Application Note

METHYLDETECT

High-sensitivity EpiMelt DNA methylation analysis using EpiMelt Real-Time PCR Master Mix

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Abstract

Epigenetic biomarkers are becoming increasingly relevant in the investigation of disease predisposition, screening and diagnosis, treatment guidance, and post-treatment surveillance. As the presence or absence of these epigenetic biomarkers greatly impacts the disease course and management, the sensitivity of the methylation detection methods is crucial.

The EpiMelt Methylation Detection Assays are based on the Methylation-Sensitive High-Resolution Melting (MS-HRM) technology. The unique primer design and specific annealing temperature ensure a robust and highly sensitive DNA methylation analysis.

In this application note, we show the performance of three EpiMelt Methylation Detection Assays using the EpiMelt Real-Time PCR Master Mix and demonstrate this combination to be a precise and sensitive method of methylation detection.

Introduction

DNA methylation is an epigenetic alteration of DNA, which plays an important role in the regulation of many biological processes, including gene expression and silencing, genomic imprinting, X chromosome inactivation, and genome stability[1,2]. An aberrant methylation pattern is a hallmark of cancer. Alteration of the established methylation pattern is typically an early event in carcinogenesis, leading to epigenetic silencing of tumor suppressor genes and transcriptional activation of oncogenes[1]. The methylation status at specific genes can have distinct consequences for the disease course, and epigenetic biomarkers have potential in all stages of disease. Before diagnosis, people can be predisposed to certain cancers through germline genetic and epigenetic alterations, and detection of DNA methylation at specific loci is one way to determine cancer predisposition.

Multiple studies have demonstrated *BRCA1* hypermethylation in peripheral blood predisposing to ovarian and breast cancer[3-5]. Epigenetic biomarkers have also been used for screening and diagnostic purposes.

In the age of personalized medicine, methylation biomarkers can also help guide treatment choices, thereby optimizing the effect for some patients, while sparing other patients from unnecessary side effects. In breast and ovarian cancer, the methylation status of *BRCA1* can guide the choice of PARP inhibitor treatment[7,8]. Finally, the detection of methylation biomarkers is a sensitive mode of post-treatment surveillance. In bladder cancer, monitoring methylation markers in urine improves the detection of recurrent disease and may spare bladder cancer patients from disease monitoring through more invasive, less sensitive methods[9].

The EpiMelt Methylation Detection Assays are based on the technology of Methylation-Sensitive High-Resolution Melting (MS-HRM). In MS-HRM, sequence-specific melting profiles of amplicons are observed across a gradually increasing temperature gradient in the presence of an intercalating, fluorescent dye[10,11]. The amplicons are amplified from bisulfite-converted template DNA, so amplicons from methylated template DNA will have a high GC content, and amplicons from the unmethylated template will have a low GC content. The amplification is performed using primers that match the methylated template and have a small mismatch towards the unmethylated template. This unique primer design is employed to overcome PCR bias[12]. Amplicons from the methylated template will then melt at a higher temperature than amplicons from the unmethylated template[10]. This principle allows the identification of methylation status based on the amplicon melting profile.

The EpiMelt Methylation Detection Assays include a locus-specific primer mix, and three ready-to-use controls: A Methylation Positive Control, an Assay Calibration Control, and a Methylation Negative Control. The method is semiquantitative, and the DNA methylation status of an unknown sample is interrogated by comparing the melting profile of the sample to the melting profiles of the controls[10,11].

The EpiMelt Real-Time PCR Master Mix uses the dsDNA intercalating dye EvaGreen® as a reporter. EvaGreen® is well suited for HRM, as it is a stable agent that becomes highly fluorescent when bound to dsDNA, is non-mutagenic and non-cytotoxic, and only has low levels of PCR inhibition[13]. This makes running HRM under saturating conditions possible, thereby preventing dye redistribution[14]. The use of EvaGreen® produces robust results with high fluorescence signals and prevents non-specific amplification[14,15].

In this application note, we demonstrate robust and sensitive methylation detection using EpiMelt Real-Time PCR Master Mix and the EpiMelt Methylation Detection Assays targeting *BRCA1*, *MLH1*, and *MGMT*.

Materials

Reagents and consumables

EpiMelt *BRCA1* Assay (MethylDetect ApS)
EpiMelt *MLH1* Assay (MethylDetect ApS)
EpiMelt *MGMT* Assay (MethylDetect ApS)
EpiMelt Real-Time PCR Master Mix (MethylDetect cat. # EPI – qPCR – 200)
LightCycler® 480 Multiwell plate 96, white (Roche, cat. # 04729692001)

Instruments

LightCycler® 480 System (Roche, cat. # 05015278001)

Methods

Amplification and high-resolution melting

Three EpiMelt Methylation Detection Assays were employed, targeting the promoter regions of *BRCA1*, *MLH1*, and *MGMT*. Each assay contains an assay-specific primer mix, and three assay-specific, ready-to-use controls: a methylated positive control, a methylated negative control, and an assay calibration control. The EpiMelt Real-Time PCR Master Mix is complete, already containing the optimum concentrations of salt and magnesium ions, and no additional preparation or optimization is necessary. For each qPCR reaction, the assay-specific controls were combined with 10.0 µL EpiMelt Real-Time PCR Master Mix 3.0 µL nuclease-free water, and 1.0 µL EpiMelt Primer Mix, according to the manufacturer's instructions (Table 1). No template controls were included for each assay, and all reactions were run in triplicates.

Reagent	Volume
EpiMelt Real-Time PCR Master Mix	10.0 µL
Nuclease-free water	3.0 µL
EpiMelt Primer Mix (10 µM)	1.0 µL
EpiMelt Control (5 ng/µL)	6.0 µL
Total	20.0 µL

Table 1: PCR mix.

The PCR amplification and subsequent HRM were performed on the LightCycler® 480 Instrument II in LightCycler® 96-well plates using the setting Detection format » SYBR Green I/HRM dye.

PCR program	Cycles	Temperature (°C)	Acquisition mode	Hold (sec)	Ramp rate (°C/sec)	Acquisitions (per °C)
Pre-incubation	1	95	None	600	4.4	-
Amplification	50	95	None	15	4.4	-
		BRCA1: 56	None	10	2.2	-
		MGMT: 59	None	10	2.2	
		MLH1: 60*	None	10	2.2	
		72	Single	15	4.4	-
High-resolution melting	-	95	None	15	4.4	-
		60	None	60	2.2	-
		95	Continuous	-	0.01	50

Table 2: PCR and HRM program. *The annealing temperature is assay-specific and may differ between platforms.

The LightCycler® 480 Instrument II PCR amplification and the HRM program are shown in Table 2. The listed annealing temperature is dependent on the assay and the platform. An annealing temperature of 56°C was used for EpiMelt *BRCA1*, 60°C for EpiMelt *MLH1*, and 59°C for *MGMT*. The resulting data were analyzed using the LightCycler® 480 Software.

Results

Using the EpiMelt Methylation Detection Assays and EpiMelt Real-Time PCR Master Mix resulted in efficient amplification and consistent melting profiles after HRM across the three EpiMelt Methylation Detection Assays (Figure 1). This is illustrated by the amplification curves (Figure 1A-C) and the relative signal difference (-d/dT) plots (Figure 1D-F) for each assay.

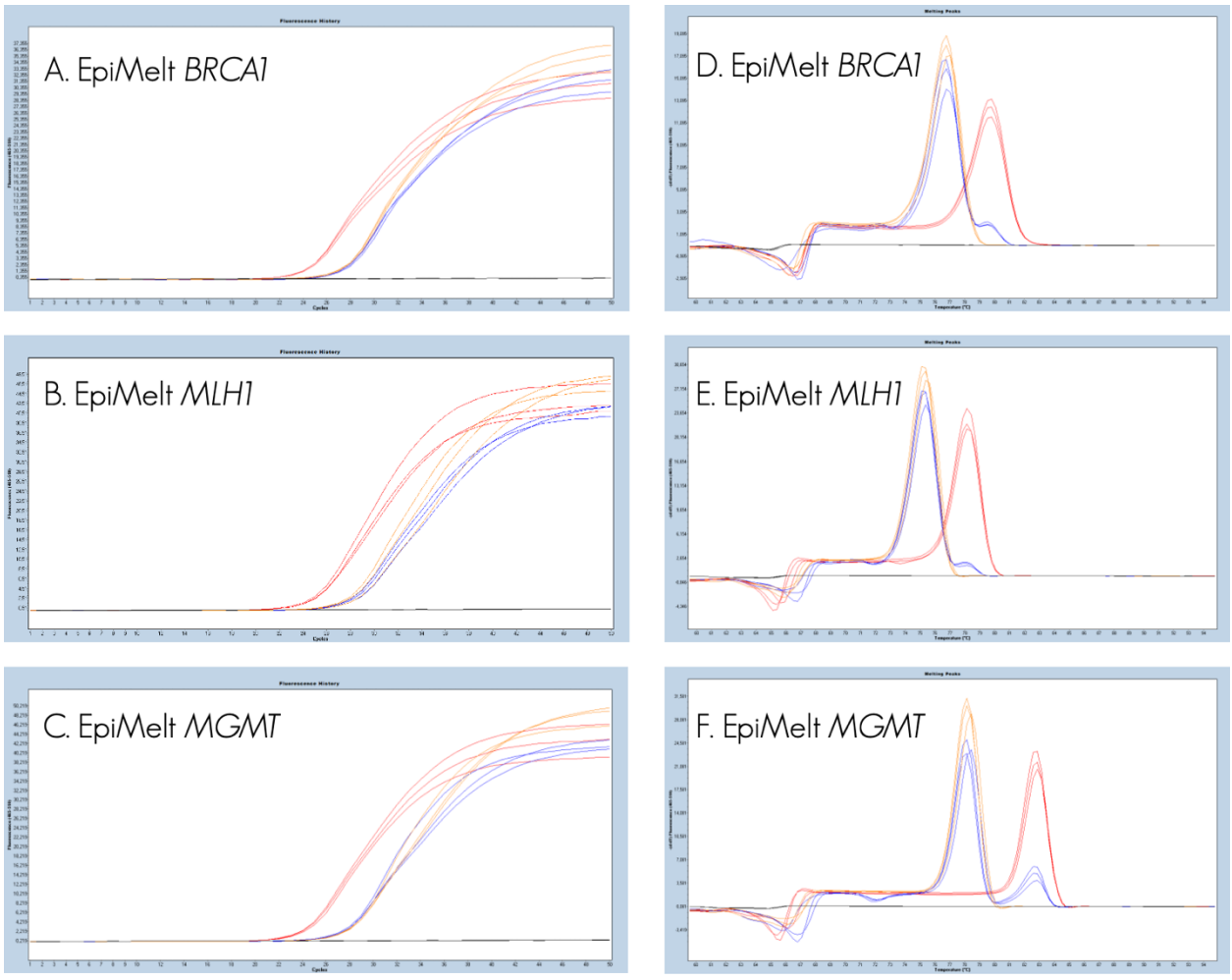

Figure 1: A-C: Amplification of the controls from (A) EpiMelt *BRCA1*, (B) EpiMelt *MLH1*, and (C) EpiMelt *MGMT* on the LightCycler® 480 Instrument II. D-F: Relative signal difference (-d/dT) plots after HRM for (D) EpiMelt *BRCA1*, (E) EpiMelt *MLH1*, and (F) EpiMelt *MGMT*. The Assay Calibration Control (blue) demonstrates the high sensitivity of the EpiMelt Methylation Detection Assays.

The amplification curves were steep and reproducible for all EpiMelt Methylation Detection Assay replicates, and the Methylation Positive Control has a lower Ct value than the Methylation Negative Control and Assay Calibration Controls. On the relative difference plots, the peaks are uniform. The extraordinary sensitivity of the EpiMelt Methylation Detection Assays is demonstrated here, as the Assay Calibration Control can be separated from the Methylation Negative Control and identified as methylated (blue curve in Figure 1).

Methylation	*BRCA1*	*MLH1*	*MGMT*
Negative	77°C	75°C	78°C
Positive	80°C	78°C	83°C

Table 3: Average Tm for the *Methylation Negative Control* and *Methylation Negative Control* from the EpiMelt *BRCA1*, *MLH1*, and *MGMT* Assays.

The EpiMelt Real-Time PCR Master Mix included a HotStart DNA Polymerase, which ensured high amplification efficiency, robust results across triplicates, and no no-template amplification.

The use of EvaGreen® DNA intercalating dye in the PCR master mix yielded high fluorescence signals, and highly specific melting profiles, allowing clear distinction between PCR products from methylated positive and methylated negative controls, and detection of DNA methylation with state-of-the-art sensitivity.

Conclusions

Here, we demonstrate the excellent performance of the EpiMelt Real-Time PCR Master Mix in combination with EpiMelt Methylation Detection Assays for robust methylation detection. The MS-HRM results allow easy evaluation of the methylation level of target loci. This application note illustrates the simplicity of methylation analysis with MethylDetect's ready-to-use EpiMelt Methylation Detection Assays, using high-quality EpiMelt Real-Time PCR Master Mix.

References

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