Cross Validation of NGS methylated targets using Droplet Digital PCR (ddPCR™)

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Introduction

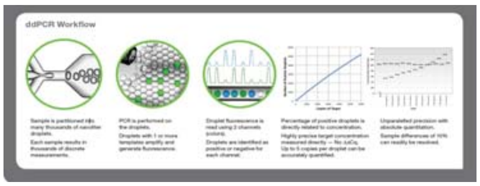
DNA methylation plays an important role in a number of physiological processes as well as common diseases such as cancer and neurodegenerative disorders. Methylation in CpG islands within gene promoters usually leads to gene silencing.

Recent data have shown the correlation of methylation state and disease status. However, there is an unmet need for a sensitive yet robust method to analyze DNA methylation in clinical samples in a rapid, inexpensive, and high throughput manner.

Herein we showcase the use of ddPCR for detection of DNA methylation in bisulfite converted gDNA samples. First we demonstrate the superior sensitivity, linearity and robustness of ddPCR methylation detection in the SNRPN promoter, a model system for an imprinted gene involved in neurological disorders. Secondly we show the detection of DNA methylation in the vimentin promoter, which is known to be methylated in cancer (Li *et al.* Nat. Biotech. 2009). We will demonstrate reproducible detection in replicate bisulfite converted samples, sensitivity, and then screen plasma samples by NGS and ddPCR.

Analysis of SNRPN promoter

Materials and Methods



High methylated and low methylated rat gDNA controls were purchased from EpigenDx. gDNA from primary rat neurons (Life Technologies) and cell lines (9/LacZ , B35, L2; all from ATCC) were prepared using the MINI Genomic DNA kit from IBI Scientific. Up to 500 ng of gDNA was bisulfite converted using the Zymo Research EZ DNA Methylation-Lightning kit. 1–3 µl of the converted DNA was subjected to ddPCR in duplicate reactions using the primers/probes shown in Figure 1 and the Droplet PCR Supermix (Bio-Rad) on the QX100™ or QX200™ Droplet Digital PCR system with an annealing temperature of 52°C.

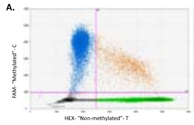
>chr1:111592360-111593159 (reverse complement)
ACTTCTTGGGAACTATTTTTAATTTTTTAAAAATATATTCAGAAATAGGTAATTGTATCCATTAGCCCAGATTGACAGCTATTATTTTTTTTT-
TAAATACTTGCTCAAATTTCCTTAGTAGGAACTCTGAAGATCAGATAGCTGACTTTTGACAGGACATTGC**AGTCAGAGAGTGACATGGA**
CCCCTGCATTGCGGCAAAAATGTGCGCATGTGCAGCCCTTGCCTTGG ACGCATGCGTAGGGAGCAGCACGAAAAACCTGAGC-
CATTGCGGCAAGTCTAGCGC**AGAGAGTAGAGGGTGC**tggagatgccagacggttggttctgaggagagatttgcaacgcaatggagcgagg

Fow Primer AGTtAGAGAGTGAtATGGA	Probe Methylated FAM-TTGGACGtATGCGTAGG
Rev Primer CAACACCCTCTACTCTCT	Probe Non-Methylated HEX-TTGGAtGtATGtGTAGGGAG

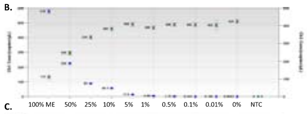
Fig. 1. Primer and probe design. Forward and reverse primers were designed to bind sequences (underlined above) flanking known methylation sites in the rat SNRPN promoter. A FAM-labeled probe was designed to bind methylated and a HEX-labeled probe to bind unmethylated CpG sites in the promoter DNA (black 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.

Sensitivity of methylation detection

A.



B.



C.

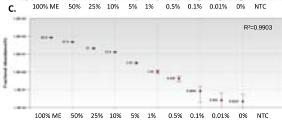


Fig. 2 Sensitivity of methylation detection by ddPCR. A. 2D ddPCR analysis plot of bisulfite converted mixed samples. B. Concentration measurements of methylated and non-methylated promoter DNA in different % mixed samples. C. ddPCR can detect levels of methylation as low as 0.5% (and 1.75 copies/ul as shown in Fig2B).

Comparison of Bisulfite Conversion kits by ddPCR

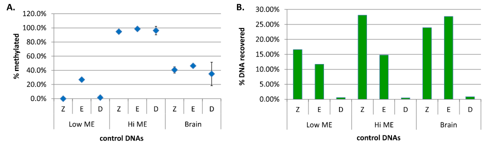


Fig. 3 Comparison of Bisulfite Conversion kits. 200ng of 3 gDNA samples were bisulfite converted using <u>Z</u>ymo Research's EZ DNA Methylation-Lightning Kit (#D0531), <u>E</u>pigentek's Methylamp DNA Modification Kit (#P-1001-1) or <u>D</u>iagenode's MagBisulfite Kit (#AF-106-0024). Percentage methylation (A) and yield (B) of bisulfite converted DNA compared between 3 kits: Zymo; E, Epigentek; D, Diagenode.

Reproducibility of Bisulfite Conversion of DNA and ddPCR

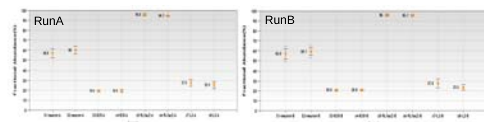


Fig. 4 Detection of DNA methylation in rat primary neurons and cell lines. gDNA samples were bisulfite converted with the Zymo kit and analyzed by ddPCR in 2 independent experiments (RunA & RunB). Duplicate experiments and repeated runs show very similar measured % of methylated DNA.

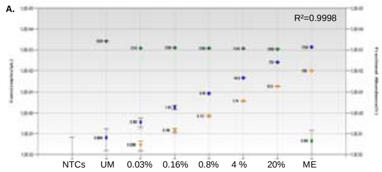
Analysis of Vimentin promoter

>chr10:17271595-17271494(reverse strand)
ccggggacgaggcgtagaggctgcggctggtgctggggcgcagcgcgcTGCCCAGGCTGTAGGTGCGGGTGGACGTAGTCACGTAGCTCCGGCTG
GAGCTCGGCCGGCTCGCGGTGCCCGGCGCCGCCGAACATCCTGCGGTAGgaggacgaggacacggacctggtggacatggctgcggagggtggcg
atggcctgggc

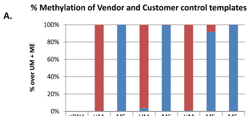
Fig. 5 Primer and Probe design of Vimentin. Initial region of probe and primers were taken from Li et al. (2009) and modified for use in ddPCR with methylation-specific primers and shorter amplicons. Primers are underlined and probe is within the box. CpGs are in red while C-to-T conversions are in blue. Methylated probe is in the FAM channel, and unmethylated signal is in the HEX channel.

Fig. 6 Sensitivity of Vimentin methylation detection by ddPCR. A. Concentration measurements of methylated and non-methylated ultramers in mixed samples. ddPCR can detect levels of methylation as low as 0.03% and 0.363 copies/ul with excellent linearity.

A.

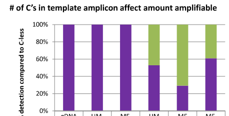


A.



	gDNA Promega	UM Uitramer	ME Uitramer	UM Moinova	ME Moinova	UM Epitect	ME Epitect	ME Zymo
% Unmethyl	0.0	100.0	0.0	96.0	0.3	98.9	8.4	0.0
% Methyl	0.0	0.0	100.0	4.0	99.7	1.1	91.6	100.0

B.

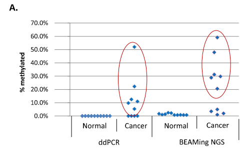


	gDNA Promega	UM Uitramer	ME Uitramer	UM Moinova	ME Moinova	ME Zymo
% additional C less	0.0	0.0%	0.0%	47.0%	70.8%	39.2%
%Vimentin	100.0%	100.0%	100.0%	53.0%	29.2%	60.8%

Fig. 7 Analysis of vendor control DNAs. A. Percentage of methylation in different purchased BSC controls. B. Comparison of measured amplifiable amount of BSC DNA between vimentin and similarly sized C-less amplicon.

Comparison of ddPCR and BEAMing-based NGS

A.



Absolute copies of input BSC DNA

B.

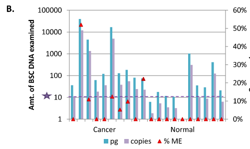


Fig. 8 Analysis of plasma cell free DNA from CRC patients. A. 4-8 ul of BSC-treated plasma DNA was analyzed by ddPCR and 5-10ul of BSC-treated plasma DNA was preamplified with 45 cycles and then analyzed by BEAMing-based NGS. B. Amts. of BSC DNA examined by ddPCR in picograms (blue), total copies (purple), and percentage methylation (red). Results above 10 copies have the highest confidence.

Conclusions

- ddPCR is a sensitive and robust validation method for NGS discoveries and provides an unbiased quantification of targets with low sample input.
- Control DNAs have variable amounts of HI or LO methylation, rather than 100% vs. 0%.
- Detection of methylation is highly robust and reproducible using ddPCR.
- ddPCR offers the ability to monitor the absolute count of target molecules examined.

References

Li et al., (2009). Sensitive digital quantification of DNA methylation in clinical samples. Nature Biotechnology 47(9):858-63.

Li LC and Dahiya R. MethPrimer: designing primers for methylation PCRs. Bioinformatics. 2002 Nov;18(11):1427-31.

Hernandez HG, et al., (2013). Optimizing methodologies for PCR-based DNA methylation analysis. BioTechniques 55:181-197.