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Research Paper

Bisulphite Differential Denaturation PCR for Analysis of DNA Methylation

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KEY WORDS

DNA methylation, polymerase chain reaction, hypomethylation, denaturation temperature, sodium bisulphite

ABBREVIATIONS

MeC	5 methyl cytosine
BDD-PCR	bisulphite differential denaturation PCR
Tm	denaturation temperature

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ABSTRACT

Differential denaturation during PCR can be used to selectively amplify unmethylated DNA from a methylated DNA background. The use of differential denaturation in PCR is particularly suited to amplification of undermethylated sequences following treatment with bisulphite, since bisulphite selectively converts cytosines to uracil while methylated cytosines remain unreactive. Thus amplicons derived from unmethylated DNA retain fewer cytosines and their lower G + C content allows for their amplification at the lower melting temperatures, while limiting amplification of the corresponding methylated amplicons (Bisulphite Differential Denaturation PCR, BDD-PCR). Selective amplification of unmethylated DNA of four human genomic regions from three genes, *GSTP1*, *BRCA1* and *MAGE-A1*, is demonstrated with selectivity observed at a ratio of down to one unmethylated molecule in 10^5 methylated molecules. BDD-PCR has the potential to be used to selectively amplify and detect aberrantly demethylated genes, such as oncogenes, in cancers. Additionally BDD-PCR can be effectively utilized in improving the specificity of methylation specific PCR (MSP) by limiting amplification of DNA that is not fully converted, thus preventing misinterpretation of the methylation versus non-conversion.

INTRODUCTION

In PCR, successive cycles of amplification usually involve incubation of reactions at three temperatures; a high temperature to melt (denature) the double-stranded DNA fragments (usually in the range 90°–100°C) followed by a temperature chosen to promote specific annealing of primers to DNA (usually in the range 50°–70°C) and finally incubation at an optimal temperature for extension by the DNA polymerase (usually 60°–72°C).[1,2] The choice of primers, annealing temperatures and buffer conditions are used to provide selective amplification of target sequences. Recently it has become common to use melting temperature profiles analytically to confirm amplification of expected products and to distinguish between amplification of different alleles, for example.[3] This approach has also been applied to the analysis of the methylation state of DNA[4] and differential melting properties of amplicons have also been used in denaturing high performance liquid chromatography for DNA methylation profiling.[5] Improved PCR performance has been described using lowered denaturation temperatures[6] and recently the use of differences in melting temperature of amplicons has been used for selective amplification of amplicons with lower Tm, being applied to differentiate bacterial species[7] or identify hypermutated HIV strains.[8] We demonstrate the potential application of differential denaturation temperature PCR of bisulphite-treated DNA (Bisulphite Differential Denaturation PCR, BDD-PCR) for the selective amplification of unmethylated sequences following bisulphite treatment of DNA.

In mammalian DNA most methylated cytosine residues (MeC) are found at CpG sites; in plants and to a more limited extent in mammals MeC is found also at CpNpG or CpNpN sites.[9-11] CpG islands should be particularly suited to BDD-PCR. CpG-rich regions (CpG islands) are commonly promoter or transcription regulatory regions of genes; methylation of such islands is associated with transcriptional repression and a lack of methylation with gene activity and an active chromatin structure. During development tissue-specific expression of genes is frequently accompanied by a lack of methylation of their promoter or regulatory regions relative to methylation in non-expressing tissues (reviewed by Ehrlich, ref. 12); similarly allele-specific gene expression as seen in imprinting and X chromosome inactivation is accompanied by alternate methylation states of expressing and non-expressing alleles. In addition to the frequently observed hypermethylation of

CpG island genes in cancer development, hypomethylation of both individual genes and repeated sequence elements is common.[13] The *MAGE-A1* gene is one example of a number of germ-line specific genes that are reactivated and demethylated in a number of cancer types.[14,15]

The methylation status of DNA is frequently studied by PCR amplification after treatment of DNA with sodium bisulphite, and this provides a pertinent example where significant differences in melting temperatures (Tms) of amplicons can arise through differences in DNA methylation levels. When DNA is treated with sodium bisulphite cytosines (Cs) are converted to uracils (Us) while methyl cytosines (MeCs) remain unreacted.[16,17] During DNA amplification by PCR, Us are replaced by thymines (Ts) and MeCs remain as Cs in the amplified DNA. Following bisulphite treatment, DNA that is unmethylated will not contain any Cs, while methylated DNA will maintain Cs at sites that were methylated. Thus amplicons derived from methylated DNA will have a higher G + C content and hence higher melting temperature. We have evaluated the potential to use this differential melting temperature for selective amplification of unmethylated DNA sequences of the human *GSTP1*, *BRCA1* and *MAGE-A1* genes.

MATERIALS AND METHODS

Primers and probes are shown in Table 1. The sequence of the *GSTP1* promoter amplicon is shown in Figure 1 and sequences of other amplicons and location of primers in Figure 2. Primers were purchased from Sigma-Aldrich (Sydney, Australia), probes PRBM32-30 and PRBW31 from Applied Biosystems (Foster City, CA, USA) and probe PRBU from Operon Technologies (Huntsville, AL, USA).

Table 1 **Primers and probes**

Primer/Probe	[nM]	Sequence
LUHF2[a]	200	5'-ACACCAAAACATCACAAAAGGTTTTAGGGAATTTTTTTT
CSPR4	200	5'-AAAACCTTTCCCTCTTTCCCAAA
PRBM32-30	100	FAM-TTGCGTATATTTCGTTGCGGTTTTTTTT-TAMRA
PRBW31	100	VIC-ACACTTCGCTGCGGTCCTCTTCC-TAMRA
PRBU	100	TET- TTGTGTATATTTTGTTGTGGTTTTTTTTTTGTTG-TAMRA
GST F52A	100	5'-GGGATTATTTTTATAAGGTTAGGAGGT
GST RC1	400	5'-AAAACCCRAACCTAATACTACRAATTAA
BRCA1-F1	200	5'-YGGATATTTAGTGTTTTTTTTTTGATT
BRCA1-R1	200	5'-TTCCRAAACTAACAAATAAATATTCTTTA
BRCA1-F3	200	5'-GTGTTTTTTTTTTGATTTTTAG
BRCA1-R3	200	5'-AAAAAAAAAACAATTTATAAATC
MAGE1-F1	200	5'-TGTTTATTTTTATTTTTATTTAGGTAGGATT
MAGE1-R1	200	5'-ACCTTACCTCCTCACAAAACCTAAA

[a]Primer LUHF2 contains a 5' "tail" that is designed to suppress amplification of unmethylated DNA,[22] but this is independent of melting temperature effects demonstrated here.

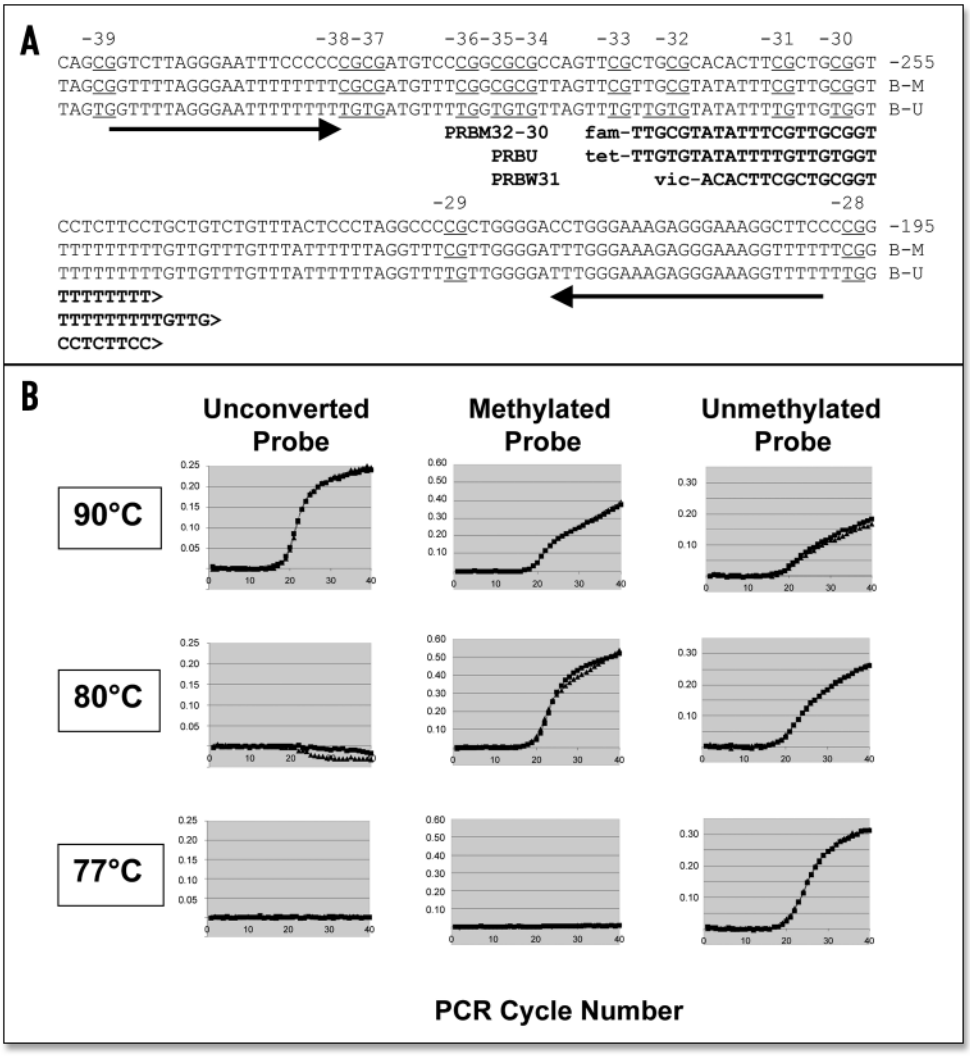

Figure 1. BDD-PCR of *GSTP1* promoter sequences. (A) Sequence of the unmodified *GSTP1* gene promoter is shown on the top line (Genbank Accession No. M24485). Underneath are shown the sequences derived after reaction with sodium bisulphite assuming the CpG sites are either unmethylated (B-U) or methylated (B-M) respectively. CpG sites are numbered relative to the transcription start site according to Millar et al.[18] The position and sequences of the TaqMan probes are indicated and primer sites shown by arrows. (B) Graphs in the same vertical column show Realtime detection using the same probe (PRB-U, for detection of unmethylated DNA, PRB-M for detection of methylated DNA and PRBW for detection of unreacted DNA). Panels in the same row show amplifications done at the denaturation temperature shown.

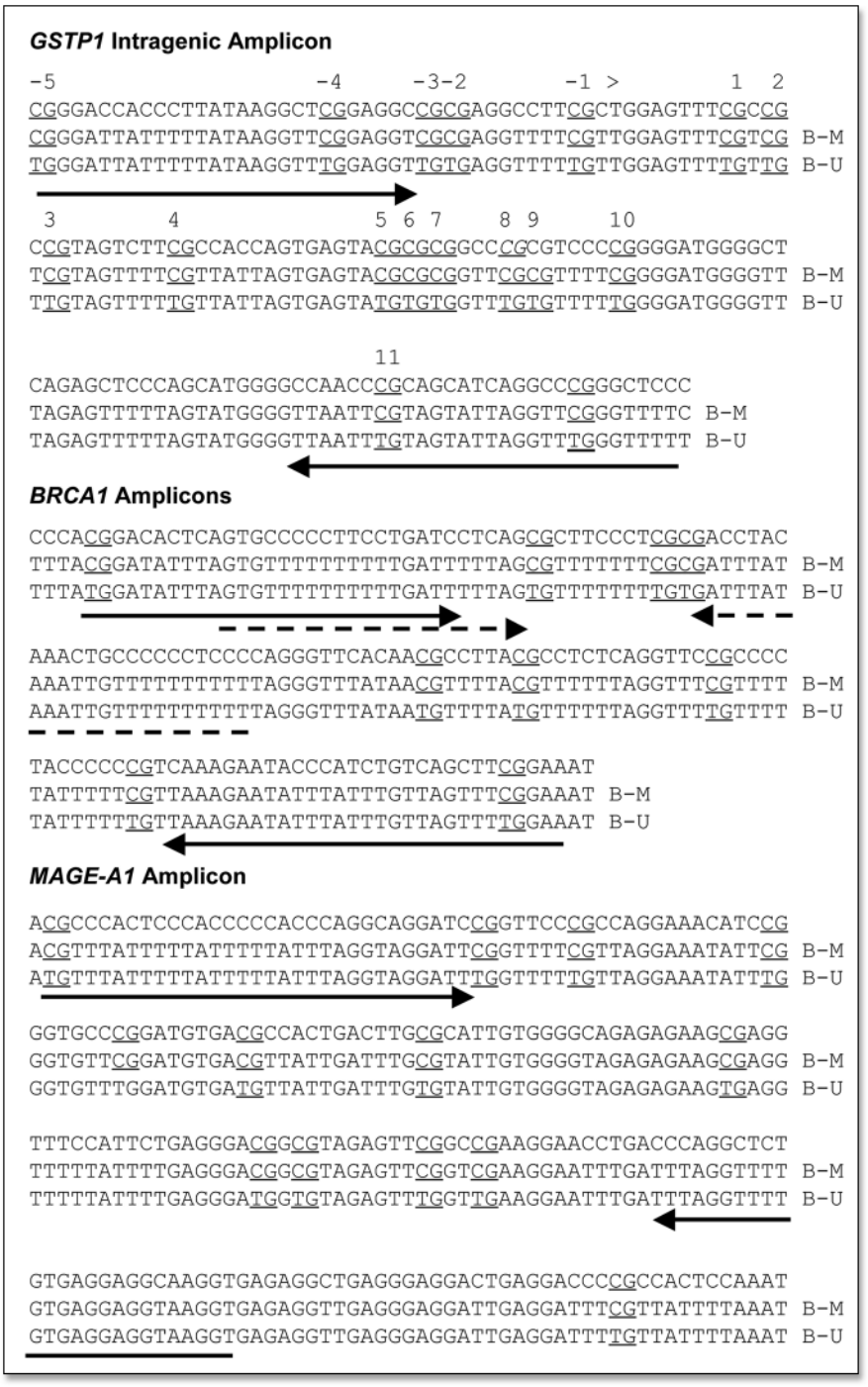

Figure 2. Amplicon sequences. The sequences of the *GSTP1* intragenic region,[22] *BRCA1* (bases 1265 to 1119, Genbank AY273801.1) and *MAGE-A1* (Bases 8389 to 8211, Genbank AC153070.1) amplicons are shown. The top row shows unmodified DNA and the second and third rows the expected DNA sequence after bisulphite conversion of methylated (B-M) or unmethylated (B-U) DNA. CpG sites are shown underlined and numbered above the sequence for the *GSTP1* intragenic region. Primer regions are shown by arrows. *BRCA1* mini-amplicon primer regions are shown by dashed arrows.

Human genomic DNA in vitro methylated at all CpG sites, CpGenome Universal Methylated DNA was purchased from Chemicon (Temecula, CA, USA) and unmethylated human genomic DNA was prepared by in vitro amplification of white blood cell DNA using a GenomiPhi kit (GE Life Sciences, Sydney, Australia). DNA was reacted with sodium bisulphite according to Clark et al.[17] Amplifications were done in an Applied Biosystems 7700 instrument (Applied Biosystems), Corbett RotorGene (Corbett, Sydney, Australia) or Biorad I-Cycler (Biorad, Hercules, CA, USA) as indicated in Table 2. All amplifications were done in 25 µl Platinum Taq PCR buffer (20 mM Tris.HCl pH 8.4, 50 mM KCl, Invitrogen, Carlsbad, CA, USA) with Platinum Taq (0.5 units), primers and probes at concentrations indicated in Table 1 and other reaction conditions as indicated in Table 2. SYBR Green (Molecular Probes) was added at 1/125,000 of stock and 10 nM fluorescein was added to reactions done on the Biorad I-Cycler. The denaturation temperature for the first five (*GSTP1* amplifications) or three (*BRCA1* and *MAGE-A1*) cycles was 95°C and for subsequent cycles the temperature indicated in the Figures and Figure legends.

Denaturation profiles of amplicons were determined by following SYBR Green fluorescence over a temperature gradient in either the ABI7700, Corbett RotorGene or Biorad I-Cycler instrument with ramp rates of 1°C per 40 sec, 2 sec or 20 sec respectively. In the Figures the Y-axis of amplification curves shows fluorescence intensity and the Y-axis of the denaturation profiles the negative differential of fluorescence.

RESULTS

Discrimination of unmethylated and methylated sequences by BDD-PCR following bisulphite treatment was evaluated using the promoter region of the *GSTP1* gene. The sequence of the amplified region of the *GSTP1* gene before and after bisulphite treatment is shown in Figure 1A. In addition to discrimination between methylated and unmethylated DNA, the use of BDD-PCR also has the potential to remove background amplification of DNA that has not fully reacted with bisulphite. Because of incomplete denaturation or secondary structure, reaction of DNA with bisulphite is not always complete and, depending on primers and PCR conditions, unmodified or partially modified DNA may be amplified.[19,20] This can particularly be the case when using methylation specific PCR (MSP) primers as they are generally designed to amplify molecules containing methylated cytosines (i.e., not converted) at PCR primer sites.[21] In amplifying methylated sequences of the *GSTP1* gene we found unwanted amplification of unconverted or incompletely converted DNA in some DNA samples and that this amplification could suppress amplification of true methylated molecules present in the population.

To demonstrate the principle of BDD-PCR, we took a mixture of amplified *GSTP1* DNA that contained approximately equal amounts of sequences corresponding to unmethylated DNA, methylated

Table 2 **PCR conditions**

PCR	dNTPs	$MgCl_2$	PCR Machine	Cycling Conditions	Detection
GSTP1 promoter	200 µM dATP, dGTP & dCTP, 400 µM dUTP	1.5 mM	ABI7700	95˚or X˚ 15 sec 60˚ 30 sec 40 cycles	Taqman probes
GSTP1 intragenic	200 µM dATP, dGTP & dCTP, 400 µM dUTP	4 mM	Corbett Rotorgene	95˚or X˚ 15 sec 60˚ 30 sec 40 cycles	SYBR Green
BRCA1	200 µM dCTP, dGTP 400 µM dATP, dTTP	5 mM	Biorad I-Cycler	95˚or X˚ 15 sec 60˚ 30 sec 65˚ 15 sec 50 cycles	SYBR Green
BRCA1 mini-amplicon	200 µM dCTP, dGTP 400 µM dATP, dTTP	2.5 mM	Biorad I-Cycler	95˚or X˚ 15 sec 50˚ 30 sec 65˚ 15 sec 50 cycles	SYBR Green
MAGE-A1	200 µM dATP, dGTP, dCTP & dTTP	1.5 mM	Biorad I-Cycler	95˚or X˚ 15 sec 58˚ 30 sec 72˚ 15 sec 50 cycles	SYBR Green

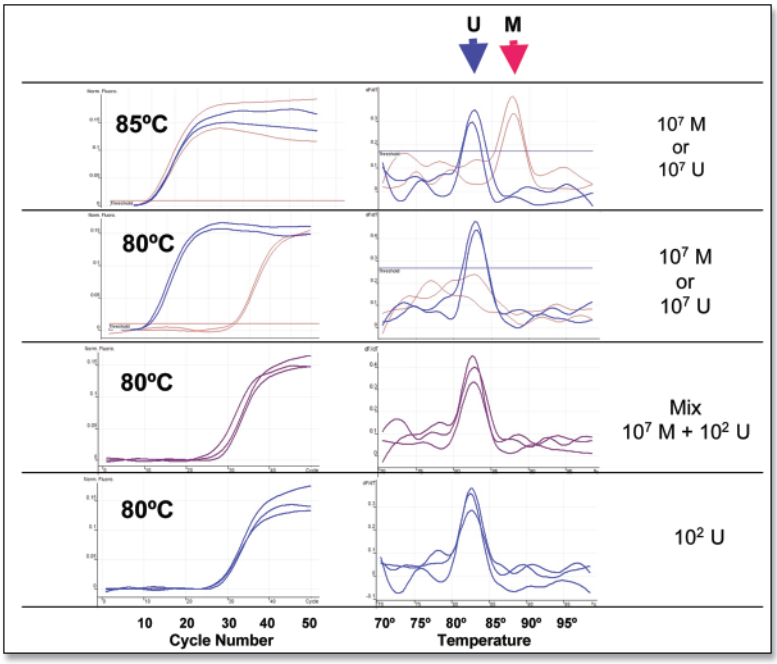

Figure 3. BDD-PCR of *GSTP1* intragenic sequences. Plasmid clones of amplicons corresponding to *GSTP1* intragenic sequences (see Fig. 2) were amplified using denaturation temperatures of 80˚C or 85˚C as indicated. Graphs to the left show Realtime detection using SYBR Green. Denaturation profiles of products are shown in the right panels. Curves are coloured red, thin line, for input methylated DNA, blue for unmethylated DNA and purple for a mixed DNA input. Input DNAs in the separate rows were (i) either 10^7 methylated DNA plasmid copies or 10^7 unmethylated DNA plasmid copies, with denaturation temperature of 85˚C. (ii) as for (i) but with denaturation temperature of 80˚C. (iii) Mixture of 10^7 methylated DNA plasmid copies and 10^2 unmethylated DNA plasmid copies, with denaturation temperature of 80˚C. (iv) 10^2 unmethylated DNA plasmid copies, with denaturation temperature of 80˚C.

DNA and unconverted DNA. This was amplified using the primers and TaqMan probes shown in Figure 1A. Separate TaqMan probes were used to identify the three different products within the same reaction. Amplifications were done using five initial cycles with denaturation at 95˚C in order that longer starting DNA molecules were fully denatured before lowering the denaturation temperature for subsequent cycles.

The results of amplifications with different denaturation temperatures are shown in Figure 1B. When PCR was performed using a denaturation temperature of 90˚C, amplification of all three templates was detected. Reduction of the denaturation temperature to 80˚C prevented amplification of unconverted DNA, while allowing amplification of both methylated and unmethylated DNA products with efficiency equivalent to that seen with a 90˚C denaturation temperature. Further reduction of the denaturation temperature to 77˚C prevented amplification of the methylated DNA product without inhibition of amplification of the unmethylated product. Clear discrimination was achieved between the methylated and unmethylated amplicons that differ by ten CpG pairs in the 141 bp.

Selectivity of amplification was further demonstrated using clones containing 156 bp fragments corresponding to methylated (M) and unmethylated (U) bisulphite-treated DNA from a separate region of the *GSTP1* gene, encompassing 13 CpG sites -3 to +10 (Fig. 2). Plasmids were amplified at different denaturation temperatures separately or mixed in a ratio of 10^5 to 1 (Fig. 3). At a denaturation temperature of 85˚C both methylated and unmethylated DNAs show equivalent amplification. With a lowered denaturation temperature of 80˚C the unmethylated sequences are selectively amplified even when present at only 100 copies mixed with 10^7 copies of the corresponding methylated sequence. Amplification was as efficient as when 100 copies were amplified alone.

Selective amplification of unmethylated sequences from genomic DNA was demonstrated using a 147 bp region of the *BRCA1* gene that contains 7 CpG sites (Fig. 2). Tms of the methylated and unmethylated amplicons were measured as 77.8˚ and 75.5˚ respectively. Using a denaturation temperature just above that of the methylated amplicon, both methylated and unmethylated DNAs were amplified equivalently (Fig. 4). At the reduced denaturation temperature of 76.9˚ amplification of the methylated amplicon was

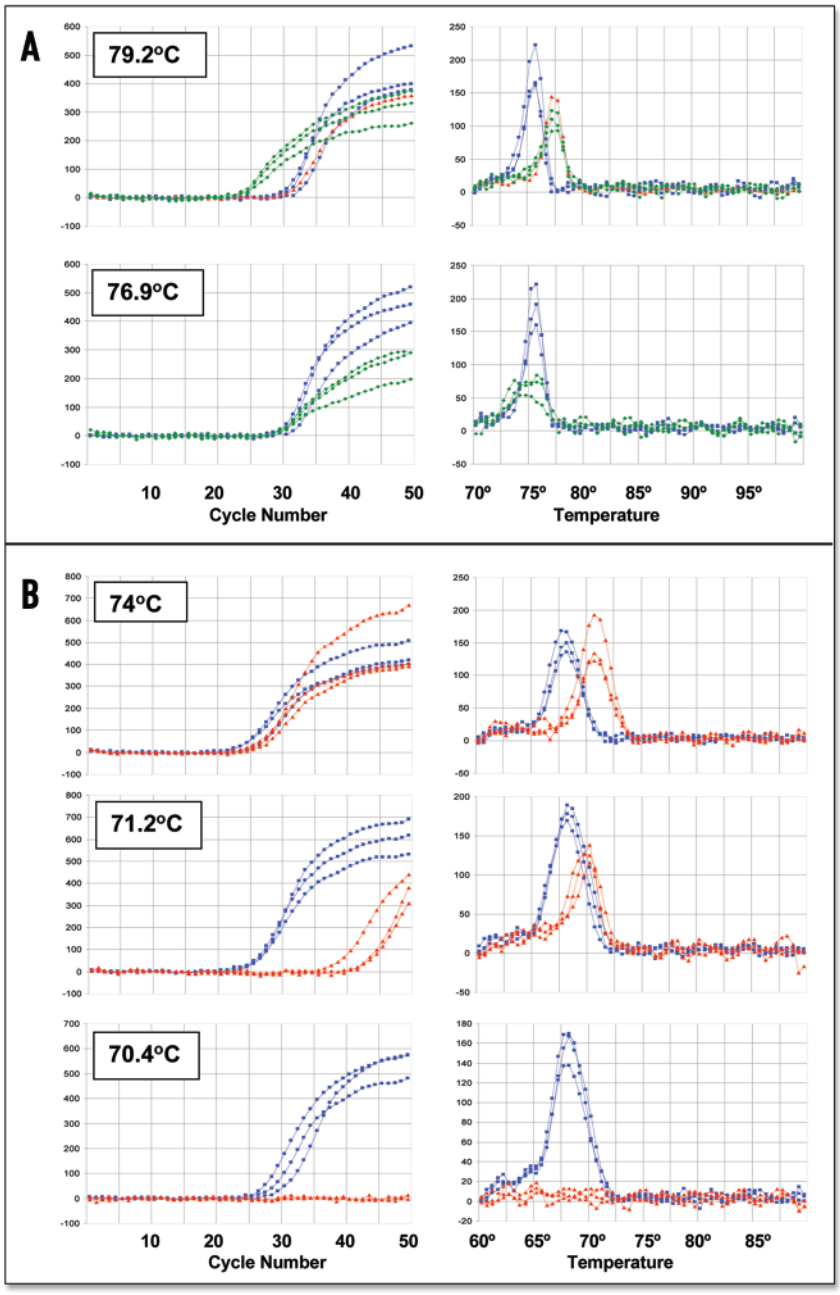

Figure 4. BDD-PCR applied to methylated and unmethylated *BRCA1* sequences. *BRCA1* sequences were amplified from fully methylated DNA (CpGenome) or white blood cell DNA (unmethylated) as described in Materials and Methods, with denaturation temperatures as shown. Graphs to the left show Realtime detection using SYBR Green. Denaturation profiles of products are shown in the right panels. The correct size of amplicons was confirmed by analysis on 10% polyacrylamide gels. (A) Input DNAs were 100 pg of methylated DNA (red line with triangles), 100 pg unmethylated DNA (blue lines with squares, triplicates) or a mix of 100 pg unmethylated DNA with 100 ng methylated DNA (green lines with diamonds, triplicates). (B) Amplification of *BRCA1* mini-amplicon. Input DNAs were 1 in 10^6 dilutions of amplicons of methylated and unmethylated DNAs. Curves are coloured red with triangles for methylated DNA and blue with squares for unmethylated DNA.

completely suppressed without any inhibition of amplification of the unmethylated DNA. Unmethylated *BRCA1* sequences are efficiently amplified at an equivalent Ct of about 31 even in the presence of a thousandfold excess of methylated genomic DNA. The breadth of the melting curve indicates the presence of other sequences amplified from the excess genomic DNA, but gel analysis demonstrated that the single major product was of the correct size. To further demonstrate the potential selectivity of amplification we took the methylated and unmethylated *BRCA1* amplicons (from Fig. 4A), diluted them 10^6 fold and subjected them to PCR using a pair of internal primers to produce a 57bp mini-amplicon that contained three CpG sites. The resultant methylated and unmethylated mini- amplicons had Tms of 71°C and 68°C respectively. Using a denaturation temperature of 70.4°C amplification of the methylated amplicon was completely suppressed, with amplification of the unmethylated amplicon being delayed by about five cycles. At an intermediate denaturation temperature of 71.2° amplification from methylated DNA was substantially inhibited and the product had a Tm between that of the methylated and unmethylated amplicons, indicating selection for partially methylated molecules present in the mix.

The *MAGE-A1* gene is an example of a gene that is differentially methylated in a tissue-specific manner, being unmethylated in DNA from expressing germline tissue, but methylated in essentially all other tissues. Additionally its demethylation and expression is associated with development of a wide variety of cancers.[14] Primers were designed to amplify a 179 bp region of the promoter region of the *MAGE-A1* gene that contained 11 CpG sites (Fig. 2). Amplification from bisulphite-treated fully methylated DNA produced an amplicon with a Tm of about 83°C, 3°C higher than that from unmethylated DNA produced by whole genome in vitro amplification of DNA (Fig. 5A). The melting profile of white blood cell DNA was indicative of fully methylated DNA as expected, while two samples of sperm DNA had Tms equivalent to or just above that of fully unmethylated DNA. Amplicons derived from either methylated or unmethylated DNA were mixed in ratios of 10^4 or 10^5:1 and amplifications done with denaturation temperatures of either 85°C or 81.9°C. At 85°C both methylated and unmethylated DNA is amplified efficiently while at 81.9°C the efficiency of amplification of the unmethylated DNA is not altered and no amplification of methylated DNA was detected up to 50 cycles.

DISCUSSION

In a previous report of the use of differential denaturation temperatures for PCR with non-bisulphite treated DNA[7] amplification of individual bands was seen to be critically dependent on differences in denaturation temperature of less than 1°C; the coamplification of multiple bands in ligation-mediated PCR might be expected to favor such fine discrimination. Similarly, Suspene et al.[8] demonstrated selective amplification of A-rich hypermutants using of human immunodeficiency virus using Tms 1°C to 4°C below that of the wild-type virus amplicon (304 bp, 34% G + C content); amplicons contained 3 to 20 G > A mutations relative to the wild-type. In the examples presented here denaturation temperature differences of 2.3°C to 5°C effectively allowed selective amplification of unmethylated amplicons in preference

to their methylated counterparts; the amplicons assessed were all about 150 bp and contained 7 to 13 CpG sites. Using a mini-57 bp amplicon, differential amplification dependent on 3 CpG sites could be demonstrated with a 2.5°C difference in denaturation temperature.

Since selection against non-denatured templates occurs in each PCR cycle, inhibition of only 50% per cycle translates into a doubling of the number of cycles needed to see the inhibited product and a major selective advantage for fully denatured amplicons. Amplicons with lower Tm can potentially arise from sequence mutation or deletion or by spurious internal priming during PCR. We have not observed this in the current examples, but it is a good precaution to monitor amplicon size by gel electrophoresis and to run replicates in order to identify potential amplification due to mispriming or deletion events in early PCR cycles.

It is notable that there is not a constant relationship between the measured Tm of amplicons and the denaturation temperature range over which inhibition of amplification is evident. This could be due to intrinsic properties of the amplicons; for example the relative melting of the central region or ends could affect primer accessibility. Properties of different instruments are likely to have a significant influence as the degree of denaturation will depend on the degree of "overshoot' of the set temperature, the ramp-rate and time at the denaturation temperature, as well (in later cycles) as the concentration of the amplicon. The measured Tm is also dependent on the ramp rate, with slower ramp rates yielding lower measured Tms. We have also noted that different software packages supplied with a single instrument give a 2°C difference in Tm. Our experience indicates that it best to take an empirical approach to determining the optimal temperature for discriminating between two amplicons. The availability of gradient blocks in PCR machines (including Real-time instruments) and the use of unmethylated and fully methylated genomic DNA makes this an efficient process.

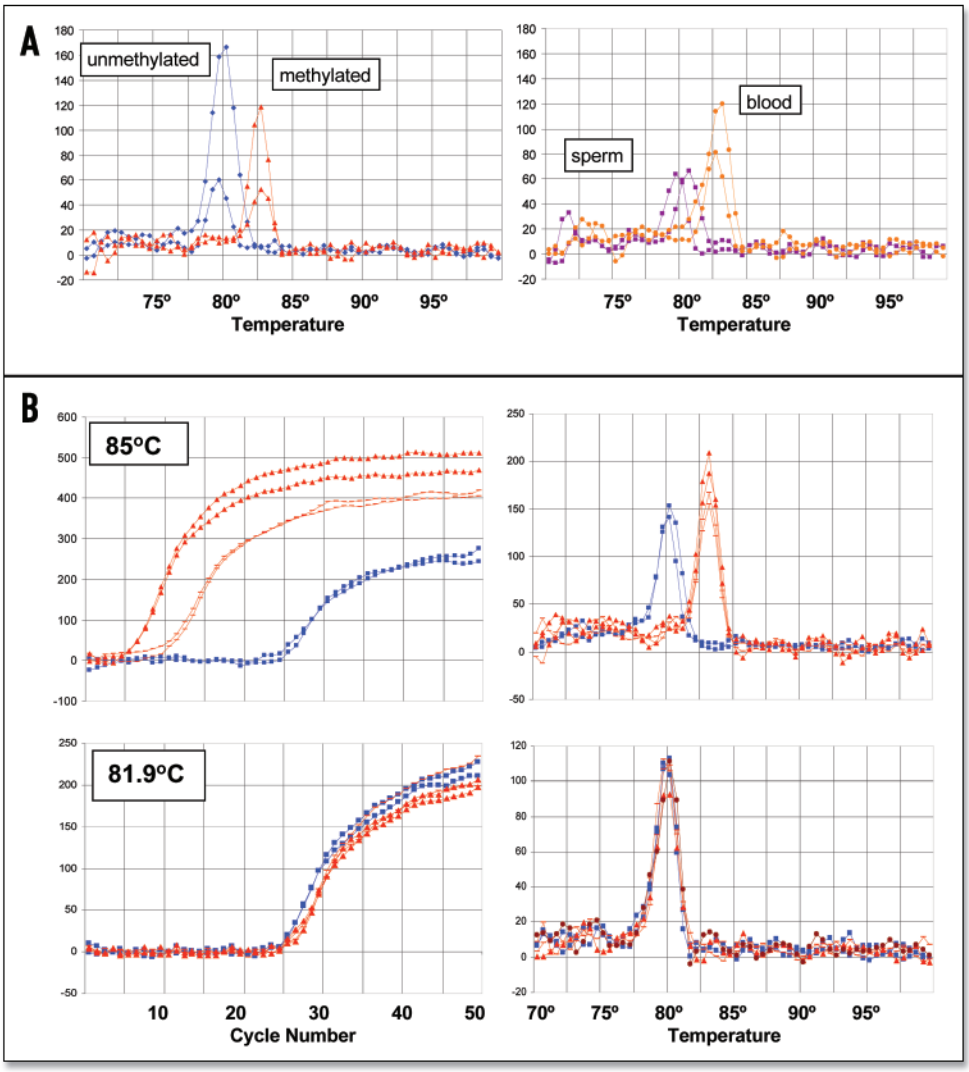

Figure 5. BDD-PCR applied to methylated and unmethylated *MAGE-A1* sequences. (A) *MAGE-A1* sequences were amplified from bisulphite-treated genomic DNAs and melting curves determined by SYBR Green fluorescence. Left panel shows melting curves of unmethylated DNA prepared by in vitro whole genome amplification and fully methylated DNA (CpGenome). The right panel shows melting curves of blood DNA and two separate sperm DNA samples. (B) Mixes of amplicons in the ratio of 10^5 or 10^4 methylated molecules to one unmethylated molecule (red lines with triangles or dashes respectively) or unmethylated molecules alone (blue lines with squares) were amplified using denaturing temperatures of 85°C or 81.9°C as indicated. Left panels show amplification curves monitored using SYBR Green and right panels show denaturation profiles. The correct size of amplicons was confirmed by analysis on 10% polyacrylamide gels.

While the use of denaturation temperature for selective amplification in PCR can be applied in a range applications, it is particularly applicable to the selective amplification of unmethylated DNA sequences following reaction with bisulphite. The same primers can be used at elevated denaturation temperature to provide an unbiased representation of the DNA population. Because amplification is dependent on the overall composition of the amplicon, it is therefore also dependent on the overall level of methylation rather than specific CpG sites and so should be useful in cases where methylation is heterogeneous. Notably, while techniques such as MSP and others[21-23] have been applied generally for the selective amplification of methylated DNA, BDD-PCR provides a simple method that can be used to selectively amplify hypomethylated sequences such as genes differentially expressed in a particular tissue, those genes such as oncogenes and tumor antigens that have become aberrantly demethylated in tumors and genes reactivated following treatment with demethylating agents such as 5-aza-2'-deoxycytidine.

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