

Methylation Analysis Using Methylation-Sensitive HRM and DNA Sequencing

Abstract

DNA methylation is a key epigenetic mechanism that regulates gene expression and chromatin structure. This application note describes a workflow for analysis of DNA methylation in specific loci by methylation-sensitive high-resolution melting (MS-HRM) followed by DNA sequencing of the reaction products (Figure 1). The workflow relies on Applied Biosystems® real-time PCR and capillary electrophoresis instruments and is supported by a comprehensive set of reagents and software tools. MS-HRM followed by Sanger-based DNA sequencing is a fast, simple method for methylation studies that can be manipulated to provide sensitivity for methylation levels as low as 0.1–2%.

Procedure Overview

MS-HRM analysis offers a simple method for quickly analyzing the methylation status of specific genetic loci. Reaction products that merit further analysis can then be sequenced directly to identify precise methylation patterns. Genomic DNA is treated with bisulfite to convert unmethylated cytosines to uracil,

then PCR primers are designed to amplify the region of interest, and the melting profiles of PCR products from the sample DNA are compared to those from fully methylated and unmethylated reference samples. PCR primers and amplicons are designed to provide the level of sensitivity required for the biological question of interest. Samples that are found to warrant further study are sequenced to identify precise methylation patterns. Applied Biosystems provides an integrated set of tools for locus-specific DNA methylation analysis using this optimized, streamlined procedure. The procedure is described in a general way in this application note. For detailed instructions, see the High Resolution Melting Getting Started Guide (P/N 4393102) and the BigDye® Terminator v1.1 Cycle Sequencing Kit Protocol (P/N 4337036).

Step 1: Bisulfite Conversion of DNA

The first step of the workflow is to treat genomic DNA with bisulfite, which deaminates unmethylated cytosines (C) to form uracil (U), but does not affect methylated cytosines.

Thus, bisulfite conversion changes the DNA sequence based on the methylation status of individual nucleotides in genomic DNA, and these changes can be detected via HRM analysis [1]. The Applied Biosystems® methylSEQr® Bisulfite Conversion Kit permits complete conversion of unmethylated cytosine to uracil in reactions that minimize DNA damage and maximize unbiased recovery of DNA.

Step 2: Primer Design

PCR primer design for MS-HRM is critical—the free online tool from Applied Biosystems, Methyl Primer Express® Software v1.0, was developed specifically for primer design in methylation studies (www.appliedbiosystems.com/methylprimerexpress). We recommend designing several sets of PCR primers for each genomic DNA region of interest, testing them for specificity and the ability to discriminate methylated from unmethylated DNA, and selecting the best-performing assay(s) for analysis of experimental samples. There are three main criteria to consider in designing

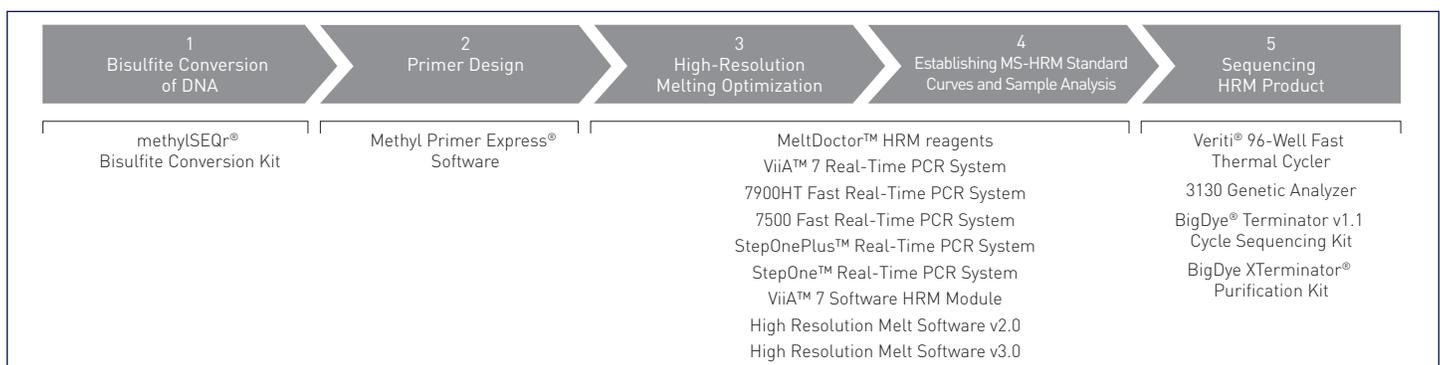


Figure 1. Applied Biosystems Workflow for Methylation-Sensitive High-Resolution Melting (MS-HRM) Analysis Followed by DNA Sequencing.

methylSEQR® Bisulfite Conversion Kit

The Applied Biosystems® methylSEQR® Bisulfite Conversion Kit is a robust method for generating high-quality bisulfite-converted DNA for optimized methylation analysis. The kit uses size exclusion column purification to increase sample yields and reduce the amount of bias seen with resin purification methods.

PCR primers: amplicon length, number of potential methylation sites in the amplicon, and inclusion of CpG dinucleotides in the PCR primer sequence. All of these factors can have a big influence on the sensitivity of the assay, and in some cases they can be manipulated to discriminate large or small differences in methylation states.

Amplicon Length and Number of Methylation Sites

The greater the number of potential methylation sites in an amplicon, the larger the T_m difference between PCR products from highly methylated and unmethylated DNA samples. This makes it easy to distinguish melt profiles; however, it may also contribute to PCR bias. Including too few CpG dinucleotides, on the other hand, may produce melt profiles that are too similar to be useful for methylation analysis. PCR bias is based on the observation that, in general, DNA fragments with high C content are not amplified as efficiently as fragments with high U/T content. After bisulfite conversion, unmethylated stretches of DNA will be significantly enriched for U/T nucleotides and may be amplified to the exclusion of their methylated counterparts [2]. PCR bias is very hard to predict; in some cases it is directly related to the number of CpG dinucleotides in the amplicon, but not always. In addition, methylation may be heterogeneous, i.e., only some potential sites are actually methylated. It is difficult to calculate accurate methylation values for fragments derived from heterogeneously methylated sequences.

The optimal number of potential methylation sites in amplicons must be determined empirically because it varies significantly among different genomic regions, for reasons that are not well understood. Successful MS-HRM assays have been published with 100–200 bp amplicons and up to 16 methylation sites.

CpG Dinucleotides in PCR Primers

When maximum sensitivity of methylation detection is needed, PCR primer sequences can be designed to include CpG dinucleotides. Since unmethylated DNA will contain mismatches to the PCR primers after bisulfite conversion, methylated fragments that match perfectly will be significantly favored in PCR [3]. When the CpG is placed near the 3' end of the PCR primer, specificity for methylated DNA will be even greater. Finally, the PCR bias can be tipped even further in favor of methylated DNA fragments by increasing the annealing temperature in the PCR to discourage amplification of mismatched primers (Figure 2). While this strategy enables detection of very low levels of methylation, the ability to discriminate differences in higher levels of methylation will be compromised. Applied Biosystems® Methyl Primer Express® Software provides options to specify the number of CpG dinucleotides to be included in the PCR primers as well as their positions.

PCR Primers Designed for This Study

We designed PCR primer pairs with CpG dinucleotides for sensitive detection of methylation in the 0% to 2% range, and without CpG dinucleotides for more uniform detection of widely ranging methylation status from 0% to 100%. Primer T_m values were 58–62°C, and

the range of amplicon sizes was 100–200 bp and included 3–22 potential methylation sites.

Step 3: MS-HRM Optimization

The goal of optimization is to identify the best PCR primer set and PCR cycling conditions for the differentially methylated region of interest. It is good practice to evaluate PCR reactions by real-time PCR, MS-HRM, and electrophoresis on high-percentage agarose gels. In real-time PCR, C_t values should be in the range of 8–30, and fluorescence should increase exponentially through the exponential phase of PCR. MS-HRM melt profiles from 0% and 100% methylated genomic DNA standards should exhibit significant differences. Finally, run the products on an agarose gel to verify that a single product of the expected size is amplified, indicating specificity for the target of interest.

Note that in some cases, adjusting annealing (or annealing/extension) temperatures in the PCR can maximize sensitivity for methylated DNA. This is illustrated in Figure 2, which shows amplification of a 110 bp fragment of the Ras protein-specific guanine nucleotide-releasing factor 1 (RASGRF1) promoter. To maximize assay sensitivity for methylation, a PCR primer pair containing 3 CpG dinucleotides was chosen. The amplicon in this case contains 9 CpG dinucleotides. Raising the annealing temperature from 60°C to 64°C maximized assay sensitivity for methylation levels in the 0–10% range.

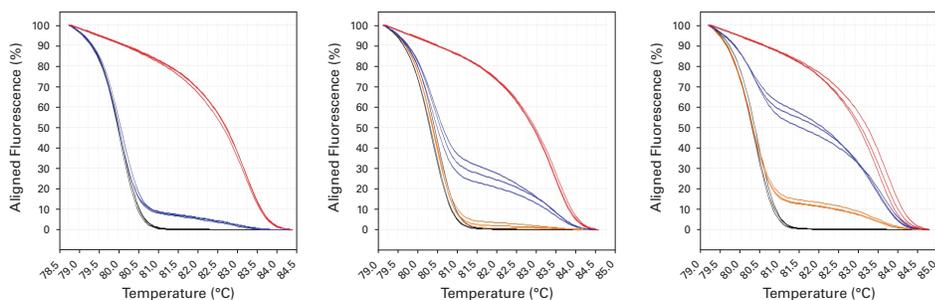


Figure 2. Raising the Annealing Temperature Can Greatly Increase the Sensitivity of the Assay When PCR Primers Contain CpG Dinucleotides. 100% methylated and 0% methylated DNA of equal concentration were mixed to mimic DNA samples with 1% and 10% methylation levels. Triplicate samples of each DNA were subjected to HRM analysis using MeltDoctor™ HRM reagents on the Applied Biosystems® 7500 Fast Real-Time PCR System. The PCR primers contain 3 CpG dinucleotides, and they amplify a 110 bp fragment of the RASGRF1 promoter containing 9 CpG dinucleotides. The annealing temperature of the PCR was increased to maximize sensitivity of the assay for methylation levels between 0% and 10%.

Step 4: MS-HRM

Standard Curve and Data Analysis

MS-HRM analysis is based on the comparison of melt profiles of experimental samples to profiles from DNA with known methylation levels. Universally (or 100%) methylated DNA is commercially available. For a source of unmethylated DNA, scientists often isolate DNA from blood mononuclear cells. For this study, DNA from human colon adenocarcinoma cell line HT29 was used as a source of unmethylated DNA because the promoters evaluated are known to be 0% methylated in this cell line. 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.

Methylation in the MT1A Promoter

To evaluate methylation in the metallothionein 1A (MT1A) promoter, a PCR primer pair without CpG dinucleotides that amplifies a 163 bp fragment containing 13 CpG dinucleotides was chosen. MS-HRM standard melt profiles were generated and used to estimate the level of methylation in 4 human tumor cell lines (Figures 3, 4). Figure 3 illustrates that different levels of methylation resulted in clearly distinguishable melting curves. The data in Figure 4 indicate that differences in MT1A promoter methylation can be clearly identified in the 4 cell lines evaluated. Reactions used Applied Biosystems® MeltDoctor™ HRM Master Mix, and were amplified and subjected to HRM using the Applied Biosystems® 7500 Fast Real-Time PCR System. Analysis was performed with High-Resolution Melt Software v2.0.

RASGRF1 Promoter Analysis

For methylation analysis of the RASGRF1 promoter, PCR primers were chosen to maximize detection of extremely low levels of DNA methylation; they 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 C_t values of 100%, 10%, and 0% methylated samples of equal concentrations. We established MS-HRM melt profiles to evaluate methylation levels below 2% by adjusting the annealing temperature of the PCR reaction to 63°C. Note that MS-HRM reactions set up to preferentially amplify

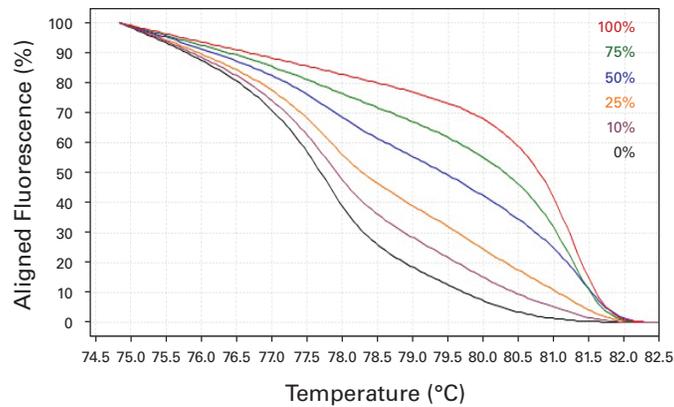


Figure 3. MS-HRM Standard Curves With Uniform Resolution From 0% to 100% Methylation. 100% methylated DNA was mixed in different ratios with DNA from HT29 cells, in which the MT1A promoter is 0% methylated. The MS-HRM curves (100%, 75%, 50%, 25%, 10%, and 0% methylation) were generated with a 163 bp PCR fragment containing 13 CpG dinucleotides.

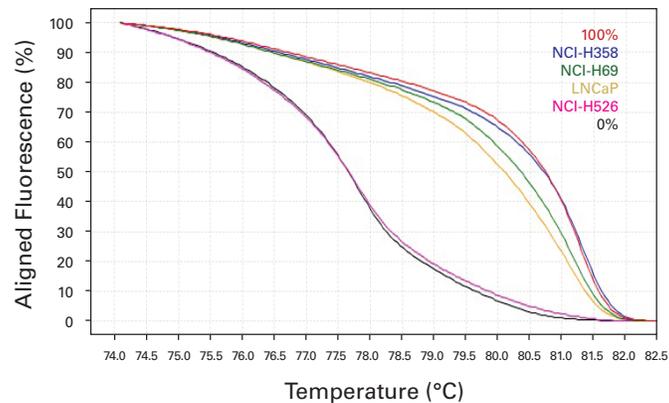


Figure 4. MS-HRM Analysis of Cell Line DNA. The methylation level of the MT1A promoter in the cell lines NCI-H358, NCI-H69, LNCaP, and NCI-H526 was analyzed by MS-HRM analysis. 100% and 0% methylated DNA are shown as well. The methylation level of the MT1A promoter can be estimated by comparing the melting 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.

MeltDoctor™ HRM Reagents

Applied Biosystems® MeltDoctor™ HRM reagents were developed for high-resolution melt analysis. They employ MeltDoctor™ HRM dye, a stabilized form of the SYTO®-9 dye—a next-generation dsDNA-binding dye developed by Molecular Probes that is designed to deliver sharp, clean melt profiles. Simplest to use is the MeltDoctor™ HRM Master Mix; it requires only the addition of template DNA and a PCR primer pair before starting the PCR. When more flexibility in reactions is needed, the MeltDoctor™ HRM Reagent Kit provides enzyme, fluorescent dye, and dNTP mix separately. Both reagent sets employ hot start-enabled DNA polymerase, minimizing nonspecific product formation and enabling reactions to be set up at room temperature.

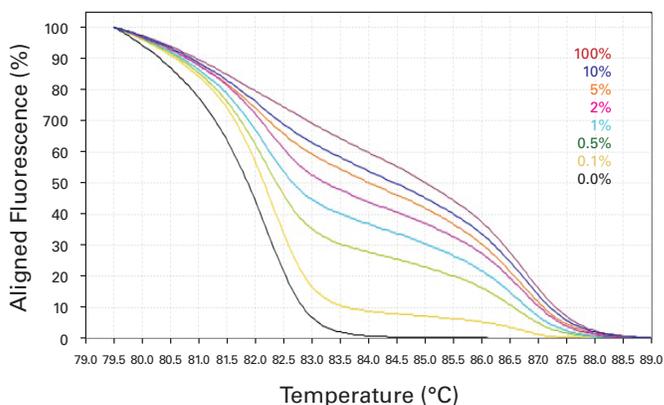


Figure 5. MS-HRM Standard Curves With High Resolution Between 0 and 2%. 100% methylated DNA was mixed in different ratios with DNA from HT29 cells, in which the RASGRF1 promoter is 0% methylated. The standard curves for methylation were generated with a 110 bp PCR fragment containing 9 CpG dinucleotides.

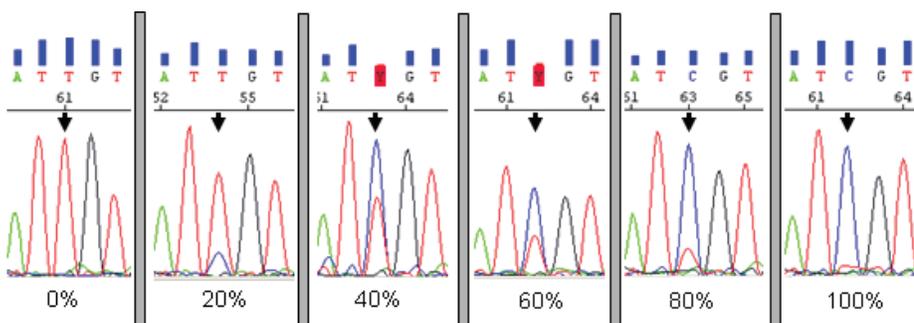


Figure 6. Sequence Data From HRM Products. Detection of the methylation status for one CpG dinucleotide by sequencing DNA samples with known methylation status.

methylated sequence require more input DNA to ensure that the unmethylated amplicons can be amplified with a C_t value <30. Figure 5 shows the MS-HRM standard curves for the RASGRF1 promoter with a PCR annealing temperature of 63°C.

Step 5: Sequencing the HRM Product

The final step in methylation analysis is to sequence the amplified PCR products to confirm overall methylation levels and pinpoint the positions of the methylated C residues. First, melt profiles for a region of the death-associated protein kinase 1 (DAPK1_1) promoter were generated by mixing 100% and 0% methylated DNA standards (Figure 6). As in previous experiments, DNA from the human colon adenocarcinoma cell line HT29 was used as a source of unmethylated DNA.

PCR products from the MS-HRM analysis were diluted 1:100 and sequenced directly using the BigDye® Terminator v1.1 Cycle Sequencing Kit and Fast cycle sequencing on the Veriti® 96-Well Fast Thermal Cycler. Because the DNA concentration in reaction products from HRM analysis is typically fairly high due to the relatively small amplicon size, dilution is necessary prior to DNA sequencing. Dilution ratios >1:20 reduce the concentrations of leftover PCR primers and dNTPs to levels that do not interfere with DNA sequencing reactions.

Sequencing reactions were cleaned up using the BigDye® XTerminator® Purification Kit and run on the Applied Biosystems® 3130 Genetic Analyzer using the 36 cm capillary array and POP-6™ polymer. Figure 6 depicts sequencing

data for one methylation site in DNA standards representing the indicated methylation levels.

A Complete Solution

Cytosine methylation of genomic DNA is known to have a significant impact in development and disease. There is also increasing interest in identification of DNA methylation-based biomarkers. MS-HRM is a simple and cost-effective method to analyze differentially methylated regions such as CGIs in gene promoters. The reaction products can then be sequenced to validate results and identify exact patterns of methylation.

Applied Biosystems offers a complete solution for MS-HRM analysis. The methylSEQR® Bisulfite Conversion Kit enables the user to obtain pure, high-quality bisulfite-converted genomic DNA. Methyl Primer Express® 1.0 Software is available at no charge on our website; it assists in the design of PCR primers targeting bisulfite-converted genomic DNA. The MeltDoctor™ HRM suite of reagents provides controls and PCR mixes designed to deliver sharp, clean melt profiles.

The StepOne™, StepOnePlus™, 7500 Fast, 7900HT Fast, and ViiA™ 7 Real-Time PCR Systems enable HRM analysis as well as precise PCR cycling conditions that are crucial for the performance and specificity of PCR with converted genomic DNA as a template. The High Resolution Melting software makes it easy to interpret and present MS-HRM data in an intuitive way. HRM is a nondestructive process; thus, HRM reaction products can be sequenced directly—typically, no additional amplification or purification steps are needed prior to sequencing. To further accelerate your time to results, the Applied Biosystems® fast resequencing system enables single-day DNA sequencing results.

Real-Time PCR Systems for HRM

ViiA™ 7, 7900HT Fast, 7500 Fast, StepOnePlus™, and StepOne™ Real-Time PCR Systems are ideal for HRM analysis applications. These instruments:

- Enable real-time PCR and high-resolution sample melt on a single instrument
- Perform an optimized, short HRM melt window to decrease time-to-results

ViiA™ 7 Real-Time PCR System

- Save time by not having to import your data into a standalone HRM software package
- Select the number of data points collected per degree of melt increment for ultimate flexibility
- Manually set the number of variants to optimize your results

7900HT Fast Real-Time PCR System

- Hands-free 96- and 384-well robotic plate loading and unloading for true walk-away automation

7500 Fast Real-Time PCR System

- Collect data in a single channel to maximize the data points acquired
- Variable excitation capacity for optimum excitation of a full range of dyes

StepOnePlus™ and StepOne™ Real-Time PCR Systems

- Continuous collection of melt curve data balances speed and resolution, and reduces the amount of time required to obtain high-quality HRM data

High Resolution Melt (HRM) Software

- No temperature-shift correction is needed—the result: clear distinction of mutant and wild type samples
- Autocalling of genotypes and automatic omission of no-template controls for rapid analysis
- Automatic grouping of unknown variant clusters minimizes subjective analysis
- Advanced Assay Settings Library for use on routine assays

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