Improved locus-specific unmethylated controls for MS-HRM analysis derived from 5-aza-2-deoxycytidine-treated DNA

Helena Čelešnik^{1,2} & Uroš Potočnik^{*1,2}

ABSTRACT

We report two restriction enzyme-based approaches for generating clean locus-specific unmethylated controls for methylation-sensitive high-resolution melting (MS-HRM) analyses. These unmethylated standards are derived from DNA treated with the demethylating agent 5-aza-2-deoxycytidine (5-Aza-dc). By using them, we overcome a limitation of 5-Aza-dc treatment – incomplete demethylation at various genomic regions. When 5-Aza-dc-treated DNA is used directly as unmethylated MS-HRM standard, partially demethylated DNA can give false methylation results. MS-HRM assay differentiates between methylated and unmethylated bisulfite-treated DNA based on the different melting profiles of PCR products amplified from them. To estimate test sample methylation levels, test sample melting profiles are compared to those of methylation standards. With our pure unmethylated controls, adequate standards of known methylation levels can be prepared for single-locus MS-HRM.

KEYWORDS:

5-Aza-2'-deoxycytidine • *DAPK1* • epigenetics • *LRPAP1* • MrcBC • methylation • methylation-sensitive high-resolution melting • MS-HRM • standard • unmethylated control

¹University of Maribor, Faculty of Chemistry & Chemical Engineering, Smetanova ulica 17, 2000 Maribor, Slovenia; ²University of Maribor, Faculty of Medicine, Center for Human Molecular Genetics & Pharmacogenomics, Taborska ulica 8, 2000 Maribor, Slovenia; *Author for correspondence: uros.potocnik@um.si

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METHOD SUMMARY

We used 5-aza-2-deoxycytidine-treated DNA to derive clean loci-specific unmethylated controls for single locus methylation-sensitive high-resolution melting analysis. One approach included digesting locus-specific PCR products amplified from 5-aza-2-deoxycytidine-treated DNA with the restriction enzymes Taq^I or Bst^{UI} to deplete the products arising from incompletely demethylated templates, followed by gel purification of the remaining unmethylated PCR population, which was used as the unmethylated control. The second approach involved depleting all contaminating methylated DNA in 5-aza-2-deoxycytidine-treated DNA by MrcBC digestion prior to bisulfite conversion.

Epigenetic mechanisms such as DNA methylation have a key role in many biological processes, including development and cancer [1]. Many techniques for assessing the methylation level of DNA samples have been developed over the past decades [2–8]. Most of them involve sodium bisulfite modification of the DNA template, which causes deamination of unmethylated cytosines within CpG dinucleotides to uracil while leaving the 5-methylcytosines intact, followed by PCR amplification of the region of interest. Bisulfite modification causes a different base composition between the methylated and unmethylated DNA, which is used to distinguish the PCR amplicons derived from these templates [3,9]. The bisulfite-converted DNA is amplified with primers designed specifically for converted DNA. Methylation-sensitive high-resolution melting (MS-HRM) can be used to analyze the amplified products and measure methylation levels at specific loci of interest. Estimation of test sample methylation level is achieved by comparing the melting profiles of PCR products amplified from test samples with the melting profiles of PCR products amplified from methylated and

unmethylated standards [9–11]. MS-HRM is fast, relatively inexpensive and can be used for screening multiple samples and for precise diagnostics [9]. The high sensitivity of MS-HRM allows detection of as low as 0.1–1% methylated alleles in an unmethylated background [11]. One key aspect for optimal performance and correct interpretation of the MS-HRM results is using a good set of methylated and unmethylated controls, since the estimated level of DNA methylation depends on the melting profiles of methylation standards. Another important aspect is choosing the optimal primers. They should be designed to amplify the bisulfite-converted region regardless of its methylation status [12,13]. In CpG methylation studies, genomic DNA purified from human cells that were treated with 5-aza-2-deoxycytidine (5-Aza-dc) is often used as a negative or unmethylated control [14–16]. The compound 5-Aza-dc is a methyltransferase inhibitor that causes genome-wide CpG demethylation [17]. For many analyzed DNA regions, bisulfite-converted 5-Aza-dc-treated genomic DNA works well in MS-HRM by serving as a negative control that can be used to generate standard curves (e.g. *APOA5* gene, Supplementary Figure 1). Standards with methylation levels ranging between 0 and 100% can be prepared by diluting fully methylated bisulfite converted DNA into a background of fully unmethylated bisulfite-converted DNA. The resulting standard dilution series (e.g., 0:100, 25:75, 50:50, 75:25, 100:0) can be used for quality control when testing new primers and for determining the methylation levels of unknown samples [9].

However, 5-Aza-dc treatment does not result in demethylation of every CpG in DNA. Some 5-Aza-dc-treated regions can remain methylated, even to a large extent [18]. The incompletely demethylated DNA can significantly affect methylation analyses and ►

Benchmarks

Figure 1. Sequence of the *LRPAP1* segment analyzed by MS-HRM. (Left) Unconverted sequence of the *LRPAP1* exon 7 segment analyzed by MS-HRM. (Right) Bisulfite-converted sequence in which non-CpG-cytosines have been replaced by thymines. The primers Fwd2 and Rev (designed for converted DNA) were used to generate the 98-bp optimized unmethylated control. The Taq^qI restriction recognition sites (TCGA) and CG (CpG dinucleotides) are underlined. The primers Fwd1 and Rev were used to amplify a larger 256-bp segment. MS-HRM: Methylation-sensitive high-resolution melting.

Figure 2. *LRPAP1* MS-HRM assay and improvement of unmethylated control. (A) The *LRPAP1* MS-HRM assay with 5-Aza-dc-treated genomic DNA as the unmethylated control (5-aza UM) and SssI-treated genomic DNA as fully methylated control (M). A substantial amount of methylation is detected when bisulfite-modified 5-Aza-dc-treated genomic DNA is amplified. Data were analyzed by using the 'Tm calling' software module. (B) Digestion of PCR products amplified from bisulfite-modified 5-Aza-dc-treated genomic DNA confirms the presence of substantial methylation. The restriction enzyme Taq^qI digests only PCR products derived from methylated bisulfite-converted templates. The Taq^qI-undigested PCR product can be gel purified and used as a negative MS-HRM control. (C) Sanger sequencing (performed at Eurofins Scientific, Germany) of the PCR product derived from 5-aza-treated DNA (left) showing the presence of fully methylated CpGs (outlined in blue); (right) sequence of the optimized UM. (D) *LRPAP1* MS-HRM assay with optimized unmethylated control DNA. The gel-purified unmethylated *LRPAP1* amplicon derived from bisulfite-modified 5-Aza-dc-treated genomic DNA served as the negative control (UM) and the gel purified *LRPAP1* product amplified from bisulfite-modified SssI-treated genomic DNA served as the positive control (M). Data were analyzed by using the 'Tm calling' software module. (E) The *LRPAP1* MS-HRM assay with optimized 100% UM (100 UM), 100% M (100 M) and standards at 75, 50 and 25% methylation (75, 50 and 25 M). Standard curves serve for determining the methylation level of test sample. Data were analyzed using the 'Gene Scanning' software module; normalized HRM curves are shown. 5-Aza-dc: 5-aza-2-deoxycytidine; M: Methylated control; MS-HRM: Methylation-sensitive high-resolution melting; UM: Unmethylated control.

► give inaccurate results, especially in single loci analyses. For example, when bisulfite-converted 5-Aza-dc-treated genomic DNA was used as the unmethylated control in MS-HRM analysis of the *LRPAP1* gene (Figure 1), a substantial signal corresponding to the fully methylated DNA was detected (Figure 2A), indicating the presence of methylated DNA in the unmethylated control. The presence was corroborated by digestion of the PCR amplicons with the restriction endonuclease Taq^qI, which specifically recognizes and cleaves products amplified from the methylated bisulfite-modified DNA (Figure 2B). Sanger sequencing of the amplicons confirmed methylation at some CpG sites (Figure 2C). We also encountered the presence of methylated DNA in 5-Aza-dc unmethylated control when several other loci were analyzed, such as *Axin1*, *CXCL12*, *DAPK1* and others (*DAPK1* depicted in Figure 3). This indicated that incomplete demethylation of individual loci is not uncommon and implied that many loci bisulfite-modified 5-Aza-dc-treated genomic DNA cannot be meaningfully used as an unmethylated control in MS-HRM. Without a completely unmethylated control, it is not possible to generate proper standard curves to test for primer bias and accurately determine the methylation status of test samples. Bypassing this problem may involve using a completely different unmethylated control, for example one generated by whole-genome amplification (WGA) and bisulfite conversion [19]. However, this method also carries some limitations. Like 5-Aza-dc-treated DNA, ►

► the unmethylated WGA products can be contaminated with methylated DNA (i.e., the original genomic template; Supplementary Figure 2). For improved performance, a second round of WGA is performed to dilute this template [9]. WGA can also show bias towards low GC regions, resulting in incomplete genome representation. The use of Phi29 polymerase has been suggested to minimize this effect [19]. In our work, however, we bypassed the issue of having methylated DNA in the unmethylated control by using 5-Aza-dc-treated genomic DNA as the source for deriving pure loci-specific unmethylated controls. Our clean controls are completely free of the methylated template, which is favorable compared with the two-step WGA controls.

In the case of *LRPAP1*, we first PCR-amplified the investigated 98-bp region of the *LRPAP1* gene (Figure 1) by

using bisulfite-converted 5-Aza-dc-treated Jurkat genomic DNA (New England Biolabs, MA, USA) as the template. Subsequently, the amplicons were digested with Taq^{AI} to deplete the PCR population arising from incompletely demethylated bisulfite-converted templates (Figure 2B). The remaining products (amplified from fully unmethylated converted DNA) were gel purified, diluted to the desired concentration and used as the optimized unmethylated control (for details, see Supplementary Material). When tested in *LRPAP1* MS-HRM analysis, the optimized unmethylated control gave a single melting peak that was easily distinguishable from the methylated control (Figure 2D). Sanger sequencing of the optimized unmethylated control verified its methylation status (Figure 2C). When mixed with fully methylated control in gradient proportions (for details, see Supplementary Material),

the optimized unmethylated control gave standard curves that were suitable for determining the methylation levels of the tested samples (Figure 2E). Methylation could be detected at levels as low as 2% (Supplementary Figure 3). Our approach therefore enabled the generation of a pure unmethylated standard that was used to prepare an accurate standard dilution range for informative methylation analysis.

To evaluate the applicability of this approach for other genomic regions, we analyzed a segment upstream of the *DAPK1* start codon (Figure 3). Using converted 5-Aza-dc-treated genomic DNA as the template, the segment was PCR-amplified and the amplicons subsequently treated with BstUI, which cleaved PCR products amplified from the methylated bisulfite-converted templates (Figure 3A). The undigested products (those amplified from unmethylated converted DNA) were gel purified and used as the optimized unmethylated control (for details, see Supplementary Material). In *DAPK1* MS-HRM analysis, the optimized unmethylated control gave a single melting peak that was easily distinguishable from the methylated control (Figure 3B) and was suitable for generation of standards with known methylation level (Figure 3C). Therefore, our approach can be adapted for measuring the methylation status of various genomic regions that present with incomplete demethylation in 5-Aza-dc-treated DNA.

We evaluated another possibility to clear 5-Aza-dc-treated genomic DNA of the remaining methylated DNA – by digesting it with methylation-specific restriction endonuclease McrBC prior to bisulfite modification. McrBC cleaves DNA containing methylcytosine on one or both strands while leaving unmethylated DNA intact. Its recognition sequence consists of two half-sites of the form (G/A)^mC, which are usually separated by 55–103 bp, sometimes more [20]. If the recognition sequence is present within the analyzed locus, McrBC treatment can help remove the methylated template. This was validated for *DAPK1* (Figure 3D), where PCR products amplified from McrBC-digested 5-Aza-dc-treated genomic DNA resisted BstUI cleavage, consistent with the absence of methylated template. It is important to note, however, that for loci that had resisted 5-Aza demethylation treatment and are ►

Figure 3. Generation of optimized unmethylated standard for *DAPK1* MS-HRM and McrBC digestion of 5-Aza-dc-treated genomic DNA. (A) Agarose gel showing BstUI-digestion of *DAPK1* PCR product amplified from bisulfite-modified 5-Aza-dc-treated genomic DNA. Gel-purified undigested PCR product served as the UM in MS-HRM. (B) MS-HRM assay with optimized *DAPK1* UM and M. Data were analyzed using the 'T_m calling' software module. (C) *DAPK1* MS-HRM assay with optimized 100% UM (100 UM), 100% M (100 M) and standards at 75, 50 and 25% methylation (75 M, 50 M, 25 M). Data were analyzed using the 'Gene Scanning' software module; normalized HRM curves are shown. (D) McrBC digestion of 5-Aza-dc-treated genomic DNA prior to bisulfite conversion removes the methylated DNA that had remained after 5-Aza-dc treatment. After bisulfite conversion, the *DAPK1* PCR product amplified from this template resists BstUI digestion, as expected for a clear UM. (E) For the 256-bp *LRPAP1* segment (primers Fwd1 and Rev), McrBC treatment results in depletion of the template for PCR or MS-HRM analysis. 5-Aza-dc: 5-aza-2-deoxycytidine; M: Methylated control; MS-HRM: Methylation-sensitive high-resolution melting; UM: Unmethylated control.

► thus still methylated, McrBC digestion can result in depletion of the template needed for PCR amplification and MS-HRM analysis. This was observed for the 256-bp *LRPAP1* region amplified with the primers Fwd1 and Rev (Figure 3E). Taken together, pretreatment of 5-Aza-dc-treated genomic DNA with McrBC before bisulfite conversion can serve as a method for generating fully unmethylated control DNA but may not be applicable for all genomic regions.

Finally, both approaches for creating clean negative controls (Taq[®]I /BstUI treatment or McrBC treatment) are dependent on the presence of suitable restriction enzyme recognition sites. Also, the presence of at least some DNA containing unmethylated CpGs is needed in 5-Aza-dc-treated genomic DNA, otherwise the restriction enzymes can cause template depletion. We therefore suggest that the utility of these approaches for specific loci be assessed on a case to case basis.

In conclusion, this work discusses the limitations of 5-Aza-dc-treated genomic DNA as an unmethylated control in MS-HRM analyses. Our results show that when difficult genomic regions are analyzed, 5-Aza-dc-treated genomic DNA can still be valuable, as it can be used to derive clean locus-specific unmethylated standards.

AUTHOR CONTRIBUTIONS

HC conceived and performed the experiments. HC and UP interpreted the results and prepared the manuscript.

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SUPPLEMENTARY DATA

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2018-0161

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