

Rapid High-Throughput Methylation Analysis Using the LightCycler® 480 System

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The LightCycler® 480 Instrument is a versatile platform for molecular studies. We assessed the performance of the instrument combined with the LightCycler® 480 High Resolution Melting Master mix for DNA methylation analysis using the methylation-sensitive high resolution melting (MS-HRM) methodology. This proved to be a robust platform for performing MS-HRM experiments, allowing rapid and high-throughput studies of DNA methylation.

Introduction

DNA methylation is the addition of a methyl group to the 5-carbon position of cytosine preceding a guanine (a CpG dinucleotide), which results in 5-methyl cytosine. The palindromic property of the CpG dinucleotide allows semiconservative replication of methylation information by DNA methyltransferase I during cell division. Regions with relatively high CpG dinucleotide density are distributed non-randomly in the human genome, with a preference for the promoter region of genes, and are called CpG islands [1].

In cancer, there is alteration in the distribution of methylation in the genome [2]. Regions that are normally methylated become unmethylated and regions that are normally unmethylated, such as promoter-associated CpG islands, become hypermethylated. This leads to a change of chromatin structure at the affected locus, resulting in gene silencing. Genes important for tumor progression are recurrently silenced by promoter hypermethylation, such as those involved in DNA repair, cell cycle control, and apoptosis. Changes in the DNA methylation status of certain genes in a tumor will be reflected by its biology. Therefore, rapid high-throughput methods to assess DNA methylation will be of value to researchers and clinicians [3].

Methylation-sensitive high-resolution melting (MS-HRM) is a technique that has been shown to be suitable for locus-specific assessment of DNA methylation [4]. It

involves PCR amplification of the region of interest from bisulfite-modified DNA using primers that amplify both methylated and unmethylated sequences. Bisulfite modification of DNA converts unmethylated cytosine to uracil but leaves 5-methyl cytosine unchanged, allowing determination of the methylation status of the region of interest following PCR amplification. In MS-HRM, PCR reactions are performed in the presence of a saturating DNA intercalating dye, and high-resolution melting (HRM) analyses take place after amplification to determine whether the amplicons originate from methylated or unmethylated variants of the template. Differences in the melting characteristics of amplified products are reflective of the differences in methylation that exist between samples. The use of a control set of defined mixtures of methylated and unmethylated DNAs allows an estimate of the degree of methylation in the sample.

MS-HRM requires real-time PCR instrumentation that has superior temperature uniformity to precisely control melting. Here, we examine the performance of MS-HRM assays for two DNA repair genes known to be affected by promoter methylation, FANCF and MGMT, on the LightCycler® 480 Instrument using the LightCycler® 480 High Resolution Melting Master Mix.

Materials and Methods

Controls and samples

CpGenome Universal Methylated DNA (Chemicon, Millipore, Billerica, MA) was used as 100% methylated control DNA. DNA extracted from peripheral blood mononuclear cells of normal individuals was used as unmethylated control DNA. Various cell line DNA samples were used as test samples. One µg of each DNA sample was bisulfite modified using the MethylEasy Kit (Human Genetic Signatures, Sydney, Australia) according to the manufacturer's instructions and resuspended in a final volume of 100 µl. Methylation standards were constructed by diluting 100% methylated control DNA (bisulfite modified) in a pool of normal DNA (bisulfite modified) at ratios of 50%, 25%, 10%, 5%, and 1%.



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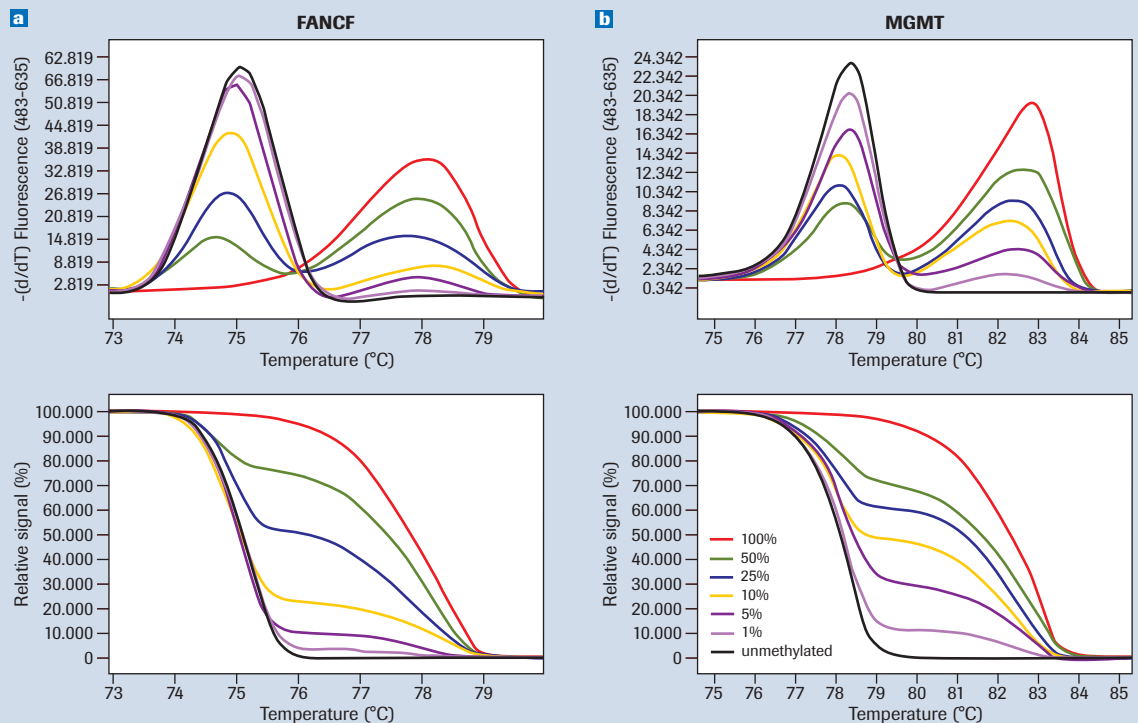


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Figure 1: (a) The FANCF MS-HRM assay with 100% methylated and unmethylated controls and methylation standards at 50%, 25%, 10%, 5%, and 1%. Data analyzed using both "Tm calling" and "Gene Scanning" software modules. **(b)** The MGMT MS-HRM assay with 100% methylated and unmethylated controls and methylation standards at 50%, 25%, 10%, 5%, and 1%. Data analyzed using both 'Tm calling' and 'Gene scanning' software modules.



MS-HRM

Primers were designed as outlined by Wojdacz & Hansen [5]. Primer sequences for the MGMT MS-HRM assay are listed in [4,5]. Primer sequences for the FANCF MS-HRM assay are available from the authors on request. Reactions were performed in 96-well LightCycler® 480 plates using the LightCycler® 480 High Resolution Melting Master mix, which contains a DNA intercalating dye, in a final volume of 10 μ l. The reaction mixture contained 1 \times LightCycler® 480 High Resolution Melting Master mix, 200 nmol/l of each primer and 1 μ l of bisulfite-modified DNA, with 2.5 mmol/l final $MgCl_2$ for FANCF and 4 mmol/l final $MgCl_2$ for MGMT. Each reaction was performed in duplicate. The cycling conditions used for both assays were as follows: SYBR Green 1 detection format; 1 cycle of 95°C for 10 minutes; 50 cycles of 95°C for 10 seconds, 58°C for 20 seconds and 72°C for 20 seconds; followed by an HRM step of 95°C for 1 minute, 50°C for 1 minute, 72°C for 5 seconds and continuous acquisition to 95°C at 30 acquisitions per 1°C.

Results and Discussion

Melting data collected using the LightCycler® 480 Instrument can be analyzed by the "Tm calling" algorithm that converts the melting profiles first into derivative plots, allowing ready distinction of methylated and unmethylated samples. Figure 1 shows that products amplified from methylated DNA have a higher Tm due to the presence of CpGs in the amplicon, whereas products amplified from

unmethylated DNA have a lower Tm due to the conversion of unmethylated C to U in the DNA sample after bisulfite treatment, which results in T in the amplicon. Where there is a mixture of methylated and unmethylated DNA two peaks are present, as seen with the methylation standards.

An alternate analysis of high-resolution melting data can be performed with the LightCycler® 480 Gene Scanning Software; it allows for correction for different fluorescence levels between samples, which arise from different amounts of PCR product after amplification. The normalized melting profiles can be directly compared and the DNA methylation level of an unknown sample can be estimated on the basis of similarities of its melting profile to standards with known methylated-to-unmethylated template ratios (Figure 1).

Both MS-HRM assays were able to detect 1% methylated DNA in a background of unmethylated DNA. Figure 1 shows that the MGMT MS-HRM assay has clearer separation of methylation standards at the lower dilutions than the FANCF assay at the primer annealing temperature (58°C) used in this experiment. As previously demonstrated, increased MS-HRM resolution of lower methylation dilutions can be achieved by increasing the primer annealing temperature [4].

Figure 2 shows various cell lines screened for methylation using the FANCF and MGMT MS-HRM assays. Figure 2 a shows that out of the nine cell lines screened for FANCF,

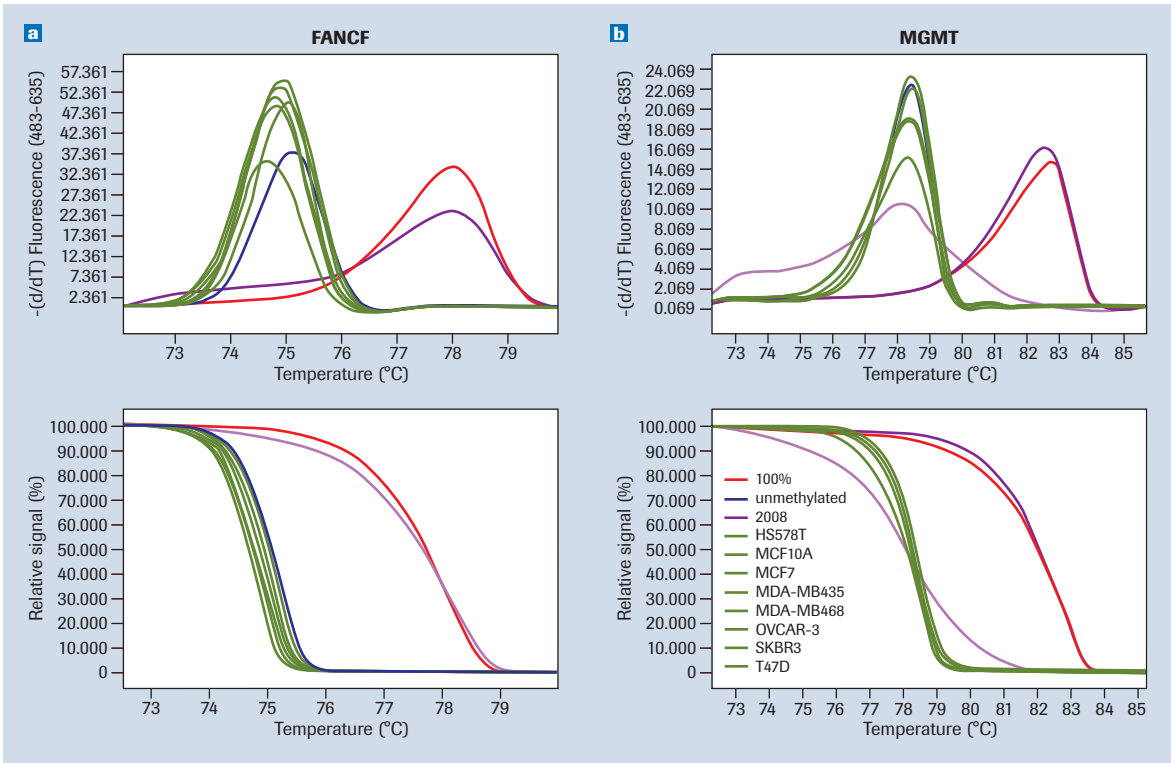


Figure 2: (a) FANCF MS-HRM assay showing the presence of DNA methylation in ovarian cancer cell line 2008. **(b)** MGMT MS-HRM assay showing the presence of methylation in breast cancer cell lines MDA-MB435 and HS578T.

only the ovarian cancer cell line 2008 showed methylation, as the melting profile for this sample resembles the 100% methylated control. All other cell lines studied were unmethylated for FANCF. Figure 2b shows that the breast cancer cell line MDA-MB435 is methylated for MGMT. The profile for this sample again closely resembles the 100% methylated control, suggesting that all CpG sites within the amplicon are methylated. The HS578T breast cancer cell line is interesting because its melt profile is distinct from both the unmethylated and methylated profiles. This profile is characteristic of heterogeneous methylation in the region examined. Because of this variable methylation, heteroduplexes are formed and this causes the PCR product to melt earlier than samples that are completely unmethylated, while the crossing over of the profile towards the fully methylated control represents homoduplexes with increased methylation levels compared with unmethylated samples.

MS-HRM allows assessment of methylation throughout the whole amplicon between the primers. As it is an closed-tube methodology, preliminary assessment of methylation can be performed very rapidly. For detailed information about methylation at specific CpG sites, bisulfite sequencing can be employed.

Conclusions

The LightCycler® 480 Instrument and the LightCycler® 480 High Resolution Melting Master mix is a robust plat-

form for performing methylation-sensitive high-resolution melting (MS-HRM) experiments, allowing rapid and high-throughput studies of DNA methylation. ■

References

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