7-Deaza-2′-deoxyguanosine allows PCR and sequencing reactions from CpG islands

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CpG islands are GC rich sequences that are found in the promoters of many genes in higher eukaryotes. They contain a high frequency of CG dinucleotides, which are substrates for DNA methylases. Methylation leads to transcriptional silencing of promoters. Owing to their high GC content CpG islands exhibit strong base–base interactions, which lead to superstructures and consequently to regions with higher melting temperatures. Therefore, Taq polymerases (especially sequenases) fall off their templates, causing premature termination of the polymerase chain reaction (PCR) or sequencing reactions. The results from such reactions are thus insufficient for further analysis. Therefore, we have evaluated the use of 7-deaza-2′-deoxyguanosine for PCR amplification of the human p16INK4A promoter and sequencing of HUMARA exon 1 PCR products. Our results show that the addition of 7-deaza-2′-deoxyguanosine significantly improves results, particularly when small amounts of poor quality DNA are available as starting material.

DNA from cultured SW1116 (human colorectal adenocarcinoma cell line; number 87071006; ECACC, London, UK) or ARH77 (human B cell lymphoma line; CRL1621; ATCC, Rockville, Maryland, USA) cells was isolated according to the instruction manual of the QIAamp DNA mini kit (Qiagen, Hilden, Germany). Both cell lines were cultured according to standard procedures in DMEM (SW1116) or RPMI 1640 (ARH77) containing 50µM 2-mercaptoethanol and 10% (vol/vol) fetal calf serum (all Life Technologies, Karlsruhe, Germany). After purification DNA concentration and quality were determined by UV photometry.

PCR conditions
We amplified the human p16INK4A promoter (accession number X94154) and HUMARA exon 1 (accession number M27423) according to previously published methods. The PCR mixtures contained 1X PCR buffer (Taq polymerase: 50mM KCl, 100mM Tris/HCl, pH 9.0, 0.1% (vol/vol) Triton-X 100, or AmpliTaq Gold polymerase: PCR Gold buffer (Applied Biosystems, Weiterstadt, Germany)), 1.5mM (p16INK4A) or 2.0mM (HUMARA) magnesium chloride, 200µM dNTP mix (both) or deaza-mix (deaza-dGTP instead of dGTP), 10 pmol of each primer (p16INK4A WT), HUMARA), 1 U Taq polymerase (Promega, Heidelberg, Germany) or for hot start PCR AmpliTaq Gold (Applied Biosystems) and 50 ng template (p16INK4A, SW1116; HUMARA, ARH77) in a final volume of 25 µl. The following PCR cycle profiles were used.

1. p16INK4A: five minutes (Taq polymerase) or 12 minutes (AmpliTaq Gold) at 94°C; 35 cycles of 20 seconds at 94°C, 20 seconds at 65°C, and 20 seconds at 72°C; followed by two minutes at 72°C.

2. HUMARA: five minutes (Taq polymerase) or 12 minutes (AmpliTaq Gold) at 94°C; 35 cycles of 20 seconds at 94°C, 20 seconds at 63.6°C, one minute at 65°C; followed by six minutes at 72°C on a Hybaid MB5 0.25 PCR machine (Hybaid, Heidelberg, Germany). The PCR products were analysed on 3% (wt/vol) 1x TBE agarose gels.

Abbreviations: BD, Big Dye; deaza-dGTP; 7-deaza-2′-deoxyguanosine; MSP, methylation specific PCR; PCR, polymerase chain reaction

MATERIAL AND METHODS
Purification of DNA templates

DNA sequence are generated. Thus, by defining appropriate primer sets methylated and unmethylated DNA can be discriminated by PCR if bisulphite treated templates are used. Because the primer pairs are specific for methylated or unmethylated DNA, respectively, MSP is a very sensitive technique. MSP has been described for the determination of the methylation status of the human p16INK4A promoter, which encodes an inhibitory protein of the cell cycle with a molecular mass of 16 kDa (inhibitor of kinase 4). Because CpG islands are GC rich they are prone to form superstructures and display higher melting temperatures. Thus, it is difficult to generate PCR products or to obtain a readable sequence from such PCR products. Here, we show that the use of 7-deaza-2′-deoxyguanosine (deaza-dGTP) in PCR reactions allows the generation of full length PCR products of the human p16INK4A promoter. Furthermore, readable sequences from PCR products of the HUMARA exon 1 were obtained. The addition of deaza-dGTP was particularly helpful when working with low amounts of DNA template of poor quality.

“Methylation of CpG islands occurs during imprinting or inactivation of X chromosomes”

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Sequencing conditions
For sequencing of HUMARA exon 1 PCR products residual primers and nucleotides were removed using ammonium acetate precipitation (one volume of PCR reaction, one volume of 4M ammonium acetate (Sigma, München, Germany), and six volumes of 100% (vol/vol) EtOH; 15 minutes of maximum speed in a microcentrifuge). The precipitate was redissolved in 20 µl water and used as a template for further sequencing using the Big Dye (BD) sequencing kit (Applied Biosystems) (4 µl BD premix, 2 µl purified PCR product, 10 pmol primer (see above)) in a final volume of 20 µl using the following cycle profile: 15 cycles of 10 seconds at 96°C and 90 seconds at 60°C on a Hybaid MBS 0.25 PCR machine (Hybaid). Excessive dye terminators were removed applying the Dye-Ex kit (Qiagen), according to the user’s instructions. Finally, 2 µl of the eluate was mixed with 18 Hi-Di formamide (Applied Biosystems), denatured for two minutes at 90°C, and loaded on to a genetic analyser ABI310 (Applied Biosystems). Separation was done using a short capillary (30 cm) and POP6 (6% performance optimised polymer; Applied Biosystems). The resulting electropherograms were analysed on a personal computer using the freely available computer software CHROMAS (http://www.technelysium.com.au/chromas.html).

RESULTS AND DISCUSSION
In the course of studies on the methylation status of the p16INK4A promoter from microdissected areas of colorectal adenocarcinomas we found it difficult to obtain PCR products using the published p16INK4A WT primers and reaction conditions. In separate experiments, we developed a clonality assay based on the HUMARA exon 1 locus, because the originally described method was too insensitive to analyse DNA from microdissected formaldehyde fixed tissue. However, both the PCR of p16INK4A and sequencing of PCR products from exon 1 of the HUMARA locus did not yield reproducible results. The 140 bp PCR product of the p16INK4A promoter was generally faint or invisible (fig 1B). Similarly, large stretches of the HUMARA exon 1 sequence were not readable (fig 2B). Variations of the standard parameters—magnesium concentration, melting temperature, denaturing temperature, hot start using AmpliTaq Gold—did not solve these problems. Because of the high GC content of these sequences (p16INK4A, 78%; HUMARA, 65%) we tried several additives, such as DMSO, formamide, and betaine, which had been reported to improve PCRs of GC rich structures. However, these did not improve the results. 7-Deaza nucleotide analogues have been reported to weaken base–base interactions, thus solving superstructures and improving PCRs. Therefore, we added deaza-dGTP instead of dGTP to the PCRs. Independently of hot start, a PCR product could be generated for the p16INK4A promoter (fig 1A). Improvement was also seen for sequencing of deaza-dGTP containing PCR products of the HUMARA exon 1 (fig 2B). All experiments were carried out at least in triplicate and the addition of deaza-dGTP is now a routine procedure in our
Situation changes when deaza-dGTP is present in the PCR. This premature termination of the sequenase reaction. This is obvious because of the beginning of the sequence (fig 2B), but intensity drops without deaza-dGTP, very strong signals can be seen at the templates, as we have shown for the HUMARA exon 1. Even cases where additional sequencing is required it can be of benefit. Therefore, many laboratories. Thus, deaza-dGTP is very useful when performing PCR and subsequent sequencing of GC rich sequences if low amounts of template are used and/or DNA quality is poor, particularly from microdissected human tissue.

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