Methylation-Sensitive High-Resolution Melting Analysis on 7500 Fast Real-Time PCR System

Abstract
DNA methylation is a key epigenetic mechanism regulating gene expression and chromatin structure. This article describes a workflow that allows the locus-specific analysis of DNA methylation by methylation-sensitive high resolution melting (MS-HRM) on the Applied Biosystems 7500 Fast Real-Time PCR System (Figure 1). MS-HRM is a fast and simple method for methylation studies in basic and clinical research. The 7500 Fast Real-Time PCR System and the High Resolution Melting software module enables scientists to run MS-HRM reactions and to analyze differentially methylated regions.

DNA Methylation
DNA methylation occurs by the covalent addition of a methyl group to position 5 of the cytosine (C) ring, creating a methylcytosine residue. In mammalian genomes, methylcytosine is almost exclusively found in CpG dinucleotides and occurs with a frequency of 2–5%. CpG dinucleotides are not equally distributed throughout the genome. They occur in either clusters of large repetitive sequences or in short CpG-rich DNA stretches known as CpG islands (CGIs) that are found preferentially in the 5' regulatory region of genes [1].

DNA Methylation in Cancer
DNA methylation plays a critical role in the regulation of gene expression in development, differentiation, and disease [2]. Changes in DNA methylation are recognized as one of the earliest genetic alterations in the development of cancer. Methylation of CGIs in promoters usually turns off gene transcription [3]. Hypermethylation of CGIs located in the promoter regions of tumor suppressor genes is the most frequent mechanism for gene inactivation in cancer [4]. In contrast, global hypomethylation of genomic DNA has been observed in tumor cells and a correlation between hypomethylated and increased gene expression has been reported for many oncogenes [5].

Step 1: Bisulfite Conversion of DNA
MS-HRM monitors the change in fluorescence as a PCR amplicon melts in the presence of an intercalating DNA dye. PCR products generated from bisulfite-treated DNA templates have different melting curves if they are differentially methylated [6]. It is challenging to achieve complete and highly selective bisulfite conversion of non-methylated Cs without significant side-reactions or extensive cleavage of genomic DNA. An incomplete conversion artificially increases the methylation level and can lead to MS-HRM results that are difficult to interpret.

Step 2: Primer Design
To analyze the methylation status of a differentially methylated region, it is recommended to design several MS-HRM assays for each region of interest. After assessing specificity and efficiency, the best performing assay is selected for the analysis of biological samples.

In an ideal situation, a PCR primer pair amplifies the methylated as well as non-methylated fragment with equal efficiency. In reality, PCR reactions have a PCR bias and often amplify the non-methylated fragment more efficiently than the methylated fragment [7]. This PCR bias limits the resolution of an HRM reaction with low levels of methylation.

An HRM reaction without PCR bias has a uniform resolution from 0% to 100% methylation. However, to detect low levels of methylation, an assay should have a higher resolution between 0% and 2%. This can be achieved by including CpG dinucleotides in the PCR primer sequence and deliberately shifting the PCR bias to preferentially amplify the methylated sequence [8]. While this allows for the detection of very low levels of methylation, the detection of higher levels of methylation will be compromised. Other factors affecting the primer design strategy include the lengths of the amplification product and the number


Figure 1. Applied Biosystems Methylation-Sensitive High Resolution Melting Workflow.
of methylation sites included in the fragment. The Applied Biosystems Methyl Primer Express® software gives researchers the option to specify the number of CpG dinucleotides to be included in the PCR primers as well as their position.

Successful MS-HRM assays have been published with amplicon sizes between 100 to 200 bps and up to 16 methylation sites. The higher the density of methylation sites, the larger the T_m difference between the methylated and non-methylated amplicons. However differentially methylated regions can display heterogeneous methylation, i.e. only some methylation sites are methylated. For PCR fragments derived from heterogeneously methylated sequences it is difficult to calculate accurate methylation values. The larger the number of methylation sites included in an amplicon, the higher the chance that the sequence is heterogeneously methylated in a biological sample.

We designed PCR primer pairs with and without CpG dinucleotides to obtain a high resolution between 0–2% and 0–100%, respectively. The T_m values were between 58°C and 62°C, the range of fragment sizes was between 100 and 200 bps, and 3 to 22 methylation sites were included in the amplicons. DNA samples were purchased from the Coriell Institute for Medical Research, Camden, NJ. 300 ng of genomic DNA was bisulfite-treated according to the manufacturers recommendations. DNA was eluted with 50 µL TE, resulting in DNA that is 100% methylated. Scientists often prepare DNA from blood mononuclear cells as non-methylated DNA samples.

To represent non-methylated DNA, we selected the human colon adenocarcinoma cell line HT29 in which the MT1A and the RASGRF1 promoters are non-methylated. 100% methylated and 0% methylated DNA of equal concentration were then mixed in different ratios to mimic DNA samples with defined levels of DNA methylation.

The reaction amplifies a 110 bp fragment from the Ras protein-specific guanine nucleotide-releasing factor 1 (RASGRF1) promoter containing 9 CpG dinucleotides. The reaction amplifies a 163 bp fragment from the metallothionein 1A (MT1A) promoter containing 13 CpG dinucleotides. For the data shown in Figure 4, a PCR primer pair containing 3 CpG dinucleotides was chosen. The reaction amplifies a 110 bp fragment from the Ras protein-

**Step 4. MS-HRM Standard Curve and Data Analysis**

To determine the MS-HRM standard curves, melting profiles need to be established for known levels of DNA methylation. Universally methylated DNA is commercially available and represents DNA that is 100% methylated. Scientists often prepare DNA from blood mononuclear cells as non-methylated DNA samples.

To represent non-methylated DNA, we selected the human colon adenocarcinoma cell line HT29 in which the MT1A and the RASGRF1 promoters are non-methylated. 100% methylated and 0% methylated DNA of equal concentration were then mixed in different ratios to mimic DNA samples with defined levels of DNA methylation.

**Methyl Primer Express® Software v1.0**

The Applied Biosystems Methyl Primer Express Software v1.0 is a complimentary online primer design tool specifically for methylation studies which assists in designing primers for both methylated and unmethylated bisulfite-modified DNA. The essential steps in designing PCR primers amplifying fragments from bisulfite converted DNA are substantially automated with Methyl Primer Express software. Users simply cut and paste in the selected genomic sequence. The software then performs an in-silico bisulfite conversion (Cs are converted to Ts) and aids in the selection of primers. Visit www.appliedbiosystems.com/methylprimerexpress
For the MS-HRM reaction analyzing the MT1A promoter, standard curves were established for 100%, 75%, 50%, 25%, 10% and 0% methylation. These primers contain no CpG dinucleotides and no PCR bias could be detected when comparing the Cₜ values of 100% and 0% methylated samples of equal concentrations. MS-HRM data were collected using the 7500 Fast Real-Time PCR System and analyzed with the Applied Biosystems High Resolution Melting v1.0 Software that converts melting curves into distinct melting profiles. Different levels of methylation result in clearly distinguishable melting curves (Figure 3). These MS-HRM standard curves were then used to estimate the level of methylation in cell line DNA samples (Figure 5).

The PCR primers amplifying a fragment from the RASGRF1 promoter hybridize to a region containing 3 CpG dinucleotides. The primer sequences are a perfect match for the methylated promoter and the PCR bias can be verified by comparing the Cₜ values of 100% and 0% methylated samples of equal concentrations. This assay design overamplifies the methylated sequence and we therefore established HRM standard curves for 100%, 10%, 5%, 2%, 1%, 0.5%, 0.1% and 0.0% methylation. By adjusting the annealing temperature of the PCR reaction, one can increase or decrease the extent of the PCR bias. For a good resolution between 0.0% and 2.0% methylation, the optimal PCR annealing temperature in this study was 63°C (Figure 4). MS-HRM reactions with a PCR bias towards the methylated sequence require more converted DNA to ensure that the non-methylated amplicons are efficiently amplified (Cₜ for 0.0% methylated DNA < 30).

Figure 4 shows the MS-HRM standard curves for the RASGRF1 promoter with a PCR annealing temperature of 63°C. These two examples illustrate how the nature of a methylation study determines the optimal primer design strategy.

**Data Analysis**

After establishing the MS-HRM standard curves, we analyzed the MT1A promoter in the four human tumor cell lines NCI-H526, NCI-H358, NCI-H69 and LNCaP. Figure 5 shows the MS-HRM curves of these four cell lines, together with the MS-HRM standard curves for 100% (red) and 0% (black) methylation, respectively. The melting profiles of NCI-H358 (blue) and NCI-H526 (purple) closely resemble the 100% and 0% HRM standard curves. The MT1A promoter is ~85% methylated in NCI-H69 cells (green), and 75% methylated in LNCaP cells (yellow).

**A Complete Solution**

Cytosine methylation is an important DNA biomarker for the study of development and disease. MS-HRM is a simple and cost-effective method to analyze the methylation level of differentially methylated regions like CGIs in gene promoters.

Applied Biosystems offers a complete solution for MS-HRM analysis. The methylSEQr Bisulfite Conversion Kit produces pure, high quality bisulfite-converted genomic DNA. Methyl Primer Express 1.0 assists in the design of PCR primers targeting bisulfite-converted genomic DNA. The 7500 Fast Real-Time PCR System enables HRM analysis as well as fast PCR cycling conditions that are crucial for the performance and specificity of a PCR with converted genomic DNA as template. Finally, the High Resolution Melting software allows researchers to interpret and present MS-HRM data in an easy and intuitive way.

![Figure 6. Applied Biosystems Provides a Complete Solution for Each Step of Successful High Resolution Melting Experiments.](image-url)
**Figure 5. MS-HRM Analysis of Cell Line DNA.** The methylation level of the MT1A promoter in the cell lines NCI-H358 (blue), NCI-H69 (green), LNCaP (yellow) and NCI-H526 (purple) was analyzed by MS-HRM analysis. The legend indicates the color associated with a specific cell line. 100%(red) and 0%(black) methylated DNA is shown as well. The methylation level of the MT1A promoter can be estimated by comparing the melting curves of the cell lines with the standard curves. As in Figure 3, MS-HRM curves were generated using a CpG-free PCR primer pair amplifying a 163 bp fragment of the MT1A promoter that contains 13 CpG dinucleotides.

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**ORDERING INFORMATION**

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**REFERENCES**


**7500 Fast Real-Time PCR System**

The Applied Biosystems 7500 Fast Real-Time PCR System is a powerful platform for laboratories requiring superior performance and maximum dye versatility. The Applied Biosystems family of real-time solutions combine innovative thermal cycling systems, powerful software, and optimized reagents.

**High Resolution Melting Software**

Use the Applied Biosystems High Resolution Melting (HRM) Software to perform more sophisticated melting analysis with an easy-to-follow workflow and minimal subjective data analysis steps with the flexibility to analyze a variation of HRM applications including methylation studies.

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