

OPTIMIZING DROPLET DIGITAL PCR ASSAY FOR PRECISE ASSESSMENT OF MEIS1 GENE PROMOTER METHYLATION

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Abstract

DNA methylation is characterized as a gene regulatory mechanism that involves the methylation of the 5-carbon (C5) position of cytosine, resulting in the formation of 5-methylcytosine. The analysis of aberrantly methylated cytosine-phosphate-guanine (CpG) dinucleotides, primarily in the promoter regions of tumor suppressor genes, can serve as promising prognostic and predictive markers of cancer development. Meis homeobox 1 (*MEIS1*) gene, crucial for cell growth and differentiation, exhibits dysregulation linked to various cancer types, acting as both a positive and negative regulator. The selection of an appropriate method for the evaluation of gene promoter methylation status is important for clinical implementation without biases regarding false positive and false negative outcomes. The study focuses on the optimization of a novel droplet digital PCR (ddPCR) assay for identifying the methylation status of *MEIS1*. Compared to traditional methods, ddPCR offers an increased sensitivity and specificity, presenting a promising tool for precise DNA methylation assessment with potential implications for cancer diagnostics and prognostics.

Keywords: methylation, *MEIS1*, ddPCR, primer design

INTRODUCTION

Epigenetic modifications, including DNA methylation, are heritable changes essential for normal development via the control of gene expression (1). These heritable changes are established during the cell cycle and cell division, leading to distinct identities of gene expression profiles while the primary sequences of DNA are the same. Failure in the proper maintenance of epigenetic machinery can lead to an alteration of gene expression and subsequent carcinogenesis (2). Recent evidence in the field of epigenetic analysis showed that cancer cells exhibit global epigenetic alterations in addition to various genetic changes observed during malignant transformation (3). DNA methylation is one of the most extensively studied epigenetic modifications characterized as the methylation of C5 position of cytosine, resulting in its modification into 5-methylcytosine. DNA methylation occurs mainly

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at the cytosine-phosphate-guanine (CpG) sites, which are localized in the promoter region of tumor suppressor genes. The transcription of genes initiates from the promoter regions characterized by relatively high frequencies of CpG dinucleotides. For this reason, aberrantly methylated CpG islands are currently defined as promising prognostic and predictive markers associated with cancer development (4).

Meis homeobox 1 (*MEIS1*) regulates cell growth and differentiation during vertebrate development. Many studies observed that *MEIS1* dysregulation is correlated with the development of numerous cancer types. Two documents emphasized the role of *MEIS1* in cancer development. *MEIS1* can act as a positive regulator of cancer proliferation observed in leukemia (5,6) or a negative regulator of several other solid tumors (7,8). The mentioned dysregulation of *MEIS1* expression suggests to be a consequence of the increased promoter methylation as was described in colorectal cancer (9).

Selecting the appropriate methods for the evaluation of gene promoter methylation status based on their capacity to distinguish 5-methylcytosine and nonmethylated CpG sites is the most crucial step toward subsequent implementation of the detecting method into clinical practice, particularly without biases regarding false positive and false negative results. Currently, several innovative methods and high-throughput molecular biology and genomics technologies are available to detect the degree of DNA methylation status (10). PCR-based methods have been widely used to evaluate DNA methylation status for decades. The first generation of PCR used methylation-specific-restricted enzymes to distinguish methylated and unmethylated alleles. These methods were followed by methylation-specific PCR (MSP) based on the amplification of sodium bisulfite-treated DNA and consecutively analyzing the presence or absence of converted unmethylated cytosine to uracil after the process of deamination (11). Droplet digital PCR (ddPCR) is a relatively novel method for estimating the absolute quantification of the target sequence of DNA. Recent data show that ddPCR manifests higher sensitivity and specificity than routinely used qPCR (12). In the present article, we describe the optimization of in-house developed ddPCR assay for the identification of the *MEIS1* methylation status.

MATERIAL AND METHODS

Designing methylation PCR primers and probes

Designing of ddPCR was conducted by the employment of free on-line application: The Eukaryotic Promoter Database (EPD), MethPrimer, and Primer3.

Firstly, EPD application was used (<https://epd.expasy.org/epd>) to find promoter regions of *MEIS1*. The nucleotide sequence with transcription start sites (TSS) (chr2: 66,434,560 - 66,435,484) is presented in Figure 1.



Fig. 1 Promoter region of *MEIS1* with marked TSS

Consequently, we used the commercially available software MethPrimer (<https://www.uro-gene.org/cgi-bin/methprimer/methprimer.cgi>) to perform digital bisulfite conversion of the input sequence with predicted CpG islands. Bisulfite converted promoter region with CpG islands (upstream to TSS) are shown in Figure 2.

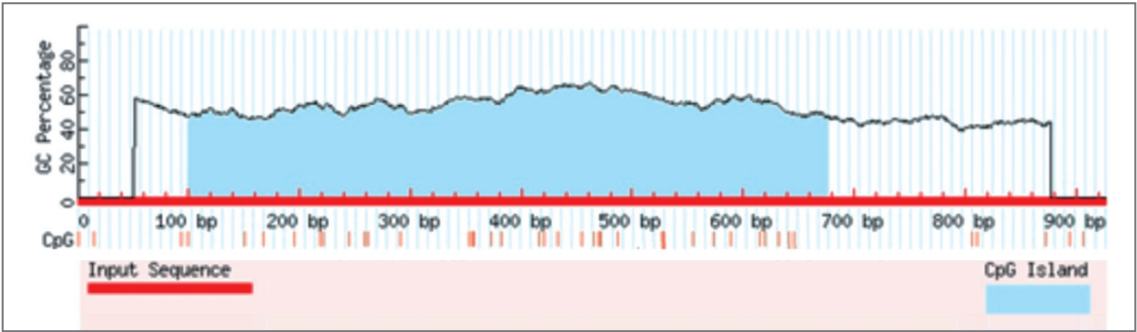


Fig. 2 CpG island prediction generated by MethPrimer software

The final step included usage of free available primer design software Primer3 (<https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>) in order to select specific methylation-independent primers (MIP) with specific methylated (labeled by FAM-BHQ1) and unmethylated (labeled by HEX-IBFQ) probes. Our designed probes targeted four nearby CpG sites (chr2:66,434,908; chr2:66,434,912; chr2:66,434,914; chr2:66,434,928) in the *MEIS1* promoter region. The primers' and probes' sequences are presented in Table 1.

Table 1 Primer and probe sequences for *MEIS1* detection

Primers	
Name	Sequence (5'→3')
MEIS1_1 Forward	TGGGGAGAGAGTTTGTAGG
MEIS1_1 Reverse	ACACAAACACCACACACC
Probes	
Name	Sequence
MEIS1_1 Methylated probe (FAM-BHQ1)	CGGTCGCGGGTTATTGTTTGC
MEIS1_1 Unmethylated probe (HEX-IBFQ)	TGGTGTTGTGGGTTATTGTTTGTGT

ddPCR analysis
Optimizing PCR conditions

The ddPCR platform (Bio-Rad Laboratories, Hercules, CA, USA) was used for the optimization of methylated (M-Probe, FAM labeled) and unmethylated (UnM-Probe, HEX labeled) probes. As a template sequence, we used commercially available fully methylated and fully unmethylated EpiTect DNA controls (Qiagen, Hilden, Germany). The reaction mix for ddPCR

contained 10 μL of Supermix for Probes (No dUTP) (Bio-Rad Laboratories), 0.45 μL of each primer, 0.45 μL of each probe, 1 μL of methylated and unmethylated control DNA adjusted with 7.75 μL of water up to a final volume of 20 μL . Subsequently, 70 μL of Droplet Generation Oil for Probes, along with the reaction mixture, was loaded into a DG8 cartridge and inserted into the QX200 Droplet Generator (Bio-Rad Laboratories) to divide each sample into 20,000 droplets. After generating oil emulsion, approximately 40 μL of the sample was transferred into 96-well PCR plates, covered with a pierceable foil, and heat-sealed by Bio-Rad's PX1 system. The first optimization step of the ddPCR assay covers estimating optimal PCR conditions. The thermal PCR consisted of enzyme activation for 10 min at 95 °C, followed by 40 cycles of denaturation for 30 sec at 94 °C, annealing/extension step for 1 min with temperature gradient, finished by one cycle of enzyme deactivation for 10 min at 98 °C. To define the optimal conditions of annealing, we performed the above-mentioned temperature gradient in the range of 50 – 62 °C. After finishing the PCR program, we loaded the plate onto the QX200 Droplet Reader (Bio-Rad Laboratories) for a final analysis.

Concentration gradient

The second optimization step was performing a concentration gradient of eight different dilutions of DNA controls to confirm the dynamic range and linearity of the method. The reaction mix for ddPCR contained 10 μL of Supermix for Probes (No dUTP) (Bio-Rad Laboratories), 0.45 μL of each primer, 0.45 μL of each probe, diluted methylated and unmethylated DNA controls in water with 8000, 4000, 2000, 1000, 500, 250, 125, and 62 copies per reaction adjusted with a variable volume (regarding to DNA input) of water up to a final volume of 20 μL . Further workflow and analysis were the same as described above.

RESULTS

Estimating optimal PCR conditions

Using commercial methylated and unmethylated DNA, we determined the best annealing temperature to be 57.1 °C for both methylated and unmethylated probes. Both DNA controls were combined with a methylated probe and an unmethylated probe. The methylated probe (Fig. 3A) manifested a sufficient sensitivity in the detection of methylated DNA and, at the same time, a perfect selectivity capacity. The unmethylated probe showed a high sensitivity for detecting unmethylated control DNA, but the same probes also manifested a cross-reactivity resulting in the generation of positive droplets detected in methylated control DNA (approx. 9%) (Fig. 3B).

Concentration gradient

Eight different dilutions of DNA controls were included in the concentration gradient to confirm the dynamic range and linearity of the method. A methylated (Fig. 4A) and an unmethylated DNA control (Fig. 4B) were used for the dilution. Similar to the concentration gradient, both controls were combined with a methylated probe and an unmethylated probe. The optimal concentration was determined by considering signal amplitude, the overall count of positive droplets, and binding specificity. In addition, thresholds were set at the level of 1500 for FAM and 1400 for HEX.

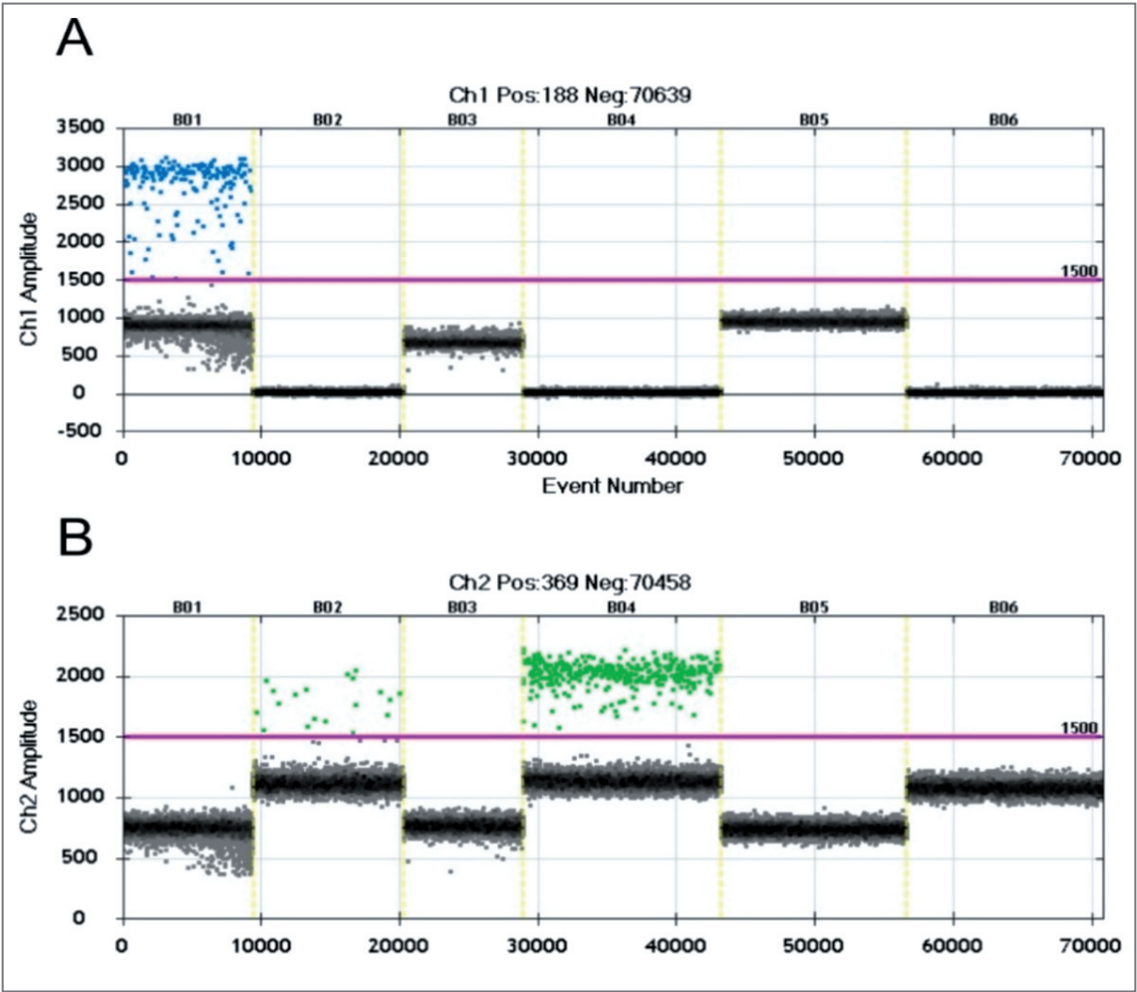


Fig. 3 PCR optimization. Figure 3A shows a positive signal generated by methylated probes using methylated control DNA (column B01); Figure 3B shows a positive signal generated by unmethylated probes using unmethylated control DNA (column B04). The positive signal generated by unmethylated probes in line B02 demonstrates the cross-reactivity of unmethylated probes with methylated control DNA.

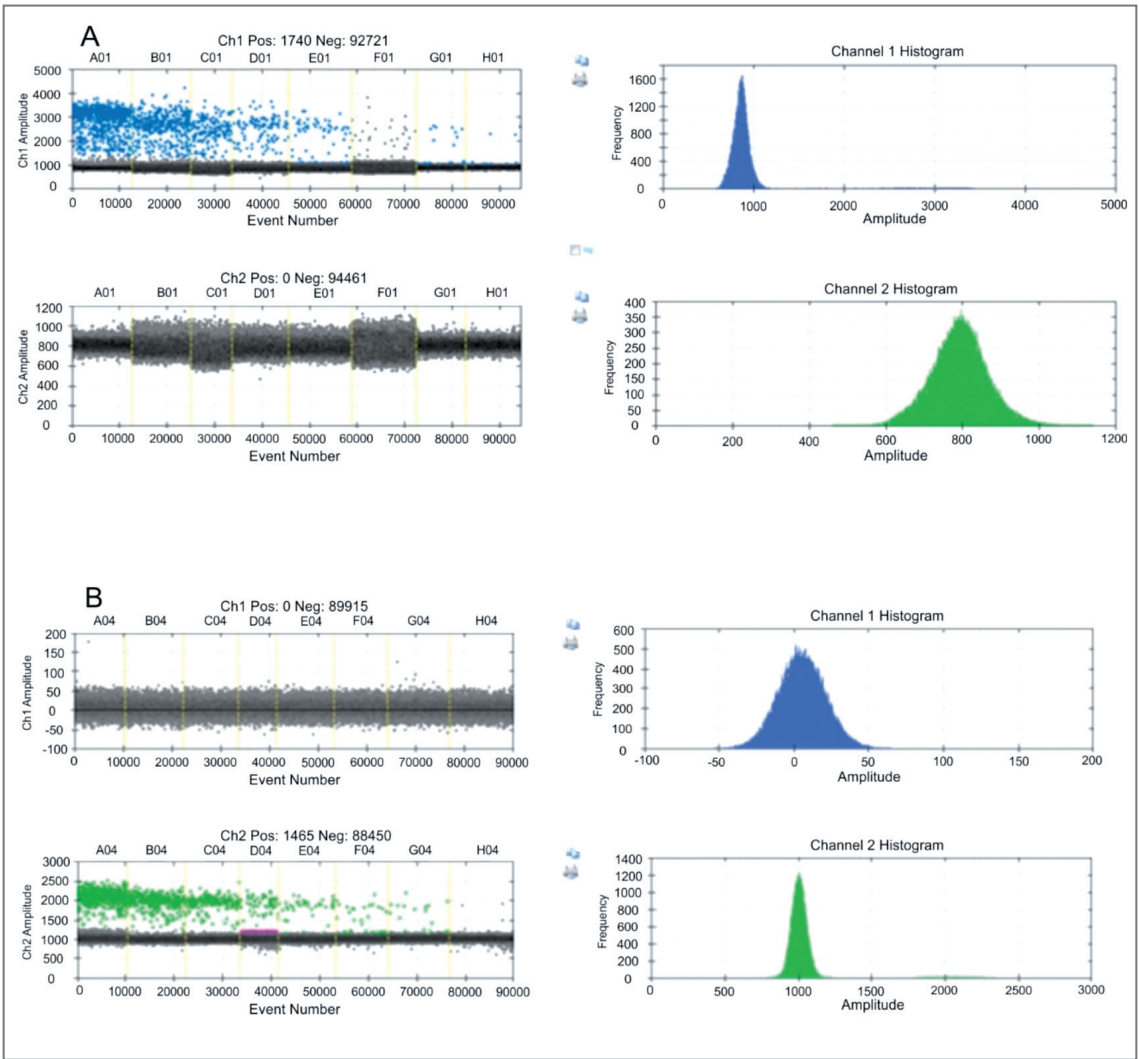


Fig. 4 Concentration gradient: A) methylated DNA control using the methylated probe. B) unmethylated DNA control using the unmethylated probe.

DISCUSSION

DNA methylation plays an essential role in cancer initiation, progression, and development of its metastatic form (13). Development and optimization of novel methods for the analysis of specific methylation patterns provides new opportunities and suggests a potential concept for clinical implementation (10). The ddPCR platform allows the precise detection and absolute quantification of targeted DNA sequences. Usage of bisulfite-treated DNA and subsequent application of specific probes for methylated and unmethylated sequences is also suitable for quantifying methylated DNA at single-base resolution utilizing the ddPCR platform (11,14).

In current study, we designed the probes with high specificity and sensitivity targeting the promoter region of the *MEIS1* gene. *MEIS1* promoter region was found using the biological

database and web resource of gene promoters EPD (15). The critical step in the methylation analysis using PCR-based methods to obtain adequate results is based on the design of optimal primers and probes targeting the gene region of interest. Nowadays, several web applications such as BiSearch (16), MSPprimer (17), or MethPrimer (18) have been developed for this purpose. In our research, we used MethPrimer to perform digital bisulfite conversion of the input sequence with predicted CpG islands. In order to design primer sequences to amplify bisulfite-modified DNA we used free-available Primer3 software (16). The software was also used to design probes in two variants to discriminate the methylation profile. Our in-house designed methylated probes recognized four CpG sites located in the promoter region. The observed data revealed that methylated probe demonstrated a good detection capacity and, at the same time, a perfect selectivity to distinguish methylated from unmethylated DNA sequences. On the contrary, unmethylated probes showed a low cross-reactivity (approximately 9%). Although the cross-reactivity of unmethylated probes was identified, these data were irrelevant for our purpose due to the fully discriminating capacity of the probe for methylated sites.

In conclusion, we described the design and optimization of the ddPCR assay recognizing methylated CpG dinucleotides in the *MEIS1* promoter region. The high sensitivity and discriminative power of our in-house developed assay proposes a potential tool for determining methylation profile of various cancer types in experimental research and subsequent clinical application.

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