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Bisulfite Sequencing with the DS3000 Compact CE Sequencer

Abstract

Of the four bases that make up genomic DNA in higher eukaryotes, the fifth carbon of cytosine can be methylated by a methyltransferase. Cytosine methylation plays an important role in various events that occur on genomic DNA, including the regulation of gene expression, and it is known as a representative epigenetic marker. Bisulfite sequencing is a commonly accepted method of analyzing methylated cytosine on a single-nucleotide basis. In bisulfite sequencing, extracted genomic DNA is treated with bisulfite (hydrogen sulfite) for a certain period. During this time, cytosine is deaminated and converted to uracil, while methylated cytosine remains as it is. Therefore, by sequencing the bisulfite-treated DNA, it is possible to distinguish between cytosine and methylated cytosine. Here, we introduce a practical example of bisulfite sequencing using the DS3000 Compact CE Sequencer (DS3000). We used genomic DNA extracted from the HCT116 cell line, which is artificially methylated by CpG methylase. The bisulfite-treated sample was subjected to electrophoresis on DS3000, and the sequences before and after bisulfite treatment were compared. As a result, 19 out of 20 methylated cytosines were detected.

DS3000
Compact CE Sequencer

Results

First, HCT116 genomic DNA was treated with bisulfite, then purified and quantified. Unmethylated cytosines were converted to uracil in this step. Next, the promoter site of the human *MLH1* gene was amplified by polymerase chain reaction (PCR). PCR amplification converts uracil to thymine, while methylated cytosine remains as cytosine. *MLH1* is involved in mismatch repair and its promoter site is strongly methylated in HCT116 cells. Next, the PCR products were treated with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®). Finally, each reaction product was analyzed by DS3000. Representative results are shown in Figure 1. Sharp and clear peaks were observed throughout the entire window of the electropherogram. A mixed base (Y) of cytosine (C) and thymine (T) can be seen at the 7th base of the electropherogram. This is presumably due to the residual cytosine in some DNA molecules. The positions of the detected methylated cytosines are summarized in Figure 2. In the *MLH1* promoter, 20 CpG sequences that serve as substrates for CpG methylase are known to be present. Among these sequences, peaks derived from methylated cytosine were identified in 19 sequences, excluding the mixed base (Y) mentioned above.

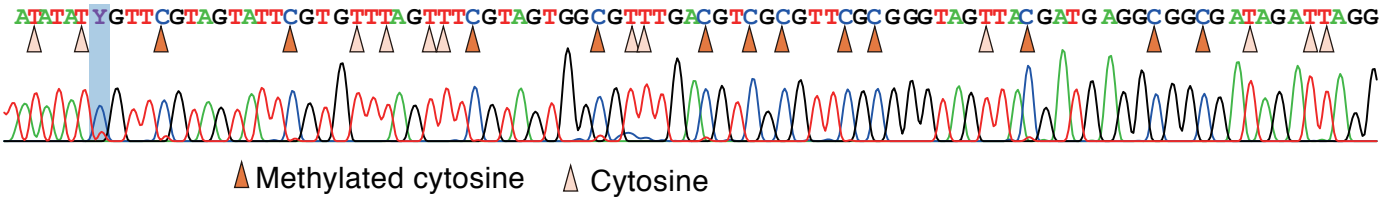

Figure 1: Representative electropherogram of bisulfite-treated DNA
Results obtained from the forward primer are shown. Please refer to the main text for details on the mixed base (Y).

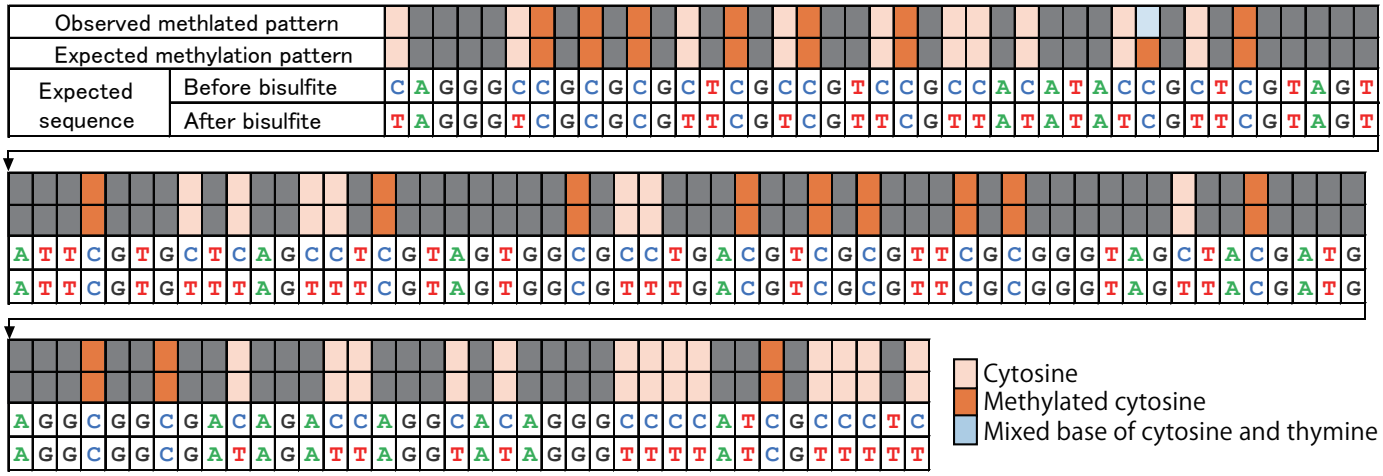

Figure 2: Locations of the methylated cytosines
The observed methylation pattern was determined using the contig sequence from the forward and reverse sequences.
The first 40 bases of each sequence were excluded. Before bisulfite: before bisulfite treatment. After bisulfite: after bisulfite treatment.

Conclusion

Here, we described a practical example of bisulfite sequencing using DS3000. By targeting the human *MLH1* gene promoter as a model system, 19 out of 20 methylation sites were detected. The remaining undetected site was thought to be due to residual cytosine molecules from incomplete bisulfite treatment. DS3000 is equipped with two electrophoresis protocols: fast sequencing and standard sequencing. The two protocols provided results of equivalent quality.

Troubleshooting

Bisulfite-treated DNA molecules become single-stranded and are unstable. We recommend handling them with extreme care. Keep the sample on ice whenever possible, and shorten the handling time.

Materials and Methods

First, 500 ng of EpiScopre® Methylated HCT116 g DNA (TaKaRa®) was treated with bisulfite using the EZ DNA Methylation-Startup™ Kit (Zymo Research). After the treatment, the DNA was purified, eluted in 10 μL of elution buffer, and 1 μL of the solution was subjected to PCR. PCR was performed with the conditions summarized in Tables 1 to 3 using control primer included in the kit. After purification with DNA Clean & Concentrator®-5 (Zymo Research), the cycle sequencing reaction was performed using 200 pg of PCR product and the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®). The resulting product was purified by ethanol precipitation, and subjected to capillary electrophoresis using DS3000 with both the fast and standard sequencing protocols (Secondary Peak Height Threshold was set to 25%). The electropherogram was visualized by SangerseqR (1) with modifications.

Table 1 : PCR cycle

Temperature (˚C)	Time (s)	Cycle
94	600	×1
94	30	×35
56	30	
72	60	
72	420	×1
4	hold	

Table 2 : PCR mixture

Reagent	Volume (μL)
2× ZymoTaq™ PreMix	12.5
Forward primer (10 pmol/μL)	2
Reverse primer (10 pmol/μL)	2
DNA (~10 ng/μL)	1
PCR-grade water	7.5

Table 3 : PCR primer sequences

Primer	Sequence
Forward	GGAGTGAAGGAGGTTACGGGTAAGT
Reverse	AAAAACGATAAAACCCTATACCTAATCTATC

Reference

1, Hill, J., T., et al. Poly peak parser: Method and software for identification of unknown indels using sanger sequencing of polymerase chain reaction products. *Developmental Dynamics.* **243**, 1632-1636 (2014)

Main specifications for the DS3000

■ Main unit specifications

Item	Details
Number of capillaries	4
Capillary length	36 cm
Sample format	8-tube strip × 4
Device control	Touch panel PC
Number of Dyes	6
Application	Sequencing analysis / Fragment analysis
Size	400 (W) × 600 (D) × 600 (H) mm
Weight	45 kg
Performance guarantee temperature	15 – 30˚C
Performance guarantee humidity	20 – 80% RH (no condensation)
Power input	100 – 240 ±10% VAC, 50/60 Hz
Rated power	260 VA
Supported secondary analysis software	• Mutation Surveyor (SoftGenetics, LLC, sold separately) • GeneMarker (SoftGenetics, LLC, sold separately) • GeneMarker HID (SoftGenetics, LLC, sold separately)

■ Run module specifications

Run Module	Application	Polymer type	Contiguous Read Length*1 (bp, QV20 CRL)	Average run time (minutes)
Fast_Sequence36_Polymer7	Sequencing analysis	Polymer7	≥600	≤32
Standard_Sequence36_Polymer7	Sequencing analysis	Polymer7	≥700	≤60
BDx_Fast_Sequence36_Polymer7	BDx sequencing analysis	Polymer7	≥600	≤32
BDx_Standard_Sequence36_Polymer7	BDx sequencing analysis	Polymer7	≥700	≤60

Run Module	Application	Polymer type	Average run time (minutes)	Sizing precision*2 (bp, 50 – 400 bp)
Fragment_Analysis36_Polymer7	Fragment analysis	Polymer7	≤35	NA
Fragment_Analysis36_Polymer4	Fragment analysis	Polymer4	≤44	<0.16

*1 Contiguous Read Length (bp, QV20 CRL) is measured with BigDye® Terminator v3.1. Sequencing Standard Kit (Thermo Fisher Scientific, sold separately)

*2 Sizing precision (bp, 50 - 400 bp) is measured with PowerPlex™ ESI17 Fast Allelic Ladder and WEN ILS 500 ESS. (Promega®, sold separately)

· BigDye is a registered trademark of Thermo Fisher Scientific Inc.

· Promega, PowerPlex is a registered trademark of Promega Corporation.

■ Consumables specifications

Product name	Part number	Details	Remarks
Capillary Cartridge 36 cm	613-0330	1 pcs	Storage temperature: 15 – 30˚C
Buffer	613-0252	Anode Buffer × 2 cartridges Cathode Buffer × 2 cartridges	Storage temperature: 2 – 10˚C
Polymer7	613-0251	4 cartridges	Storage temperature: 2 – 10˚C
Polymer4	613-0250	4 cartridges	Storage temperature: 2 – 10˚C
Septa for Cathode Buffer Cartridge	613-7231	10 pcs	
Retainer for Cathode Buffer Cartridge	613-7233	4 pcs	
Septa for 8 well tubes	613-7230	24 pcs	
Base and Retainer for 8 well tubes	613-7232	4 pcs	
Anode Electrode Assembly	613-7263	1 pcs	

Specifications in this catalog are subject to change with or without notice,
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CAUTION:For correct operation, follow the instruction manual when using the instrument.

NOTICE:The system is For Research Use Only, and is not intended for any animal or human therapeutic or diagnostic use.
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