Enabling cutting-edge neuroscience research with Droplet Digital™ PCR: Detection of DNA methylation, RNA editing, and alternative splicing

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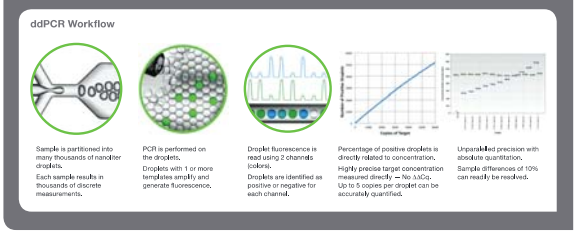
Abstract

Two decades after the original demonstration of digital PCR, Bio-Rad's Droplet Digital PCR (ddPCR™) system has finally made this method widely accessible, facile, and affordable. ddPCR partitions traditional PCR reactions, including hydrolysis probes or intercalating dye, into tens of thousands of highly uniform nanoliter droplets and performs endpoint 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. Moreover, multiple sequence-specific targets can be detected simultaneously in a sample by using different fluorophores and fluorescence amplitudes. Many basic research, clinical, and industrial questions that were previously hampered by insufficient sensitivity, precision, and/or reproducibility, are now addressable.

Droplet digital PCR enables a wide variety of applications commonly used in neuroscience research, such as:

- Determination of gene copy number variation (CNV)
- Detection of rare single nucleotide polymorphisms (SNPs)
- Absolute quantitation of DNA/RNA, e. g. for detection of viruses, miRNA, and gene expression analysis
- Preparation and quantitation of next generation sequencing libraries
- Quantitative measurement of RNA editing and RNA splice variants
- DNA linkage studies (e.g. haplotyping)

Here we discuss ddPCR applications for detection of **DNA methylation, alternative splice variants, and RNA editing.**



Example 1: Detection of DNA Methylation

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)
ACTTCTTGGGAACTATTTTTAATTTTTAAAAATATATTCAGAAATAGGTAATTGTATCCATTAGCCCAGATTGACAGCTATTATTTTTTTTTT-
TAAATACTTGCTCAAATTTCCTTAGTAGGAACTCTGAAGATCAGATAGCTGACTTTTGACAGGACATTGC**AGTCAGAGAGTGACATGGA**
CCCCTGCATTGCGGCAAAAATGTGCGCATGTGCAGCCCTTGCC[TTGG ACGCATGCGTAGGGAGC]AGCACGAAAAACCTGAGC-
CATTGCGGCAAGTCTAGCGC**AGAGAGTAGAGGGTGCt**ggagatgccagacggttggttctgaggagagattttgcaacgcaatggagcgagg

Fow Primer AGTtAGAGAGTGAtATGGA
Rev Primer CAACACCCTCTACTCTCT
Probe Methylated FAM-TTGGACGtATGCGTAGG
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

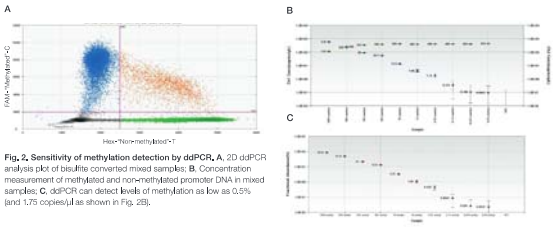


Fig. 2, Sensitivity of methylation detection by ddPCR. **A**, 2D ddPCR analysis plot of bisulfite converted mixed samples; **B**, Concentration measurement of methylated and non-methylated promoter DNA in mixed samples; **C**, ddPCR can detect levels of methylation as low as 0.5% (and 1.75 copies/µl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

Detection of DNA methylation in rat primary neurons and cell lines
Bisulfite Conversion of DNA and ddPCR

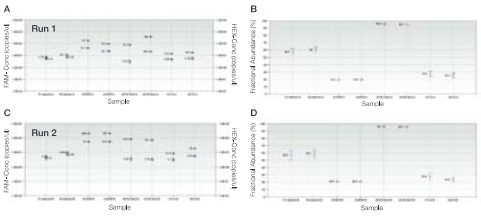


Fig. 3. Detection of DNA methylation in rat primary neurons and cell lines. gDNA samples were bisulfite converted and analyzed by ddPCR in 2 independent experiments (Run 1 and Run 2). Concentration measurement of methylated (FAM) and non-methylated (HEX) DNA from Run 1 (**3A**) and from Run 2 (**3C**). Levels of methylated DNA from Run 1 (**3B**) and from Run 2 (**3D**). Duplicate experiments and repeated runs 1 and 2 show very similar measured % of methylated DNA.

Summary — Methylation

- Highly methylated control DNA contains ~20% non-methylated sites
- ddPCR-based detection of methylation is highly robust and reproducible
- Using 150 ng of bisulfite converted gDNA per well, at least 0.5% of methylated DNA can be detected by ddPCR

Example 2: Detection of Alternative Splicing using EvaGreen ddPCR

Materials and Methods

Human primers were designed for the following 3 genes that are alternatively spliced in mouse brain (Gehman et al.):

Camta1: Fow aatgaggtttcttcgccgct, Rev TCCTTGGCCTTTTTCAATTCTt

Ablim1: Fow CTCCATCAACTCCCCTGTGT, Rev TGGGTGGCTTTCGGTAAAT

Tpm3 : Fow CGTGCTGAGTTTGCTGAGAG, Rev GCTCCTCTTTGGTGCATTTC

RNA (Ambion) was reverse transcribed using iScript™ Advanced cDNA synthesis kit (Bio-Rad), cDNA was quantified by ddPCR using the QX200 ddPCR system and EvaGreen Supermix at an annealing temperature of 57°C. Alternatively spliced forms depending on splice factors (Gehman et al, 2011) are highlighted in bold font.

B

Gene	Predicted Length (bp)	Length difference (bp)
Camta-1 (calmodulin binding transcription activator 1)	57, **88**	31
Tpm-3 (tropomyosin 3)	**88**, 167, (1415)	79
Ablim-1 (actin binding LIM protein 1)	113, **218**, (338)	105

Fig. 4. Expected Splicing Products. A. Primer design; B. Expected sizes of splice variants. Alternatively spliced forms are highlighted in bold font.

ddPCR detection of splice variants

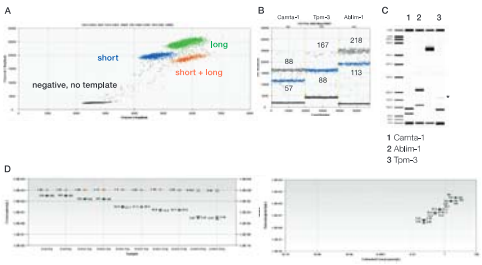


Fig. 5. ddPCR detection of splice variants. **A**, 2D fluorescence plot analysis of EvaGreen ddPCR results allows discrimination between different length amplicons. Brain cDNA containing both long and short forms of Ablim-1 was analyzed using the Ablim-1 primers. Droplets with no PCR amplification are shown in **black** (negative); droplets with short form of Ablim-1 are shown in **blue**; droplets with long form of Ablim-1 are shown in **green**; droplets with both long and short form of Ablim-1 are shown in **orange**; **B**, 1D plot of ddPCR using Camta-1, Tpm-3 and Ablim-1 primers: the intensity of fluorescence in channel 1 is proportional to the length of the amplicon (in bp), e.g. the long 88bp amplicon from Camta-1 shows similar fluorescence intensity as the short form of Tpm3 (also 88bp); **C**, DNA was extracted from parallel PCR reactions shown in B and analyzed on the Experion™ system from Bio-Rad. Detected PCR products show the expected length. Asterix indicates a weak band of the long amplicon of Tpm3; **D**, cDNA was diluted as indicated and analyzed by ddPCR using Camta-1 primers. The short form is detected in channel1, the long form in channel 2. There is a linear relationship between input and measured concentration (right panel). The ratio between short and long form is constant regardless of input amount indicating that the detection of either form is not biased.

Alternative splicing in tissue samples

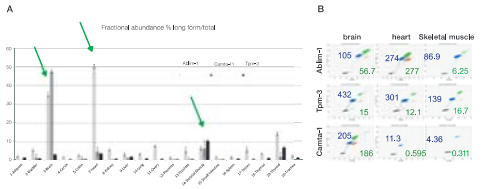


Fig. 6. Detection of alternative splicing in tissue samples. A panel of human tissue RNA was reverse transcribed and analyzed in ddPCR for expression of long and short splice forms of Camta-1, Ablim-1, and Tpm-3. **A**, Brain, heart, and skeletal muscle samples showed highest relative levels of the long forms (arrows). The corresponding 2D plots for these tissues samples are shown in **B**. Droplets amplifying the short version of the transcript are shown in **blue**, the long version in **green** and droplets with both forms in **orange**. The concentration measurements for the short and long forms are indicated in **blue** and **green** font respectively (copies/µl).

Summary — Alternative Splicing

- Splice variants with length difference from 30–100bp can be detected by EvaGreen ddPCR in single wells
- We identified differential expression of alternative splice variants of Ablim1, Camta-1, and Tpm-3 in a human tissue panel. Specifically, the long variant of Camta-1 is highly expressed in the brain, but not other tissues, suggesting a brain-specific function

Example 3: RNA Editing

Materials and Methods

Primers (Fow GGGATTTTTAATAGTCTCTGGTTTTCC, Rev AGAGAGGGATCTTGGCGAAA) and probes (GluR2-A-Q: TGCCTTTATGCAGGNNGGATGC, GluR2-G-R: TGCCTTTATGCGGCNNGGAT; N-5-Nitroindole) were designed for the detection of the Q/R editing site of the AMPA-type glutamate receptor (GluR2). Two promiscuous nucleotides (N) were placed in the probes in secondary editing positions to allow detection of all variants. RNA (BioChain) was reverse transcribed using the iScript Advanced cDNA synthesis kit (Bio-Rad). cDNA was subjected to ddPCR on the QX100 system using the Droplet PCR supermix. Annealing temperature for thermocycling was 57°C.

Editing on the Q/R site of GluR2

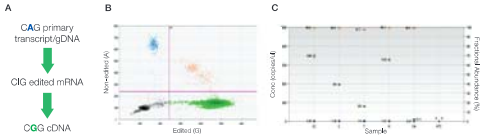


Fig. 7. Editing on the Q/R site of GluR2. **A**, Schematic view of Q/R editing of GluR2; **B**, RNA from various brain tissues was reverse transcribed and analyzed in ddPCR for levels of edited and non-edited GluR2. 2D ddPCR plot indicates levels of edited (green) and non-edited (blue) transcripts; **C**, Abundance of edited RNA in cerebral cortex (CC), cerebellum (C), thalamus (T), hippocampus (H), and cerebral meninges (CM) samples. Fractional abundance shows percent of edited form in the total sample.

Summary — RNA Editing

- GluR2 mRNA is edited in human brain samples
- ddPCR is able to detect very small changes in the levels of edited mRNA
- 99% of the transcript is edited in cortex, cerebellum, meninges, and hippocampus samples as previously reported (Sommer et al.1991)
- 97% of the thalamus sample is edited. Further studies are needed to determine whether this is due to the donor or a tissue specific effect

References

Gehman et al. (2011). The splicing regulator Rbfox1 (A2BP1) controls neuronal excitation in the mammalian brain. Nature Genetics 43(7).

Sommer B, Kohler M, Sprengel R, Seeburg PH (1991) RNA editing in brain controls a determinant of ion flow in glutamate-gated channels. Cell 67: 11–19.

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