DNA Methylation Detection Using Droplet Digital™ PCR

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Abstract

The year 2023 marks 12 years since the advent of Droplet Digital PCR (ddPCR™). In that time, ddPCR technology has revolutionized the absolute quantification of nucleic acids. With a host of technical advances and enhanced automation in recent years, Droplet Digital PCR is moving from a specialized research technique to a robust tool in clinical and translation research applications. This white paper reviews the use of Droplet Digital PCR for detection of DNA methylation. We review ddPCR technology, compare different workflow strategies, and explain critical parameters for optimal assay design to get unprecedented sensitivity, linearity, and robustness when detecting DNA methylation by Droplet Digital PCR.

Introduction

DNA methylation plays a critical role in many normal developmental and sustaining physiological processes. However, aberrant methylation can lead to a variety of disease states, including several types of cancer. For instance, global hypomethylation can contribute to the activation of oncogenes while hypermethylation within tumor suppressor genes can result in silencing. Mounting evidence demonstrates the utility of analyzing methylation status as an indicator of early-stage cancer development and as a method for monitoring tumor response to therapy. Detecting methylation changes in cell-free DNA (cfDNA) is especially attractive given the noninvasive nature of blood draws. Unfortunately, sensitive, robust, and cost-effective methods to perform methylation analysis on cfDNA samples remain an unmet need. Here we demonstrate how common methylation detection methods combine with Droplet Digital PCR to enhance analysis of these precious samples.

DNA Digestion Using Methylation-Sensitive Restriction Enzymes (MSRE)

By far, the fastest and easiest method of DNA methylation quantification incorporates MSRE digestion. Further, this method does not have the drawbacks of bisulfite conversion, such as extensive DNA degradation and high starting material input requirements.

As shown in the schematic below, methylation-sensitive restriction enzymes do not cleave restriction sites in the presence of a methylated cytosine, leading to PCR amplification (Figure 1), while cleavage of an unmethylated site inhibits amplification.

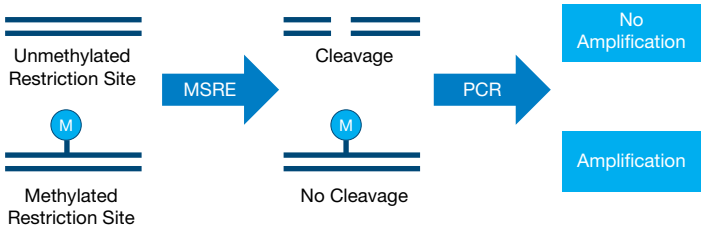


Fig. 1. Schematic overview of methylation-sensitive restriction enzyme digestion. M, methyl; MSRE, methylation-sensitive restriction enzyme.

There are a variety of commercially available MSREs available (Table 1). Specific primers can be designed to amplify a specific sequence of interest that contains an MSRE. See the Primer Design section for recommendations on how to design primers.

Table 1. Overview of MSREs and restriction site sequences.

Restriction Enzyme (all 4-base cutters)	Restriction Site
AccII (BstUI, Bsh1236I)	CG↓CG GC↑GC
AciI (SsiI)	C↓CGC GGC↑G
HpaII (HapII)	C↓CGG GGC↑C
HhaI (CfoI)	GCG↓C C↑GCG

To detect the methylation status at a specific locus, the sample is digested by a methylation-sensitive restriction enzyme and analyzed with Droplet Digital PCR (Figure 2). When using probe-based detection methods, multiplexing several methylation sites or targets is possible. For best results, a duplex reaction with a reference target (no restriction sites) is advised (e.g., *RPP30*). The reference assay is used to normalize and correct for small input differences caused by pipetting or samples with unstable copy number.

Methylation percentages are calculated with high precision via Droplet Digital PCR by measuring DNA concentrations in samples with (MSRE+) and without (MSRE–) enzyme. The percentage of methylation, or methylation fraction, is calculated using the precise ddPCR concentrations measured for samples either digested (MSRE+) or undigested (MSRE–), as follows:

MSRE+ ratio = target concentration/reference concentration
MSRE– ratio = target concentration/reference concentration

% methylation = MSRE+ ratio/MSRE– ratio

Note that copy number results are available from the undigested (MSRE–) samples and can be used to detect differences in samples (assuming the use of a stable reference gene).

Samples are prepared as follows (incubations can be done in a thermal cycler such as the T100 Thermal Cycler):

- MSRE+ samples — 50–100 ng of DNA is incubated for 1 hr at 60°C with 2–10 U of BstUI and 0.5 µl 10x CutSmart Buffer (New England Biolabs, Inc., discontinued; rCutSmart Buffer [#B6004S] is a suitable alternative) in a total volume of 5.0 µl.
- MSRE– samples — 50–100 ng of DNA is incubated for 1 hr at 60°C with 0.5 µl 10x CutSmart Buffer in a total volume of 5.0 µl.

In a 22 µl experiment, 1–100 ng of incubated DNA samples can be analyzed using 11 µl ddPCR Supermix for Probes (No dUTP) (Bio-Rad Laboratories, Inc., catalog #1863024) and primers and probes in a final concentration of 900 and 250 nM, respectively.

PCR mixtures are partitioned into ~20,000 droplets using the Automated Droplet Generator (Bio-Rad, #1864101). Subsequent PCR is performed in a thermal cycler using the the protocol outlined in Table 2.

Table 2. Thermal cycling protocol. Ramp rate set to 2°C/sec for all steps.

Cycling Step	Temperature, °C	Time	Number of Cycles
Enzyme activation	95	10 min	1
Denaturation	95	30 sec	
Annealing/extension	55	1 min	40
Enzyme deactivation	98	10 min	
Hold	12	<48 hr	1

Droplet reading can be performed on a QX200™ or QX600™ Droplet Reader. All the data shown in this white paper were generated using a QX200 Droplet Reader.

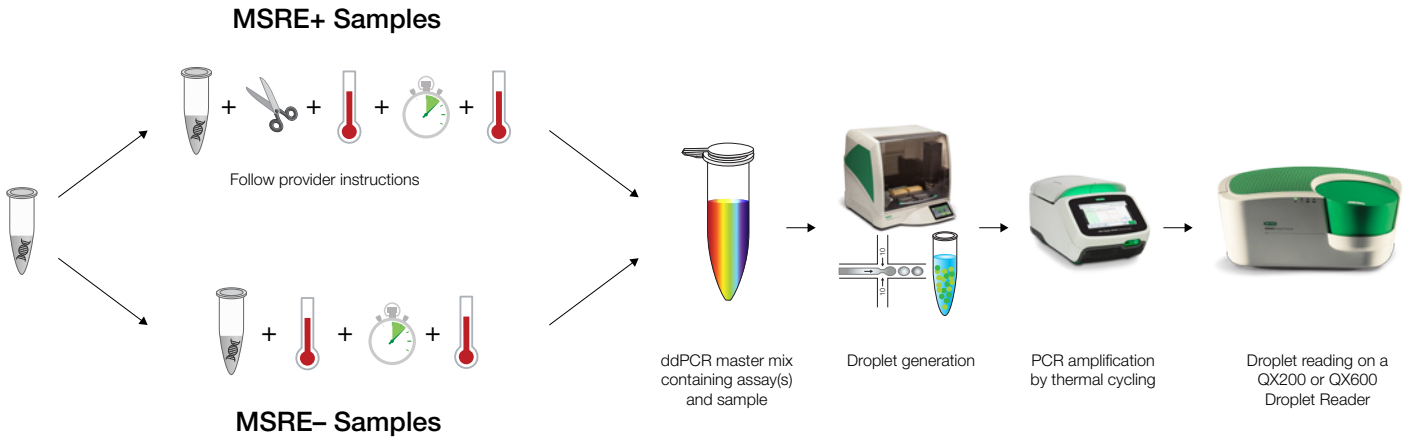


Fig. 2. Typical ddPCR MSRE workflow. Sample is split. One aliquot is digested with a restriction enzyme and the other without restriction enzyme. Both aliquots are run as individual samples using a standard ddPCR setup. MSRE, methylation-sensitive restriction enzyme.

Examples of Assay Performance

In this section, we highlight assay performance using a few examples (Figures 3–5).

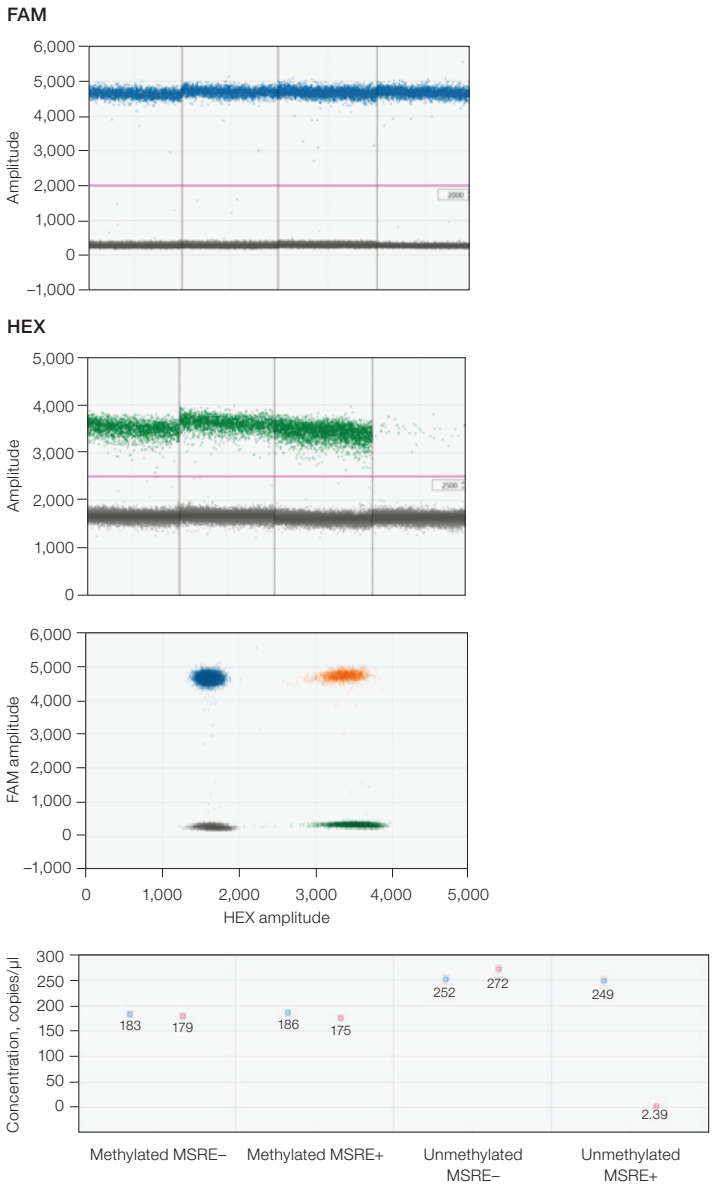


Fig. 3. *PTGER4* promotor (FAM) and *RPP30* (HEX). One-dimensional plots show the positive and negative droplet clusters from two targets run in duplex, *PTGER4* (FAM, AssayID dHsaEXD82842910) and *RPP30* (HEX, AssayID dHsaCP2500350). Two-dimensional plot shows the four clusters from the same reaction. HpaII is used for digestion. Concentrations (copies/µl) are shown in the bottom graph and used for calculating the percentage of methylation from each sample (see calculations). MSRE, methylation-sensitive restriction enzyme.

PTGER4 Promoter (FAM) and *RPP30* (HEX)

The percentage of *PTGER4* promoter methylation was calculated as shown in Table 3.

Table 3. *PTGER4* promoter methylation calculations.

	Methylated	Unmethylated
MSRE+ Ratio	175/185 = 0.946	2.39/249 = 0.0096
MSRE– Ratio	179/183 = 0.978	272/252 = 1.079
Methylation, %	0.946/0.978 = **97%**	0.0096/1.079 = **0.9%**

SEPT9 CGI3 Promotor (FAM) and *RPP30* (HEX)

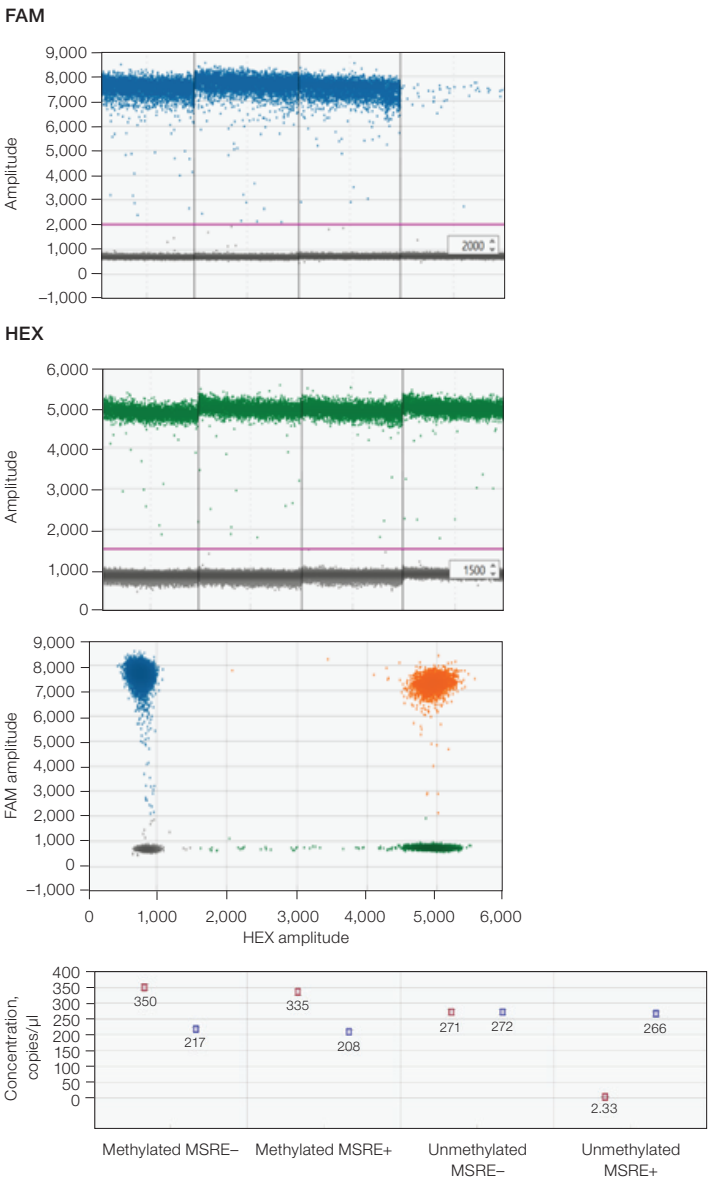


Fig. 4. *SEPT9* CGI3 promotor (FAM) and *RPP30* (HEX). One-dimensional plots show the positive and negative droplet clusters from two targets run in duplex, *SEPT9* CGI3 (FAM, AssayID dHsaEXD18126781) and *RPP30* (HEX, AssayID dHsaCP2500350). Two-dimensional plot shows the four clusters from the same reaction. BstUI is used for digestion. Concentrations (copies/µl) are shown in the bottom graph and used for calculating the percentage of methylation from each sample (see calculations). MSRE, methylation-sensitive restriction enzyme.

The percentage of *SEPT9* promoter methylation was calculated as shown in Table 4.

Table 4. *SEPT9* promoter methylation calculations.

	Methylated	Unmethylated
MSRE+ Ratio	335/208 = 1.611	2.33/266 = 0.0088
MSRE– Ratio	350/217 = 1.613	271/272 = 0.996
Methylation, %	1.611/1.613 = **100%**	0.0088/0.996 = **0.9%**

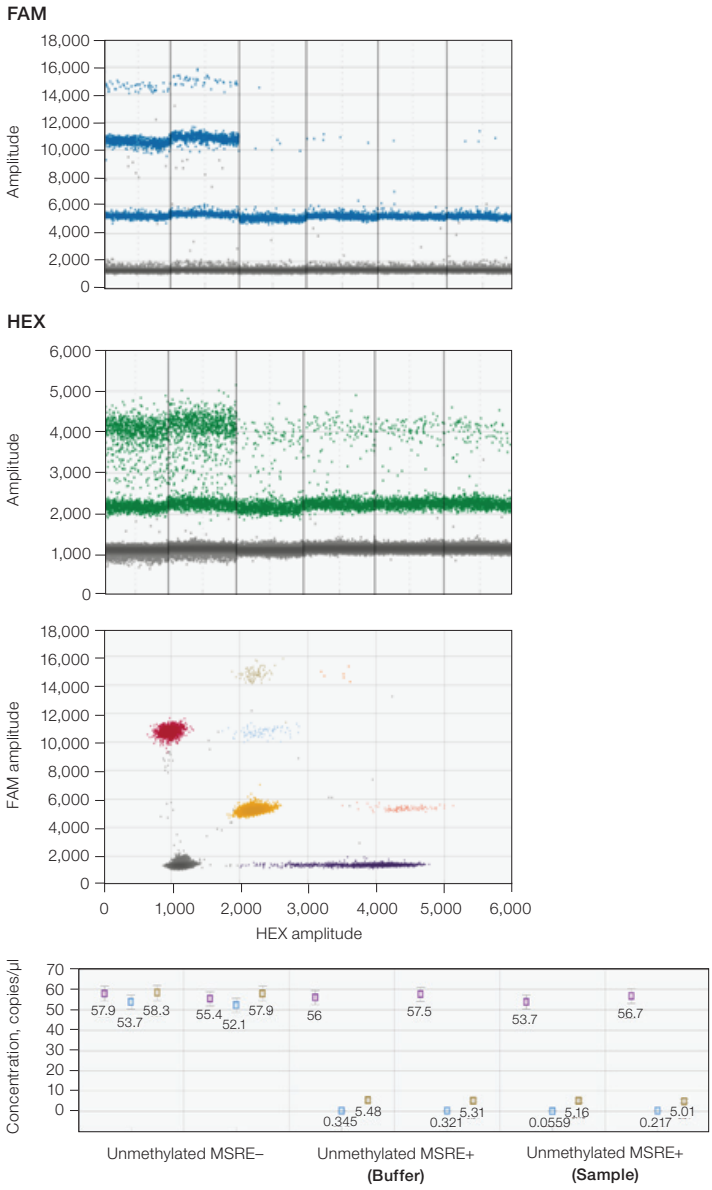


Fig. 5. Probe Mix Triplex *WIF1* (FAM), *NPY* (FAM), and *RPP30* (0.4x FAM, 0.25x HEX). One-dimensional plots show the positive and negative droplet clusters from three targets run in triplex. WIF1 (FAM, AssayID dHsaEXD31159278), NPY (FAM, AssayID dHsaEXD18238370), and RPP30 (0.4x FAM, AssayID dHsaCP2500313, 0.25x HEX, AssayID dHsaCP2500350). Two-dimensional plot shows up to eight clusters from the same reaction. A mixture of AciI and HhaI was used for digestion. Two replicate reactions of an unmethylated control sample were used to compare the digestion in sample (buffer: digestion overnight) with the digestion in master mix (sample: 45 min at room temperature). Concentrations (copies/µl) are shown in the bottom graph and used for calculating the percentage of methylation from each sample (see calculations). MSRE, methylation-sensitive restriction enzyme.

WIF1 and *NPY* methylation was calculated as shown in Table 5.

Table 5. *WIF1* and *NPY* methylation calculations.

	Overnight Digestion	ddPCR Master Mix Digestion
WIF1		
MSRE+ Ratio	0.345/56 = 0.0062	0.0559/53.7 = 0.00104
MSRE– Ratio	53.7/57.9 = 0.927	53.7/57.9 = 0.927
Methylation, %	0.0062/0.927 = **0.7%**	0.00104/0.927 = **0.1%**
NPY		
MSRE+ Ratio	5.48/56 = 0.0979	5.16/53.7 = 0.0961
MSRE– Ratio	58.3/57.9 = 1.007	58.3/57.9 = 1.007
Methylation, %	0.0979/1.007 = **9.7%**	0.0961/1.007 = **9.5%**

ddPCR Assay Design

Since analysis is based on restriction enzyme cleavage, the amplicon sequence must include cleavage sites. This approach provides high specificity, however only specific restriction sites can be analyzed, which can be a limitation. To reliably measure DNA methylation, we recommend including at least two but not more than four restriction sites in the amplicon. If a single MSRE cannot cut at least two sites in an amplicon, combining multiple MSREs that cut within the amplicon is a good alternative. For an extended overview on how to design specific ddPCR assays, please refer to the Assay Design for Droplet Digital PCR section in bulletin 6407.

Consider the following when designing primers:

- Check for restriction sites within the area of your target sequence
- Look for restriction enzymes that have 2 or more restriction sites close together
- Design primers outside these restriction sites to produce an amplicon with 2 or more restriction sites
- A restriction site can be included in the primers but only in the last 4 nucleotides of the 3' end of each of the primers. Restriction sites outside the primers are preferred
- Short amplicons (<100 nucleotides) enable fragmented sample analysis (e.g., formalin-fixed paraffin-embedded [FFPE] or circulating tumor DNA [ctDNA])
- Combining different restriction enzymes is possible and used to perform double digestions in the same reaction

Example Assay Design

Figure 6 shows an example assay design for the transcription factor one cut homeobox 2 (ONECUT2).

Amplicon: 64 bp, location hg19|chr18:55103715-55103778:+

Sequence: AAATGCTCAGCCCCAACTTCGA**CGCG**CACCACACT GCCATGCTGACC**CGCG**GTGAGCAACACC

Fig. 6. Example assay design for the transcription factor ONECUT2 (BstUI restriction sites). Bio-Rad PrimePCR AssayID: dHsaEXD31605634. BstUI sites are bolded, primer sites are underlined, and the probe site is highlighted yellow. Chr, chromosome; hg, human genome.

Cytosine Deamination

While MSRE digestion protocols are rapid and reliable, sodium bisulfite conversion is the current gold standard of methylation analysis. In this method, DNA is denatured and treated with sodium bisulfite. This process deaminates unmethylated cytosine in DNA to uracil and leaves methylated cytosines unchanged. Amplifying the treated DNA with PCR converts uracils further to thymines while the methylated cytosines remain cytosines. An example of cytosine deamination is shown in Figure 7.

Although bisulfite treatment is relatively fast and straightforward, it requires elevated temperatures and high pH that can damage DNA, resulting in fragmentation and depyrimidination. Unmethylated cytosines are especially sensitive to degradation with this method, resulting in challenges amplifying GC-rich areas.

A less damaging method for cytosine deamination was recently developed by New England Biolabs, Inc. (NEB). In this method, APOBEC enzyme is used to convert cytosine to uracil, resulting in far less DNA degradation. However, APOBEC can also deaminate 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC). To protect the methylated cytosine from deamination, NEB uses TET2 enzyme and Oxidation Enhancer (#E7129) to modify 5mC and 5hmC to forms that are not substrates for APOBEC.

When deciding which method to utilize, the required starting material should be considered. Sodium bisulfite protocols typically require between 0.1 and 2 µg of DNA (depending on the manufacturer) while the enzymatic approach can be performed with much less.

Since both methods rely on the conversion of unmethylated DNA to thymine, primers and probe designs are the same for both.

Template	**A:** 5'–GACCGTTCCAGGTCCAGCAGTGCGCT –3' **B:** 3'–CTGGCAAGGTCCAGGTCGTCACGCGA –5'
Cytosine Deamination	**A:** 5'–GATCGTTTTAGGTTTAGTAGTGCGTT –3' **B:** 3'–TTGGCAAGGTTTAGGTTGTTATGCGA –5'

Fig. 7. Example of cytosine deamination on template DNA strand. Underline denotes methylated cytosine. Note that sense and antisense strands are no longer complementary after deamination.

Primer Design

There are two main design strategies available to specifically amplify converted (unmethylated) and unconverted (methylated) sequences with Droplet Digital PCR: methylation-specific primers and methylation-independent primers (Figure 8).

Primer design considerations for methylation-specific primers:

- Include as many CpG sites as possible, especially at the 3' end of the primer
- Consider the same CpG sites in the primer sequence for methylated DNA and unmethylated DNA
- Maximize specificity by using a detection probe including CpG sites

Primer design considerations for methylation-independent primers:

- No CpG sites within the primer sequence
- Use an adequate number of C (no CpG)
- Spanning a maximum number of CpG sites in the amplicon
- Maximize specificity by using a detection probe including CpG sites

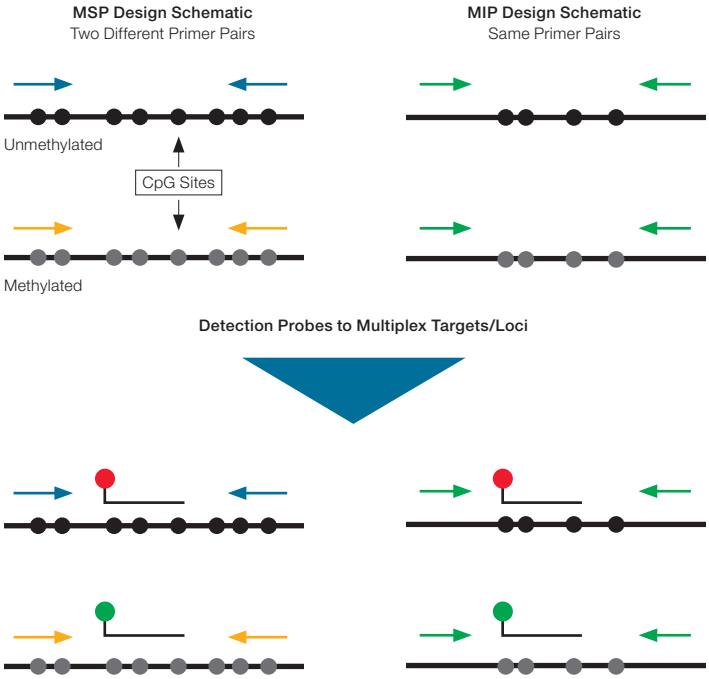
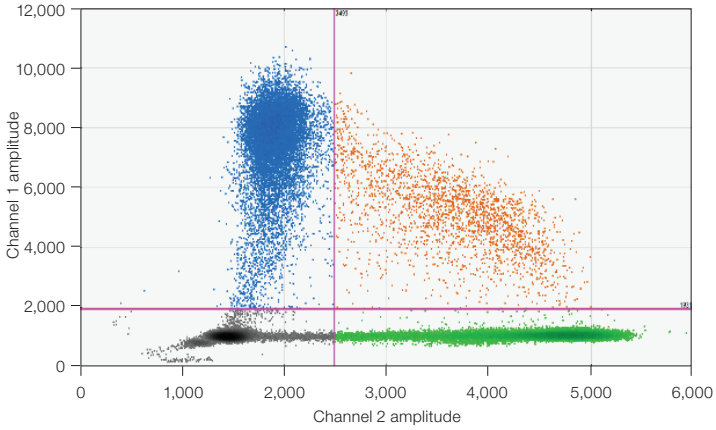


Fig. 8. Comparison of methylation-specific and methylation-independent design strategies. CpG, 5'-cytosine-phosphate-guanine-3'.

Example Performance (Rat *SNRPN*)

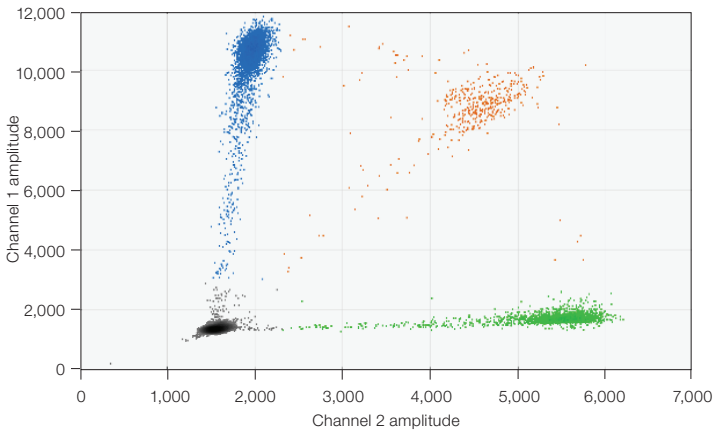
Figure 9 shows examples of the performance of methylation-specific and methylation-independent primers.

Ch1+Ch2+:2284, Ch1+Ch2–:14314, Ch1–Ch2+:59623, Ch1–Ch2–:189385



Methylated sense forward	TATTGTAGTTAGAGAGTGATATGGATT
Methylated sense reverse	CTAAACTTACCGCAATAACTCAAAT
Methylated probe	FAM-ACTACTCCCTACGCATACGTCCAAA
Unmethylated sense forward	TTGTAGTTAGAGAGTGATATGGATT
Unmethylated sense reverse	ACTAAACTTACCACAATAACTCAAAT
Unmethylated probe	HEX-ATACTACTCCCTACACATACATCCAAAACA

Ch1+Ch2+:420, Ch1+Ch2–:3851, Ch1–Ch2+:2306, Ch1–Ch2–:340439



Methylation-independent primer forward	AGTtAGAGAGTGAtATGGA
Methylation-independent primer reverse	CAACACCCTCTACTCTCT
Methylated probe	FAM-TTGGACGtATGCGTAGG
Unmethylated probe	HEX-TTGGAtGtATGtGTAGGGAG

Fig. 9. Two-dimensional plot showing the duplex reaction for rat *SNRPN* promotor from a mixture of methylated and unmethylated control samples. **A**, methylation-specific primers (target: chr1:111592360-111593159 [reverse complement]); **B**, methylation-independent primers (target rn7| chr1:111123593-111123731). Chr, chromosome.

Summary

Methylation-sensitive primers have the advantage of detecting methylation levels at the primer-binding region but have the disadvantage of needing two different primer sets.

Methylation-insensitive primers have the advantage of a single primer set, which should compensate for PCR bias and can be combined with high resolution melt analysis, resulting in methylation information at the regional level (amplicon).

Neither method gives information on individual CpG resolution levels.

Conclusions

Droplet Digital PCR has been utilized in many new genetic analysis applications. Adding methylation-based detection tools to its already large portfolio of applications highlights the continuously growing utility of Droplet Digital PCR.

While many conventional methods are available for cytosine deamination–based methylation analysis (methylation-specific PCR, high resolution melt analysis, pyrosequencing, etc.), several publications have demonstrated the added value that Droplet Digital PCR provides (van Wesenbeeck et al. 2018, Cho et al. 2020, Hagelans et al. 2017, Lehmann-Werman et al. 2018, Larsen et al. 2018, Jensen et al. 2019).

Methylation detection using MSRE-based digestion in combination with Droplet Digital PCR (MSRE ddPCR) is a relatively new technique (Nell et al. 2020, Wang et al. 2021, van Zogchel et al. 2021, van de Leemkolk et al. 2022). Advantages for this method include negligible DNA degradation and a minimum amount of required starting material. These parameters make this method well suited for applications that require liquid biopsy samples. Additionally, because the DNA is preserved, other genetic alterations such as copy number variants and single nucleotide variants can be performed on the same sample.

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