RainDrop® Digital PCR System Quantifies Methylated DNA Biomarkers

This application note illustrates the RainDrop digital PCR System's capability to quantify small changes in DNA methylation. Highlighted is analysis using Zymo Research's OneStep qMethyl™ kit with low amounts of tumor DNA in single- and multiplex assay formats.

DNA Methylation

DNA can be modified by methylation of cytosine bases, particularly cytosines preceding guanines (CpG dinucleotides). DNA methylation is an important epigenetic modification involved in the regulation of gene expression and stable gene silencing, and can be used as a biomarker in human disease profiling. Various methods have been developed to analyze DNA methylation levels, both across the genome and at specific loci, in order to discover and interrogate disease relevant loci for methylation-based transcriptional control. Methods able to quantitatively measure differences in DNA methylation between normal and cancer cells provide promising sources for biomarker identification and assessment.

Bisulfite Not Required

Bisulfite treatment of DNA is commonly performed in methylation analysis but PCR amplification of bisulfite-treated DNA can sometimes pose technical challenges, including low recovery of amplifiable material and challenges to conventional polymerases due to the presence of uracils in the treated material. Zymo Research's OneStep qMethyl™ Kit supports the detection of locus-specific DNA methylation by digestion of the sample with methylation-sensitive restriction enzymes, using standard qPCR assay reagents. Combining the proven advantages of digital PCR with this bisulfite-independent approach creates a simple quantitative methylation analysis platform.

Successful Start with Single- and Duplex dPCR Assays

MGMT promoter DNA methylation was able to be quantified using the OneStep qMethyl™ kit "right out of the box" in a single-plex format with only 20 ng input DNA and no optimization using the RainDrop Digital PCR System (Figure 1).

Next, a duplex assay using RAB25-VIC and MGMT-FAM probes was developed, and blends of methylated and non-methylated control DNA (15%, 25%, 30% and 100%) were tested. Example fluorescence scatter plots are show in Figure 2-A and the quantitative results are shown in Figure 2-B. High linearity and quantitative power were clearly demonstrated using the droplet-based dPCR method.

Figure 1
Fluorescence Intensity Scatter Plot

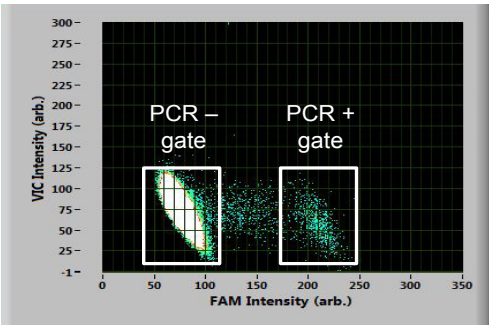
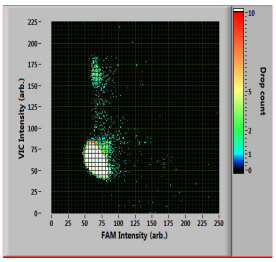
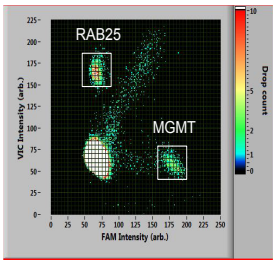


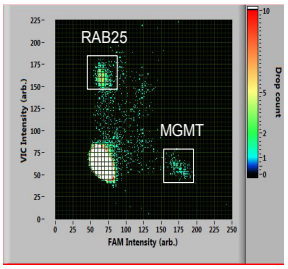
Figure 2-A



20ng
100% non-methylated

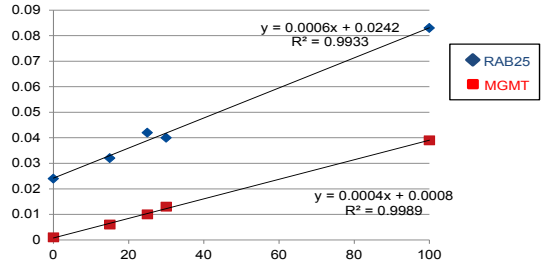


20ng
100% methylated



20ng
25% methylated
75% non methylated

Figure 2-B



Multiplex and Reference Assay Inclusion

The RainDrop dPCR System enables multiplexing of additional assays by inclusion of appropriate probes and primers[2]. An example 4-plex assay cluster plot that includes target promoters for Cyclin D2 (CCDN2) and Retinoic Acid Receptor B (RARB) is shown in Figure 3. A Reference assay targeting a non-methylated region can also be added, allowing normalizing ratios based on input genome equivalents (see Figure 4).

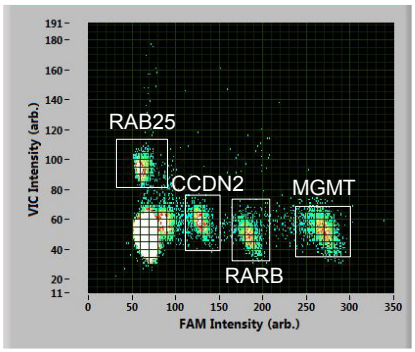
Validation Using Breast Cancer Samples

Two duplex digital PCR assays for CCDN2 and RARB were developed including a methylation-independent REF assay to provide normalized quantification. Stage II and Stage IV breast cancer samples were processed with the OneStep qMethyl™ Kit and quantified by dPCR. The cluster plots and count data in Figure 4 demonstrate the difference in Target/ REF ratio for tumor versus control between Stage II and Stage IV samples. CCDN2 and RARB promoters show higher methylation values in the tumor samples compared to the adjacent normal samples with CCDN2 showing a large increase in methylation in Stage II. These results were confirmed by qPCR (data not shown) and are consistent with the literature.

Conclusion

The RainDrop Digital PCR System provides an open analysis platform to rapidly deploy new advances in genomic analysis, including methylation quantification using bisulfite-treated or methylation-sensitive restriction enzyme-treated input. In this example, the droplet-based system demonstrated multiplex analysis of methylated DNA using the Zymo Research's OneStep qMethyl™ Kit. The combined system delivers high sensitivity and accurate DNA methylation quantification with low quantities of starting material, opening new opportunities for biomarker research.

Figure 3



References

1. Michael L. Samuels, Ryan Kemp, Frances Long, Jill Petrisko, Lam Nguyen, Manuel Krispin, Quantitative digital PCR analysis of cancer gene promoter methylation using low amounts of input DNA, Poster Presentation, AACR Conference, 2013.

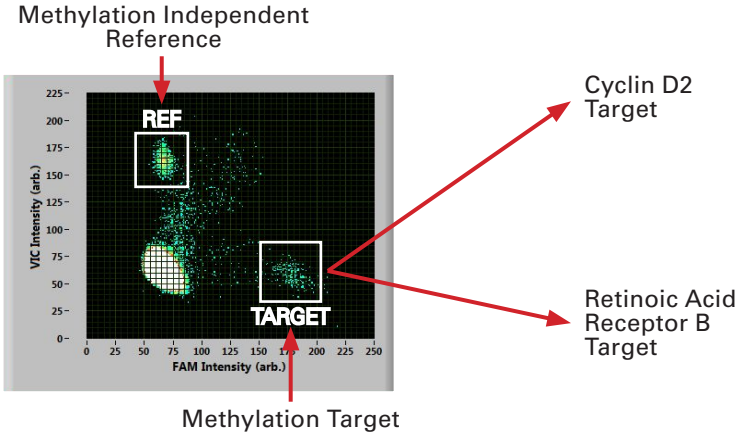
2. Zhong Q, et al, Multiplex PCR: breaking the one target pr color barrier of quantitative PCR, DOI: 10.1039/c1lc20126c.

4. Levenson VV. DNA methylation as a universal biomarker. Expert Rev Mol Diagn. 2010:10(4):481-8

5. Sunami E., Shinozaki M., et.al. Estrogen receptor and HER2/neu status affect epigenetic differences of tumor-related genes in primary breast tumors. Breast Cancer Research 2008:10(3) r46.

The RainDrop Digital PCR System is for Research Use Only; not for use in diagnostic procedures.

Figure 4



	STAGE II		STAGE IV	
Assay VIC/FAM	REF/CCDN2	REF/CCDN2	REF/CCDN2	REF/CCDN2
Template	Tumor	Control	Tumor	Control
ng Input	20	20	40	20
ul Input	25	25	25	25
# Droplets	2821297	2695102	2777435	2459884
# NEG	2802492	2682575	2768332	2443456
# REF	6424	8066	7339	12673
# CCDN2	720	61	96	28
%CCDN2/REF	11.2	0.8	1.3	0.2

	STAGE II		STAGE IV	
Assay VIC/FAM	REF/RARB	REF/RARB	REF/RARB	REF/RARB
Template	Tumor	Control	Tumor	Control
ng Input	20	20	40	20
ul Input	25	25	25	25
# Droplets	2210248	2164475	2486639	2485516
# NEG	2198442	2148965	2464390	2473954
# REF	3403	5450	4981	6203
# RARB	490	374	536	381
%RARB/REF	14.4	6.9	10.8	6.1

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