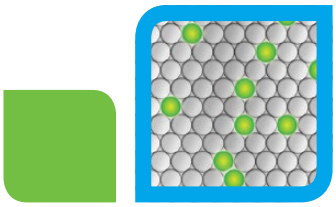
Droplet Digital™ PCR: Detection of DNA Methylation

C Litterst, S Marrs, D Shelton, and M Patil
Digital Biology Center, Bio-Rad Laboratories, Inc., 5731 W Las Positas Blvd, Pleasanton, CA 94588



Droplet Digital PCR

Bulletin 6554

Abstract

Two decades after the original demonstration of digital PCR, Bio-Rad's QX100™ and QX200™ Droplet Digital PCR (ddPCR™) Systems have finally made this method widely accessible, facile, and affordable. ddPCR partitions traditional PCR reactions into tens of thousands of highly uniform nanoliter-sized droplets and performs end-point PCR. Target molecules of interest can then be counted as PCR-positive and PCR-negative droplets in the droplet reader and their concentration in the sample computed without reliance on a standard curve.

Here we showcase the use of ddPCR for detection of DNA methylation in bisulfite-converted genomic DNA (gDNA) samples. We demonstrate unprecedented sensitivity, linearity, and robustness of DNA methylation detection by ddPCR in the *SNRPN* promoter, a model system for an imprinted gene involved in neurological disorders.

Introduction

DNA methylation is one of the most studied epigenetic modifications. DNA methylation plays an important role in a number of physiological processes as well as common diseases such as cancer and neurodegenerative disorders. In mammals, DNA methylation occurs at the C5 position of cytosine in CpG dinucleotide sequences, which are mainly concentrated in regions known as CpG islands. Methylation in CpG islands within gene promoters usually leads to gene silencing. Recent data have shown a correlation of DNA methylation and disease status. However, there is an unmet need for a sensitive yet robust method to analyze DNA methylation in clinical samples.

We chose *SNRPN* as a model gene for methylation detection by ddPCR since it is a known imprinted gene. The maternal *SNRPN* gene is methylated (silenced), whereas only the paternal gene is expressed. We developed a ddPCR protocol for detection of methylation sites in the *SNRPN* promoter based on bisulfite-converted gDNA samples. Our method is very sensitive, linear, and robust, and is suitable for low concentrations in biological samples.

Methods and Results

High- and low-methylated rat gDNA controls were purchased from EpigenDx. gDNA from primary rat neurons (Life Technologies Corporation) and cell lines from American Type Culture Collection (9L/lacZ, B35, and L2) were prepared using the MINI Genomic DNA Kit from IBI Scientific. Up to 500 ng of gDNA was bisulfite converted using the EZ DNA Methylation-Lightning Kit (Zymo Research Corporation). Converted DNA (1–3 µl) was subjected to ddPCR in duplicate reactions using the primers/probes shown in Figure 1 and ddPCR Supermix for Probes (No dUTP) (Bio-Rad) on a QX100 ddPCR System with an annealing temperature of 52°C. DNA methylation detection results are shown in Figures 2 and 3.

>chr1:111592360–111593159 (reverse complement)
ACTTCTTGGGAACTATTTTTAATTTTTTAAAAATATATTCAGAAATAG-
GTAATTGTATCCATTAGCCCAGATTGACAGCTATTATTTTTTTTTTTA-
AATACTTGCTCAAATTTCCTTAGTAGGAACTCTGAAGATCAGATAGCT-
GACTTTTGACAGGACATTGC**AGTCAGAGAGTGACATGGA**-
CCCCTGCATTGCGGCAAAAATGTGCGCATGTGCAGCCCTTGCC-
TTGGACGCATGCGTAGGGAGCAGCACGAAAAACCTGAGCCATT-
GCGGCAAGTCTAGCGC**AGAGAGTAGAGGGTGCt**ggagatgccagac-
ggttggttctgaggagagattttgcaacgcaatggagcgagg

Forward primer: AGTtAGAGAGTGAtATGGA
Reverse primer: CAACACCCTCTACTCTCT

Methylated probe: FAM-TTGGACGtATGCGTAGG
Nonmethylated probe: HEX-TTGGAtGtATGtGTAGGGAG

Fig. 1. Primer and probe design. Forward and reverse primers were designed to bind sequences (underlined above) flanking known methylation sites in the rat *SNRPN* promoter. A FAM-labeled probe was designed to bind methylated and a HEX-labeled probe to bind nonmethylated CpG sites in the promoter DNA (black box, covering two CpG sites). CpG methylation sites are shown in red. The C in CpG is resistant to bisulfite conversion and remains a cytosine upon bisulfite treatment. Blue font indicates cytosines that will be changed to uracil/thymidine by bisulfite treatment.

BIO-RAD

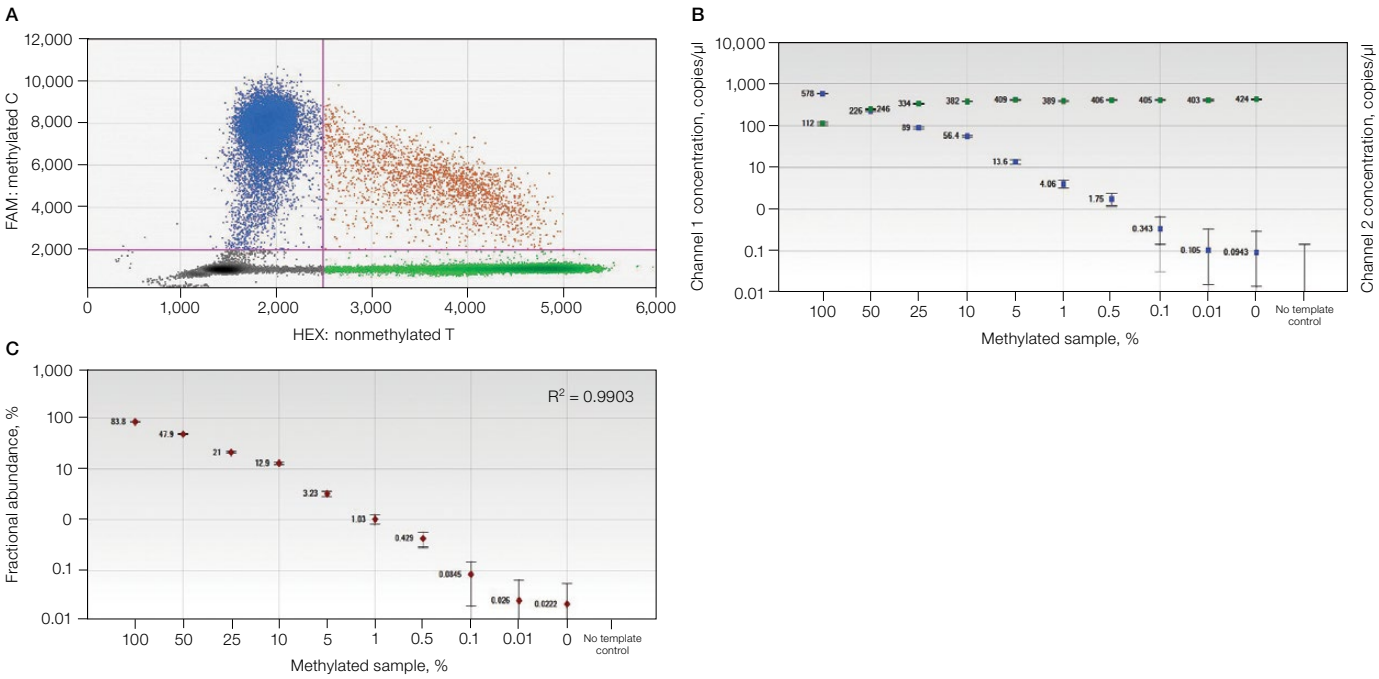


Fig. 2. Sensitivity of methylation detection by ddPCR. High- and low-methylated control gDNA were mixed according to the percentages indicated in **B** and **C** (methylated sample, %) and subjected to bisulfite conversion and ddPCR. **A**, 2-D ddPCR analysis plot; **B**, concentration measurement of methylated and nonmethylated promoter DNA in mixed samples; **C**, ddPCR can detect levels of *SNRPN* promoter methylation as low as 0.5% (and 1.75 copies/µl as shown in **B**). R^2 value shows good correlation between expected and measured percentages of methylated DNA.

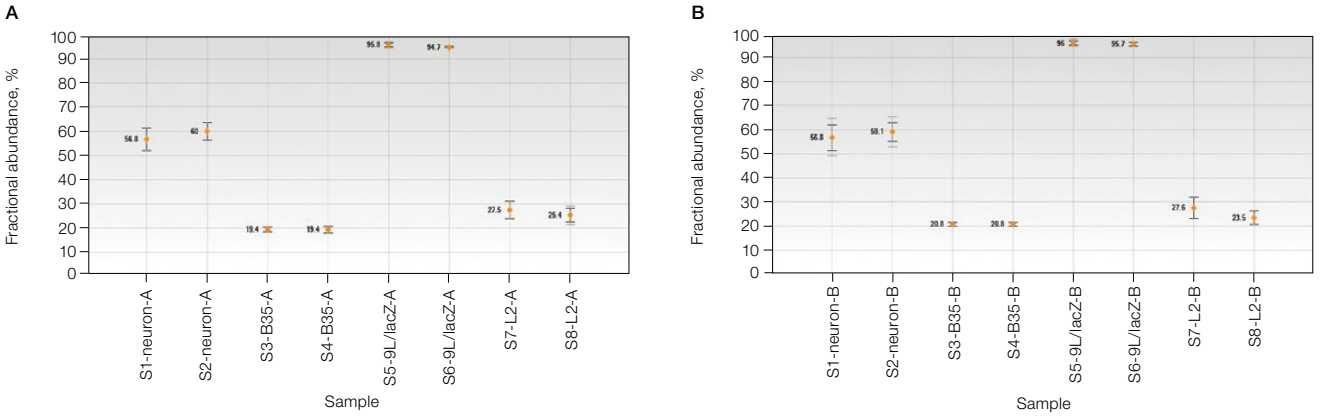


Fig. 3. Detection of DNA methylation in rat primary neurons and cell lines. gDNA samples were bisulfite converted and analyzed by ddPCR in two independent experiments (runs 1 and 2). Levels of methylated (FAM) and nonmethylated (HEX) DNA from run 1 (**A**) and run 2 (**B**) are shown. Duplicate experiments and repeated runs 1 and 2 show a very similar measured percentage of methylated DNA.

Summary

- Highly methylated control DNA contains ~20% nonmethylated sites
- Detection of methylated DNA using ddPCR is a highly robust and reproducible method
- At least 0.5% of methylated *SNRPN* promoter DNA can be detected by ddPCR using 150 ng of bisulfite-converted gDNA per well

Conclusions

We designed a ddPCR duplex assay to detect two adjacent methylation sites in the *SNRPN* promoter. Using high- and low-methylated control gDNA, we show that our method can detect amounts as low as 0.5% methylated DNA in the background of a nonmethylated control DNA. We also demonstrate superior sensitivity, linearity, and robustness of DNA methylation detection by ddPCR. Mixed cortical neurons show 56–60% of *SNRPN* promoter methylation, which indicates that our method is suitable for measuring methylation in biological samples with low concentrations.

References

Hernández HG et al. (2013). Optimizing methodologies for PCR-based DNA methylation analysis. Biotechniques 55, 181–197.

Zeschnigk M et al. (1997). Imprinted segments in the human genome: Different DNA methylation patterns in the Prader-Willi/Angelman syndrome region as determined by the genomic sequencing method. Hum Mol Genet 6, 387–395.

Visit bio-rad.com/web/ddPCRMethylation for more information.

BIO-RAD

Bio-Rad Laboratories, Inc.

Life Science Group

Web site *www.bio-rad.com* ***USA*** *800 424 6723* ***Australia*** *61 2 9914 2800* ***Austria*** *01 877 89 01* ***Belgium*** *09 385 55 11* ***Brazil*** *55 11 3065 7550* ***Canada*** *905 364 3435* ***China*** *86 21 6169 8500* ***Czech Republic*** *420 241 430 532* ***Denmark*** *44 52 10 00* ***Finland*** *09 804 22 00* ***France*** *01 47 95 69 65* ***Germany*** *089 31 884 0* ***Greece*** *30 210 9532 220* ***Hong Kong*** *852 2789 3300* ***Hungary*** *36 1 459 6100* ***India*** *91 124 4029300* ***Israel*** *03 963 6050* ***Italy*** *39 02 216091* ***Japan*** *81 3 6361 7000* ***Korea*** *82 2 3473 4460* ***Mexico*** *52 555 488 7670* ***The Netherlands*** *0318 540666* ***New Zealand*** *64 9 415 2280* ***Norway*** *23 38 41 30* ***Poland*** *48 22 331 99 99* ***Portugal*** *351 21 472 7700* ***Russia*** *7 495 721 14 04* ***Singapore*** *65 6415 3188* ***South Africa*** *27 861 246 723* ***Spain*** *34 91 590 5200* ***Sweden*** *08 555 12700* ***Switzerland*** *026 674 55 05* ***Taiwan*** *886 2 2578 7189* ***Thailand*** *1800 88 22 88* ***United Kingdom*** *020 8328 2000*