[Epigenetics 4:4, 231-234; 16 May 2009]; ©2009 Landes Bioscience

Brief Report

Primer design versus PCR bias in methylation independent PCR amplifications

Tomasz K. Wojdacz,†,* Tanni Borgbo† and Lise Lotte Hansen

Institute of Human Genetics; University of Aarhus; Aarhus, Denmark

†These authors contributed equally to this work.

Key words: methylation, MS-HRM, primers, PCR bias, melting

Many protocols in methylation studies utilize one primer set to generate a PCR product from bisulfite modified template regardless of its methylation status (methylation independent amplification MIP). However, proportional amplification of methylated and unmethylated alleles is hard to achieve due to PCR bias favoring amplification of unmethylated relatively GC poor sequence. Two primer design systems have been proposed to overcome PCR bias in methylation independent amplifications. The first advises against including any CpG dinucleoteides into the primer sequence (CpG-free primers) and the second, recently published by us, is based on inclusion of a limited number of CpG sites into the primer sequence. Here we used the Methylation Sensitive High Resolution Melting (MS-HRM) technology to investigate the ability of primers designed according to both of the above mentioned primer design systems to proportionally amplify methylated and unmethylated templates. Ten "CpG-free" primer pairs and twenty primers containing limited number of CpGs were tested. In reconstruction experiments the "CpG-free" primers showed primer specific sensitivity and allowed us to detect methylation levels in the range from 5 to 50%. Whereas while using primers containing limited number of CpG sites we were able to consistently detect 1–0.1% methylation levels and effectively control PCR amplification bias. In conclusion, the primers with limited number of CpG sites are able to effectively reverse PCR bias and therefore detect methylated templates with significantly higher sensitivity than CpG free primers.

The ability of sodiumbisulfite to selectively deaminate cytosines under conditions where 5-methylcytosines remain intact allowed the introduction of PCR amplification into methylation studies. PCR products derived from bisulfite modified DNA have different sequences depending on the methylation status of the template subjected to bisulfite treatment. The origin of the PCR product and hence the methylation status of the locus of interest can be determined by post PCR analyses of amplification products.

Direct sequencing was one of the first post-PCR methods applied to the investigation of the methylation status of the locus of interest.[1] Bisulfite sequencing allows the investigation of the methylation status of each CpG dinuclotide within the analyzed region of interest and therefore is considered as a gold standard technique in methylation studies. Nevertheless, the costs and labor intensiveness of this method has prompted the development of other approaches that allow for efficient screening of large number of the samples.

Methods based on melting curve analyses[2] and more recently High Resolution Melting (HRM),[3] DHPLC,[4] restriction digestion (COBRA)[5,6] or single strand conformation analyses[7] are examples of technologies, which allow for high throughput and cost efficient analyses of a large number of samples. However, while sequencing reveal the methylation status of single cytosines, the majority of other techniques determine the overall methylation status of the amplicon.

All above techniques rely on PCR amplification with only one primer set that simultaneously generates PCR products from both methylated and unmethylated bisulfite modified templates. After exposure to bisulfite the methylated variant of the allele with preserved cytosines at 5-methylcytosines sites has higher GC content than the unmethylated one. The templates with different GC content tend to amplify with different efficiencies during PCR. The preferential amplification of one of the templates is referred to as "PCR bias." The PCR bias in methylation studies has long been recognized and shown to be directed towards the unmethylated (GC-poor) allele.[8,9]

Over-amplification of the unmethylated allele may mask the presence of methylated allele during post-PCR analyses and therefore compromise the sensitivity and specificity of the methylation screening method.

Many attempts have been made to overcome the PCR bias in methylation experiments. Increased annealing temperature of PCR amplification and different PCR additives have in some cases been

*Correspondence to: Tomasz K. Wojdacz; Institute of Human Genetics; University of Aarhus; The Bartholin Building; Wilhelm Meyers Allé, Bygn. 240; Aarhus C DK-8000 Denmark; Tel.: +45.89421682; Fax: +45.8612.3173; Email: wojdacz@humgen.au.dk

Submitted: 04/14/09; Accepted: 05/14/09

Previously published online as an *Epigenetics* E-publication: http://www.landesbioscience.com/journals/epigenetics/article/9020

Table 1 **The specifications and the sensitivity of the primers used in experiments**

Gene name/Ref	Primers	100%	50%	10%	5%	1%	0.1%
MGMT assay 1 (14)	F: 5'-GGTTTGGGGGTTTTTGATTAG-3' R: 5'-CCTTTTCCTATCACAAAAATAATCC-3'	✓	✓	✓	-	-	-
MGMT assay 2 (14)	F: 5'-GGTATTAGGAGGGGAGAGATT-3' R: 5'-TATACCTTAATTTACCAAATAACCC-3'	✓	✓	-	-	-	-
MGMT assay 3 (10)	F: 5'-TTGGTAAATTAAGGTATAGAGTTTT-3' R: 5'-AAACAATCTACGCATCCT-3'	✓	✓	-	-	-	-
BLU (14)	F: 5'-AAGGATTTGGAGTTTAGGAGAGATT-3' R: 5'-CCAAAATCTAAAACAAAACAATTAC-3'	✓	✓	✓	✓	+/-	-
RASSF1A (14)	F: 5'-AGTTTTTGTATTTAGGTTTTTATTG-3' R: 5'-AACTCAATAAACTCAAACTCCCC-3'	✓	✓	✓	+/-	-	-
RFC (13)	F: 5'-CTAATAACACCCCAAAATACTAAC-3' R: 5'-GTTTTATTTTTGGGGTAGTTTTTTGATTTT-3'	✓	✓	-	-	-	-
DBCCR1 (11)	F: 5'-GGGAGGTAGAGGGAGTAGTGAT-3' R: 5'-AAAATACCTAACTCCTAACAACCTACC-3'	✓	✓	✓	✓	-	-
CDH13 (10)	F: 5'-TTGGGAAGTTGGTTGGTTG-3' R: 5'-ACAACCCCTCTTCCCTACCT-3'	✓	✓	✓	✓	-	-
DBC1 assay 1 (12)	F: 5'-TAAATACTGTTAAATATTTATAGAGAGA-3' R: 5'-CCCGAAATCCTAATACCCTTAAA-3'	✓	✓	✓	✓	+/-	-
DBC1 assay 2 (12)	F: 5'-AGAGAAGTTTTTGTTTTATTTTG-3' R: 5'-CCCGAAATCCTAATACCCTTAAA-3'	✓	✓	✓	✓	-	-
MS-HRM DAPK1	F: 5'-GCGCGGAGTTGGGAGGAGT-3' R: 5'-CTCCGAACTACCCTACCAAACC-3'	✓	✓	✓	✓	✓	+/-
MS-HRM HIC1	F: 5'-GGCGGTTCGGGTAGTAAGTAGTT-3' R: 5'-AACGAAACAACAAAACCCCCAACC-3'	✓	✓	✓	✓	✓	✓
MS-HRM RASSF1A	F: 5'-GTTTTAGATGAAGTCGTTATAGAGGT-3' R: 5'-CCCCCACGACAACTAATCCCTAA-3'	✓	✓	✓	✓	✓	✓
MS-HRM BSG	F: 5'-GGTTTTGTAGGGGTCGGGAATG-3' R: 5'-CGCCGAAACCCCAAACTCCC-3'	✓	✓	✓	✓	✓	✓
MS-HRM ESR1	F: 5'-GCGTTCGTTTTGGGATTGTATTTGTTT-3' R: 5'-TCTAACCCCGACCCTACCCC-3'	✓	✓	✓	✓	✓	+/-

Ticks indicate that PCR product has been generated at respected dilution point, "+/-" indicates that PCR product was obtained but without 100% reproducibility. For primers containing CpG sites 5 examples are shown in the bottom of the table. Further examples of the primers tested can be found in refs. 15–17, and are available on request.

shown to improve amplification of the methylated allele, nevertheless, the data in the literature are contradicting.[8,10]

Guidelines for design of methylation independent primers (MIP), which allow for proportional amplification of methylated and unmethylated templates have been proposed by Clark et al.[1] These guidelines advise the avoidance of CpG sites in the primer sequence and if impossible to do so, a C from a CpG dinuclotide should be mismatchd with a T to ensure an unbiased amplification.

We selected ten primer pairs from the literature (Table 1) designed to bind to CpG free sequences.[10-14] The primers were previously used for methylation independent amplifications for different experimental protocols. For each primer set we aimed to evaluate the extent of PCR bias as well as the influence of the annealing temperature on the efficiencies of the amplification of both methylated and unmethylated alleles. As a testing system, developed by us the Methylation Sensitive High Resolution Melting protocol (MS-HRM) has been chosen.[15] MS-HRM combines PCR amplification using methylation independent primes with subsequent HRM analyses of the PCR products. The PCR product generated from methylated (bisulfite modified) template has a relatively higher GC content and therefore higher melting temperature than PCR product generated from unmethylated (GC-poor) variant of the same template. The HRM analyses allow for highly sensitive monitoring of the melting temperature of PCR product and hence distinction methylated and unmethylated PCR products.

To visualize preferential amplification of the methylated or unmethylated alleles, the analyses were performed on the mixes of methylated (Millipore, CpGenome^{TM} Universal Methylated DNA) in unmethylated (Peripheral blood DNA or Millipore, CpGenome^{TM} Universal Unmethylated DNA) bisulfite modified templates. The mixes ranged from 0.1% methylated template in unmethylated background, through 1, 5, 10, 50 to 100% unmethylated template. The EpiTect Bisulfite Kit (Qiagen) was used to bisulfite modify 200 ng of template DNA. The LighCycler®480 platform (Roche) was used for both PCR amplifications and the subsequent HRM analyses. The PCR mixes consisted of

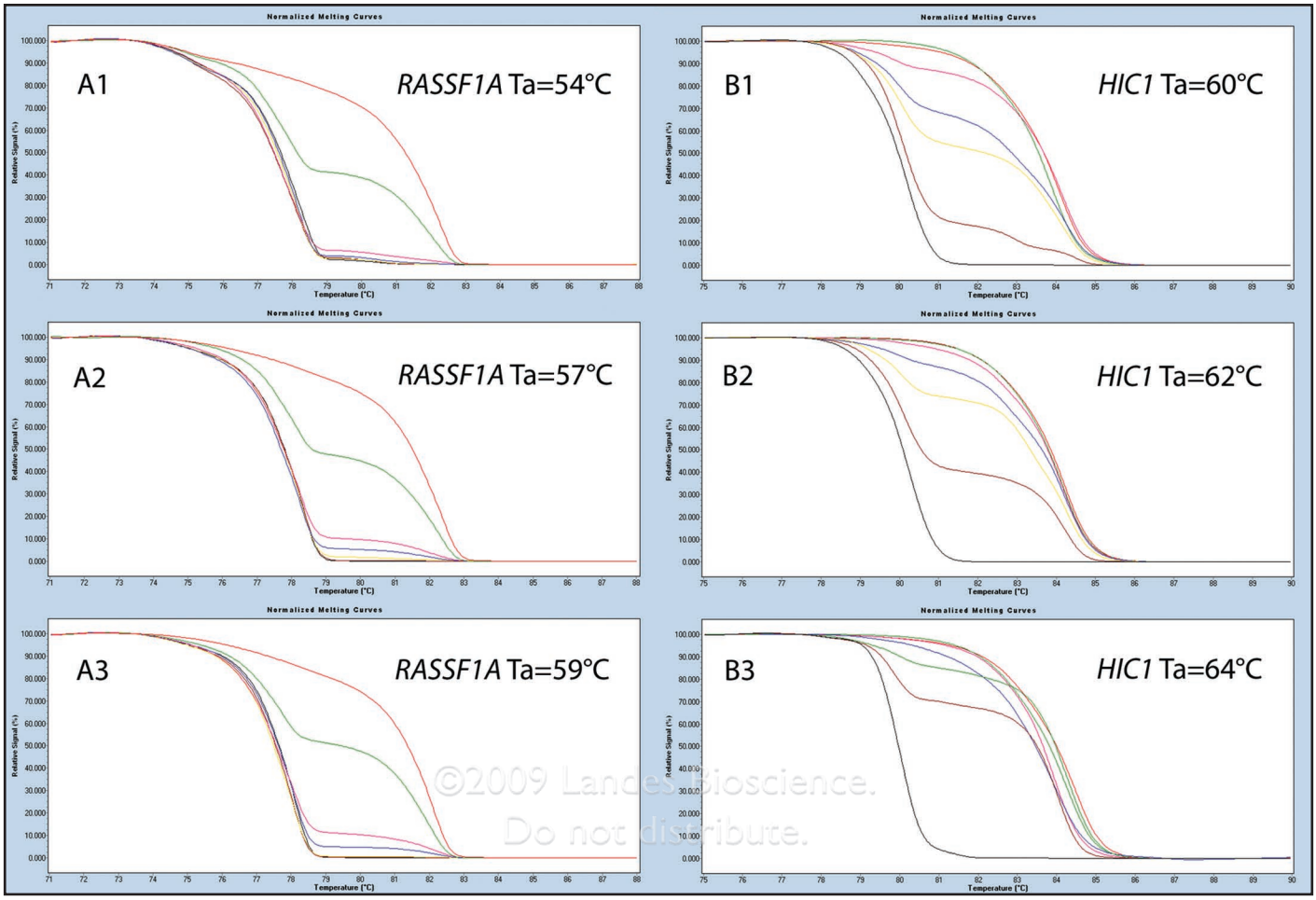

Figure 1. MS-HRM scans obtained after melting of the PCR products amplified using CpG free primers. The Gene Scanning software was used to analyze the data. The melting curves represent the melting of PCR product obtained from amplification of mixes: black—0%, brown—0.1%, yellow—1%, blue—5%, pink—10%, green—50%, red—100% of methylated template in unmethylated background. In (A), HRM scans of amplification product with CpG-Free primers at 3 different annealing temperatures (A1–A3) are depicted. The scans show minimal influence of increased annealing temperature on the amount of PCR product amplified from methylated template. (B) displays HRM scans of amplification products obtained in PCR with primers containing limited number of CpG sites. Scans (B1–B3) show an annealing temperature dependent increase in amount of PCR product originating from the methylated variant of the template. For further examples see: Figures 1 and 2 in Supplementary Data section and refs. 15–17.

1x LightCycler®480 HRM Master Mix (Roche), 3 mM Mg^{+2}, 250 nM of each primer and 6 ng of template. All the PCR amplification and HRM melting were run in triplicates and repeated at least once.

The results of our experiments showed that each of the primer sets chosen from the literature could detect the methylated sequence with different sensitivity (Figure 1, parts A1–3, Table 1 and Supplementary Data Figure 1). Half of the primers (5 out of 10) were only able to reproducibly detect 5% methylation levels. Three of the primer sets generated only detectable PCR product from the mixes containing 50% of methylated template and two allowed for reproducible detection of 10% methylation levels Table 1. We anticipated that the difference in the sensitivity of the primers was attributed to the PCR bias occurring during amplification and favoring amplification of the unmethylated template. The significant differences in the sensitivity of the primers also suggest that the PCR bias is not only GC content dependent but also sequence specific. Increasing the annealing temperature of the amplifications did not have a significant influence on the sensitivity of the primers (Figure 1, parts A1–3 and Suppl. Data Figure 1).

Recently we proposed a new primer design system, based on the principle of inclusion of a limited number of CpG sites in to the primer sequence.[16] Annealing of the primers containing a limited number of the CpGs to the bisulfite modified template is temperature dependent. At low temperatures, primers bind both methylated and unmethylated templates allowing for amplification of both alleles with PCR bias. However, at higher temperature the primers preferentially anneal to the methylated template allowing for the reversal of the PCR bias.

We have designed a panel of 20 assays according to our new primer design rules and tested their ability to reverse PCR bias.

The experimental settings were the same as used in the tests of the primers designed according to Clark et al.[1] Each of our primer sets at relatively low annealing temperatures showed different amplification efficiencies for methylated and unmethylated variant of the template (Figure 1, parts B1–3 and Suppl. Data Figure 2).

However, with the increase of the annealing temperature the sensitivity of the primers increased significantly (Figure 1, parts B1–3 and Suppl. Data Figure 2). Overall, by tuning the annealing temperature for each of the primer sets from our new panel we were able to detect the methylation levels of 1–0.1% with most of the primers allowing for reproducible detection of 0.1% methylation levels (Figure 1, parts B1–3, Table 1 and Suppl. Data Figure 2). The same pattern of annealing temperature dependent efficiency of amplification of methylated/unmethylated allele was observed for all of the primer sets.

It is important to notice that the observed sensitivity of our primers was the same for each primer set regardless of the amplified sequence and is similar to the sensitivity levels of Methylation Specific PCR.[17]

The comparison of two different primer design systems showed that the primers designed according to our newly proposed guidelines allow for correction of PCR bias in amplification where one primer set amplifies both the methylated and unmethylated template. Despite the fact that it is impossible, to design two primer sets (with and without CpGs) targeting the same sequence and directly compare performance of the primers. Our strategy based on comparison of patterns of overall behavior of primers with and without CpGs showed clearly that primers with limited number of CpGs allow for highly sensitive detection of methylated template in unmethylated background.

We hypothesize that the main difference between our and previously reported primer design with regard to the ability to reverse PCR bias depends on the fact that primers without CpGs as opposed to primers containing CpGs can not selectively bind to methylated or unmethylated alleles. The CpG-free primers always bind with the same efficiency to both methylated and unmethylated templates and therefore cannot correct for PCR bias that seems to occur during the DNA elongation step of the PCR. The primers containing a limited number of CpG sites bind to methylated and unmethylated templates with different efficiencies and the binding efficiency is annealing temperature dependent. At low annealing temperature the primers bind, both methylated and unmethylated templates equally, but with an increase of the temperature, the primers anneal more efficiently (perfect matching) to methylated template allowing for its preferential amplification. Hence, at a certain primer specific annealing temperature the preferential binding of the primers to the methylated sequence corrects for PCR amplification bias.[3,9,15]

With the increasing awareness of the significance and power of methylation changes not only in development of the disease but also in clinical diagnostic and treatment, reliable and robust methodologies are critical for the progress in the field. The superior sensitivity of the assay is of high importance for the applications aiming to detect methylated biomarkers in environment where the concentration of the biomarker is very low e.g., detection circulating tumor DNA in plasma samples.

The above findings illustrate the significance of careful evaluation of the sensitivity of each PCR primer set used in the methodologies based on methylation independent amplifications. Careful evaluation is especially important when the method is being considered for use in clinical settings and PCR bias when underestimated may significantly compromise both the sensitivity and the specificity of the protocol.

Acknowledgements

We would like to acknowledge Dr. Alexander Dobrovic for a critical review of the manuscript and Britta B. Thestrup for the technical assistance with the experiments. This work was financed with grants from Lundbeck and Toyota Foundations. We would also like to thank Dr. Michael Hoffmann and Roche Diagnostics for the support of the research reported in this publication.

Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/WojdaczEPI4-4-Sup.pdf

References

1. Clark SJ, Harrison J, Paul CL, Frommer M. High sensitivity mapping of methylated cytosines. Nucleic Acid Res 1994; 22:2990-7.
2. Worm J, Aggerholm A, Guldberg P. In-tube DNA methylation profiling by fluorescence melting curve analysis. Clin Chem 2001; 47:1183-9.
3. Wojdacz TK, Dobrovic A. Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation. Nucleic Acid Res 2007; 35:41.
4. Couvert P, Poirier K, Carrie A, Chalas C, Jouannet P, Beldjord C, et al. DHPLC-based method for DNA methylation analysis of differential methylated regions from imprinted genes. BioTechniques 2003; 34:356-62.
5. Xiong Z, Laird PW. COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acid Res 1997; 25:2532-4.
6. Eads CA, Laird PW. Combined bisulfite restriction analysis (COBRA). Methods Mol Biol 2002; 200:71-85.
7. Bianco T, Hussey D, Dobrovic A. Methylation-sensitive, single-strand conformation analysis (MS-SSCA): A rapid method to screen for and analyze methylation. Hum Muta 1999; 14:289-93.
8. Warnecke PM, Stirzaker C, Melki JR, Millar DS, Paul CL, Clark SJ. Detection and measurement of PCR bias in quantitative methylation analysis of bisulphite-treated DNA. Nucleic Acid Res 1997; 25:4422-6.
9. Wojdacz TK, Hansen LL. Reversal of PCR bias for improved sensitivity of the DNA methylation melting curve assay. BioTechniques 2006; 41:274-8.
10. Shen L, Guo Y, Chen X, Ahmed S, Issa JP. Optimizing annealing temperature overcomes bias in bisulfite PCR methylation analysis. BioTechniques 2007; 42:48-50.
11. Gao S, Worm J, Guldberg P, Eiberg H, Krogdahl A, Sorensen JA, et al. Loss of heterozygosity at 9q33 and hypermethylation of the DBCCR1 gene in oral squamous cell carcinoma. Br J Cancer 2004; 91:760-4.
12. Gronbaek K, Ralfkiaer U, Dahl C, Hother C, Burns JS, Kassem M, et al. Frequent hypermethylation of DBC1 in malignant lymphoproliferative neoplasms. Mod Pathol 2008; 21:632-8.
13. Guldberg P, Worm J, Gronbaek K. Profiling DNA methylation by melting analysis. Methods 2002; 27:121-7.
14. Lorente A, Mueller W, Urdangarin E, Lazcoz P, von Deimling A, Castresana JS. Detection of methylation in promoter sequences by melting curve analysis-based semiquantitative real time PCR. BMC Cancer 2008; 8:61.
15. Wojdacz TK, Dobrovic A, Hansen LL. Methylation-sensitive high-resolution melting. Nat Protocols 2008; 3:1903-8.
16. Wojdacz TK, Hansen LL, Dobrovic A. A new approach to primer design for the control of PCR bias in methylation studies. BMC Res Notes 2008; 1:54.
17. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 1996; 93:9821-6.