stained master slide was used as the template to outline the area of tumor tissue. Tumor tissue was microdissected by scraping with a sterile scalpel blade. Normal tissue was first removed, and then tumor tissue was transferred into a 1.5-mL microcentrifuge tube to which 1 mL Histoclear had been added. The sample was mixed on a rotor (RMS 6; Ratek Instruments, Boronia, Victoria, Australia) at room temperature for 10 min and then centrifuged at 16 000× *g* for 10 min. The supernatant was removed, the pellet was resuspended in fresh Histoclear, and the rotary mixing and centrifugation were repeated twice.

The pellet was then sequentially rehydrated first in 1 mL absolute ethanol and then in 1 mL of 80% ethanol. On each occasion, the sample was mixed on the rotor for 10 min, followed by centrifugation at 16 000× *g* for 10 min. After the removal of the supernatant, 200 µL InstaGene™ matrix (Bio-Rad Laboratories, Hercules, CA, USA) and 80 µg Proteinase K were added to the pellet and incubated for 16 h at 55°C. The sample was then heated at 100°C for 10 min to inactivate the Proteinase K, and the supernatant was collected after spinning at 16 000× *g* for 15 min.

The supernatant was extracted using phenol chloroform; 1/10 volume of 3 M sodium acetate (pH 5.2) was added to give a final concentration of 0.3 M. Two and a half volumes of absolute ethanol were added before an overnight precipitation at -20°C. The DNA was then spun at 16 000× *g* for 30 min, washed in 70% ethanol, air dried, and resuspended in 17 µL Ultrapure™ water (Fisher Biotec, Perth, Australia).

In the latter part of the methodology, it was necessary to adapt the bisulfite conversion procedure for DNA extracted from formalin-fixed, paraffin-embedded sections. The limited amounts of DNA required the use of carrier DNA and a co-precipitant (3,5). Our previous protocol, which worked well with genomic DNA prepared by standard means, gave incomplete conversion with section-derived DNA. Accordingly, a more extensive denaturation protocol was adopted that involved the use of urea and repeated cycling up to 95°C (6).

Salmon sperm DNA (1 µg; Sigma, St. Louis, MO, USA) was added as a carrier to the isolated DNA (3). The DNA was denatured using 2 µL freshly prepared 3 M NaOH in a final volume of 20 µL and then incubated for 30 min at 37°C. Then, 278 µL of a 6.24 M urea/4 M sodium bisulfite and 2 µL freshly prepared 100 mM hydroquinone (Sigma) were added. The solution was made by dissolving 7.5 g urea (Ajax Chemicals, Sydney, Australia) in 10 mL sterile water. This was followed by 7.6 g sodium bisulfite (Sigma) and then adjusting the pH to 5.0 with 10 M NaOH

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and adding sterile water to a final volume of 20 mL. The final concentrations of the urea, sodium bisulfite, and hydroquinone were 5.8 M, 3.7 M, and 0.67 mM, respectively. The DNA was subjected to 20 cycles of 55°C for 15 min, followed by denaturation at 95°C for 30 s in a PTC-200™ thermal cycler (MJ Research, Waltham, MA, USA).

The modified DNA was then desalted using CONCERT™ rapid PCR purification columns (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The bisulfite-modified DNA was eluted from the column using 50 µL TE (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) preheated to 55°C. A desulfonation step followed where 5.5 µL of 3 M NaOH was added to the 50 µL eluate and the DNA was incubated at 37°C for 15 min. One microliter of molecular biology-grade mussel glycogen (5 µg/µL) (Roche Molecular Biochemicals, Mannheim, Germany) was added to the sample as a co-precipitant. This was followed by 1/10 volume of 3 M sodium acetate and 2.5× volume of absolute ethanol. The

sample was mixed and then precipitated at -20°C for 16 h and spun at 16 000× *g* for 25 min. The pellet was then resuspended in 50 µL Ultrapure water and stored at 4°C until used.

To validate this protocol, we used our previously developed methylation-sensitive, single-strand conformation analysis (MS-SSCA) methodology (1). MS-SSCA is based on electrophoretic detection of sequence differences of bisulfite-modified DNA that has undergone PCR amplification. The PCR product is denatured to give single strands and then subjected to non-denaturing gel electrophoresis where it adopts sequence-dependent secondary structures (conformers) with different migration rates.

Primers were designed to examine the promoter methylation status of connexin 26. It was not possible to totally avoid CpG dinucleotides in the design of these primers, but they were placed as far 5' in the primer sequence as was possible. We chose to maintain the C at CpG dinucleotides. This slightly favored the amplification of methylated sequences, but it should be noted that PCR amplification often favors the unmethylated sequence (8). A pair of primers [5'-ATTCGGGAAGTTTGTAGGATTGAGGT-3' (sense) and 5'-GTTAAATCTCTACGCTAAACTCCTAC-3' (antisense)] was used to amplify a 245-bp target fragment of the connexin 26 promoter region (GenBank® accession no. U43932) from bisulfite-modified DNA samples. PCR was performed in a final volume of 50 µL using 5–10 µL sodium bisulfite-modified DNA, 100 ng each primer, 2 mM MgCl₂, and 0.5 U HotStarTaq™ DNA polymerase in buffer supplied by the manufacturer (Qiagen, Valencia, CA, USA). The first cycle was preceded by an activation step of 95°C for 15 min. Amplification was then performed for 45 cycles with an initial 10 cycles of 94°C for 1 min, touch-down annealing temperature step from 72°C to 62°C for 45 s and 72°C for 45 s, followed by 35 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min. SSCA was carried out as previously reported (1). Briefly, the PCR product was denatured in freshly prepared loading buffer (95% formamide, 10 mM NaOH, 0.25% bromophenol blue, and 0.25% xylene cyanol) by heating at 95°C for 5

min and chilled on ice for 3 min. It was then electrophoresed on a 0.75× MDE™ polyacrylamide gel (BMA, Rockland, ME, USA), stained with SYBR® Gold (Molecular Probes, Eugene, OR, USA) and visualized on a transilluminator (TVD 1000 R/F; Spectronics, Westbury, NY, USA) (Figure 1).

In this communication, we have a new protocol for the preparation of DNA suitable for PCR amplification from formalin-fixed, paraffin-embedded tissues and have adapted the bisulfite conversion procedure. Current methodologies for the extraction of DNA from paraffin-embedded sections use highly flammable and carcinogenic solvents such as xylene or octane in the initial deparaffinization step. We used Histoclear in the deparaffinization step to avoid exposure to these highly carcinogenic organic solvents. The procedure can be safely performed without the use of a fume hood. Histoclear, a plant-derived alkyl hydrocarbon, has been classified as safe by the US Department of Labor. Histoclear has a further advantage in that it facilitates the formation of a visible pellet after centrifugation, thus minimizing the loss of tumor tissue on aspiration, unlike xylene where tissue fragments remain afloat in the solvent. To our knowledge, this is the first report of the use of Histoclear to prepare DNA from paraffin-embedded sections for DNA analysis.

We used InstaGene during the Proteinase K digestion of DNA from formalin-fixed, paraffin-embedded sections. InstaGene is a Chelex®-type biotechnology-grade resin specially formulated for the purpose of preparing genomic DNA for PCR. It functions to remove inhibitors of PCR amplification. Nevertheless, we found it necessary to include a subsequent phenol chloroform extraction step to improve the yield of PCR product (results not shown).

The quality of modified DNA is suitable for methylation studies. We were routinely able to amplify DNA from a single 10-µm section using the technology described above. Furthermore, we were able to extract PCR-quality DNA from sections that other laboratories had found to be refractory to amplification using xylene-based protocols. We thus carried out a comparative MS-SSCA study using Histoclear and xy-

Figure 1. MS-SSCA of Cx26 promoter from paraffin-embedded section of breast tumors. DNA extracted from paraffin-embedded sections from seven breast cancer patients were sodium bisulfite-modified, PCR amplified, and electrophoresed on an MDE gel as described in the text. (Figure is an inverse image.)

lene on paraffin sections using five unselected patient sections. Except for the solvent, the adopted protocol was performed as described above. Where Histoclear was used, amplification of the correct size product of 245 bp was detected in the sodium bisulfite-modified DNA from all patients. By contrast, when xylene was used, amplification was observed only for four patients. MS-SSCA of the PCR product from Histoclear and xylene-extracted, sodium bisulfite-modified DNA of this patient revealed no difference in banding patterns (results not shown). It is thus clear that not only is Histoclear a less toxic alternative to xylene but it may be more reliable in producing amplifiable DNA. We have now been able to extract PCR-quality DNA from 50 of 54 (93%) paraffin-embedded breast tumor sections ranging in age from a few weeks to seven years old.

We overcame the problem of having only limited amounts of genomic DNA by amending our bisulfite modification protocol. First, we used salmon sperm DNA as a carrier during the initial bisulfite conversion step (3). Also, to reduce the loss of DNA after modification, molecular biology-grade glycogen was added as a co-precipitant (5). As the extracted DNA was in some instances not completely converted in the bisulfite modification step, we have also included in our MS-SSCA protocol the use of high urea concentrations and repeated thermocycling to maintain single-strand

separation and aid conversion (6).

The use of CONCERT rapid PCR purification columns significantly reduced the time required and simplified the desalting of samples after bisulfite conversion. In comparison, previously reported desalting methodologies were more complex and time consuming (2,3,6).

Figure 1 shows banding patterns of individual patient breast tumor samples. Lanes 1, 5, and 7 show a pattern typical of an un methylated sequence. Lanes 2, 3, 4, and 6 are methylated with little or no normal pattern, as would be expected from microdissected tumor samples. Variant bands were stabbed and sequenced to verify the complete conversion of un methylated cytosines to thymines from DNA extracted from paraffin-embedded sections. Interestingly, there is a complexity of methylation, especially in the latter three samples. This level of complexity was not observed by us in bulk DNA from tumor samples and may indicate that the analysis of paraffin sections may more accurately reflect tumor heterogeneity.

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