Application Note

Quantifying CpG methylation using MS-HRM qPCR

Pedro Quintas PhD & Constantine Garagounis PhD

Introduction

DNA methylation is an epigenetic process through which nucleobases (mainly cytosines) are modified by the addition of a methyl group. This mechanism has essential roles in cellular processes including regulation of gene expression, development, and disease. In this application note we describe a workflow for the analysis of DNA methylation in specific loci using methylation-sensitive high-resolution melting (MS-HRM) qPCR.

This method relies on the treatment of DNA with sodium bisulfite [1], which converts unmethylated cytosines to uracils and leaves methylated cytosines unchanged. These uracils are then replaced by thymines during PCR amplification (Figure 1). Amplicons originating from methylated DNA templates therefore contain a higher number of the more stable C-G base pairs than those originating from unmethylated ones. This results in an increased melting temperature of methylated targets.

This shift in melting temperature is determined using high-resolution melting (HRM) qPCR [2]. During this process an intercalating dye present in the mix binds to double-stranded DNA produced during the PCR and generates fluorescence. Post amplification, the reaction mix is exposed to increasing temperature in order to generate a melting profile. As the temperature reaches the product melting point the DNA strands dissociate, the dye is released, and a sharp decrease in the fluorescence is observed allowing the accurate estimation of the product's melting temperature. How much a products' melt point is affected by bisulfite treatement is an indicator of how many methylated cytosines it contains.

The main advantage of this method is the adaptability to high throughput workflows, thus providing a cost-effective, quick, and accurate way of measuring the methylation level of many samples simultaneously.

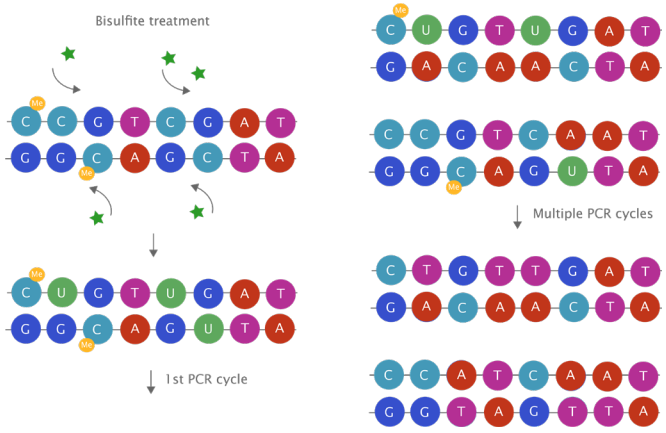

Figure 1: Bisulfite treatmend of DNA converts unmethylated cytosines to uracil

During PCR, uracil acts as a template for adenines to be incorporated in the nascent DNA strands. In subsequent cycles, these adenines act as a template for thymine incorportation in positions corresponding to the unmethylated cytosines in each strand. After multiple PCR cycles, all unmethylated cytosine-guanine basepairs are replaced with thymine-adenine base pairs.

Method

Using PCR Biosystems Clara™ HRM Mix and a thermocycler capable of performing HRM experiments we can measure the level of methylation in DNA samples. In this application note we describe the experimental conditions used to determine the level of methylation in the human FN3K gene.

Primer design

Primer selection is critical for MS-HRM (methylation-sensitive HRM) experiments. When developing a new assay, it is recommended that several primer sets are tried before selecting the ones that give better results. For this application we used a set of published primers (see Table 1). When designing primers for a new assay a few considerations should be kept in mind:

1. The amplicon should be around 100 bp and contain 3-10 CpG sites between the primers. The higher the number of methylation sites the greater the Tm difference between methylated and unmethylated sequences. However, if the number of methylation sites is too big, it may lead to PCR bias towards one of the amplicons.
2. Design primers with a melting temperature between 56–60 °C and within 1-2 °C of each other. Try to keep the GC content between 35-65%.
3. Include one or two CpG sites close to the 5' end of each primer.
4. Primers should be complementary to the methylated template, after bisulfite treatment. Thus, they should contain T in the place of any non-methylated C residues.

FN3K target fragment:	
GTTCGTGGGGTTGTTTTTAGGGTTAGACGTACGGCGAGTTGTG-TACGGACGTTTTTGCGGCGATTTAGGGGAAACGTACGCGGAT-TCGGTAGTTAAGGTTAAAGTCGGCG	
Primer name	**Primer sequence**
FN3K Forward	GTTCGTGGGGTTGTTTTTAGGG
FN3K Reverse	CGCCGACTTTAACCTTAACTAC

Table 1: Target sequence (after bisulfite treatment) and primers used in this MS-HRM assay

Cytosines in CpG sites are marked in magenta and thymines that originate from non-CpG cytosines upon bisulfite treatment are marked in turquoise. The primer locations are underlined. Adapted from [3].

Template preparation

To generate the standard curve, methylated and unmethylated DNA (commercially available) of equal concentration were mixed in different proportions, to cover the desired range. In this case we wanted to cover the full range of methylation, so we prepared samples corresponding to 0%, 20%, 40%, 60%, 80%, and 100% methylation. 20 µL of each sample were then treated with bisulfite using the Promega MethylEdge® Bisulfite Conversion System, which deaminates unmethylated cytosines (C) to form uracil (U) but does not affect methylated cytosines. The same treatment was also performed on the samples to be tested.

Reaction setup

Reactions were set up using a Qiagen QIAgility robot as reported in table 2, with a final volume of 20 µL.

Reagent	Volume	Final concentration.
2x Clara HRM Mix	10 µL	1x
Forward primer (10 µM)	0.8 µL	400 nM
Reverse primer (10 µM)	0.8 µL	400 nM
DNA template (1 ng/µL)	5 µL	0.25 ng/µL
PCR grade H_2O	3.4 µL	-

Table 2: Reaction setup and composition

Cycling conditions

Thermocycling was carried out in a Roche LightCycler 96 qPCR machine with the cycling parameters outlined in Table 3. The annealing temperature is a crucial parameter for MS-HRM experiments and needs to be determined empirically. The goal is to obtain good separation of the melt curves at each methylation level. Increasing the temperature results in more specific binding of the primers to the methylated template (assuming they are designed to include a CpG methylation site), thereby achieving a better separation of the templates with a lower level of methylation. Care must be taken not to lower the annealing temperature too much, as there's an increased risk of primer-dimer formation.

Cycles	Temperature	Time
1	95 °C	2 minutes
45	95 °C 60 °C 72 °C	15 seconds 20 seconds 10 seconds
High resolution melt	Refer to instrument instructions	-

Table 3: Cycling conditions

Results

The results were analysed using the HRM functionality of the instrument software, which normalises the start and end fluorescence level and converts the melt curves into melt peaks (Figure 2). The maximum fluorescence of each peak was then plotted against the level of methylation to generate a standard curve (Figure 3), which can be used to estimate the level of methylation of the test samples (Table 4).

The PCR products can be further analysed by sequencing to confirm the level of methylation and to identify the cytosine residues that are methylated.

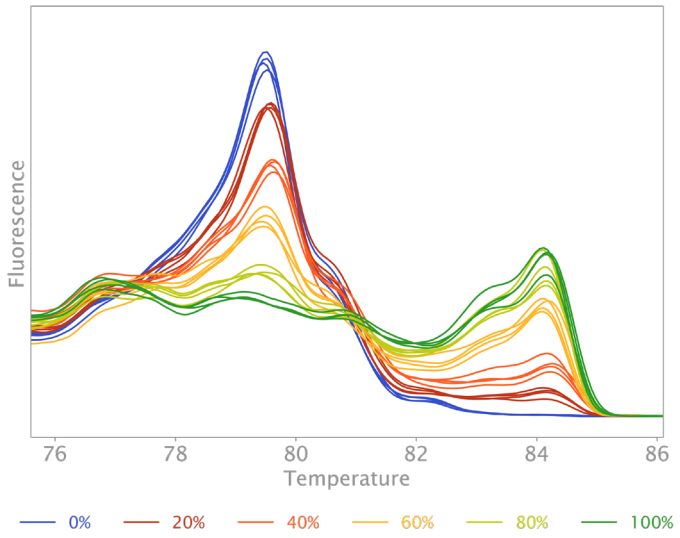

Figure 2: Melt peaks resulting from the MS-HRM analysis of FN3K gene standard curve

The graph was created using different samples with a known percentage of methylation (indicated below graph).

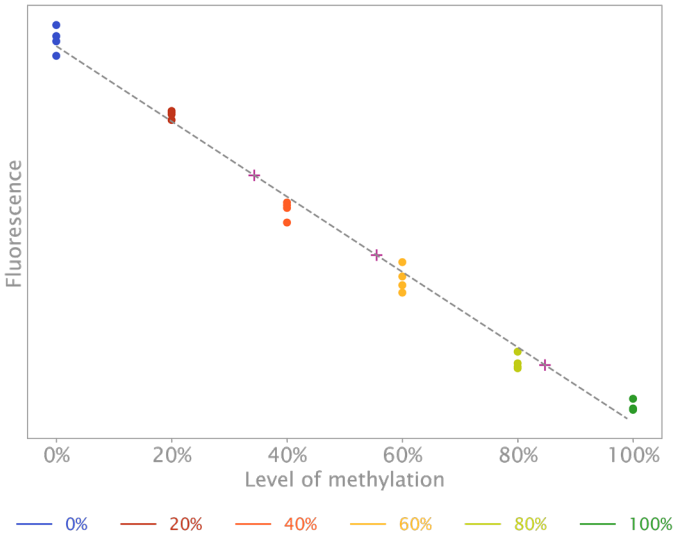

Figure 3: Quantifying % methylation

The standard curve was obtained by plotting the fluorescence of standard curve samples at 79.5 ˚C versus their known methylation percentage (indicated), while the estimated methylation of test samples is represented by + signs.

Measured Fluorescence	Calculated Methylation
0.147	84.7%
0.220	55.5%
0.273	34.3%

Table 4: Calculated levels of methylation in test samples

Discussion

Cytosine methylation of genomic DNA has a significant impact in development and disease. Methylation-sensitive high-resolution melting analysis provides a quick, sensitive, and accurate way of quantifying the level of methylation in DNA samples. PCR Biosystems Clara™ HRM Mix offers best-in-class performance for both SNP genotyping and MS-HRM.

Product use

PCR Biosystems products, including Clara™ HRM Mix, alone do not provide diagnostic results and are supplied for research use only. However, all products are manufactured under an ISO 13485-compliant management system and are suitable for use as components in molecular diagnostic assays, where applicable country laws allow and after clinical validation of an assay itself.

If you would like to discuss which products are best suited to your application or need further technical advice on how to use Clara™ HRM Mix for MS-HRM analysis, contact our team of experts at technical@pcrbio.com.

References

1. M. Frommer, L. E. McDonald, D. S. Millar, C. M. Collis, F. Watt, G. W. Grigg, P. L. Molloy and C. L. Paul, “A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands,” Proc Natl Acad Sci U S A, vol. 89, no. 5, pp. 1827-31, 1992.
2. T. K. Wojdacz and A. Dobrovic, “Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation,” Nucleic Acids Res, vol. 35, no. 6, p. e41, 2007.
3. D. Hussmann and L. L. Hansen, “Methylation-Sensitive High Resolution Melting (MS-HRM),” in DNA Methylation Protocols. Methods in Molecular Biology, vol. 1708, J. Tost, Ed., New York, NY, Humana Press, 2018.