A droplet digital PCR assay for detection of methylated *BCAT1* and *IKZF1* in circulating tumor DNA

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Introduction

Circulating tumor DNA (ctDNA) can be detected in patients with colorectal cancer (CRC) using a qualitative real-time PCR test (qPCR) assaying for methylated *BCAT1* and *IKZF1* DNA in blood. Quantitative assessment of ctDNA levels may have clinical utility, including informing effectiveness of tumor debulking and response to chemo/radio therapy. The aim of this study was to analytically and clinically evaluate a quantitative test for *BCAT1* and *IKZF1* using droplet digital PCR (ddPCR).

Methods

PCR Assays: PCR assays were developed for either the QX200 ddPCR (Bio-Rad) or LightCycler 480 II (Roche) platforms. The qPCR method is a triplex assay, detecting a control gene, *ACTB, and* methylation within the genes *BCAT1* and *IKZF1*. The ddPCR method is a duplex assay that does not detect *ACTB*. Refer to table 1 for assay conditions. Results were reported as methylated DNA (*BCAT1+IKZF1*) per mL of plasma input.

Analytical testing: Analytical samples included pooled plasma, from presumed normal donors (age < 30yo), spiked with two-fold dilutions of fully methylated DNA (Millipore) from 2.3 to 300 pg/mL (30 sample replicates per concentration). For each concentration, the resulting bisulphite treated DNA was pooled and tested as 40 PCR replicates using either ddPCR or qPCR.

Clinical testing: Clinical samples were obtained from colonoscopy confirmed subjects. DNA extracted from 4mL of plasma was bisulphite treated and assayed for methylated *BCAT1* and *IKZF1* DNA by triplicate analyses of 7µL bisDNA input into ddPCR and qPCR, the equivalent of 2mL of plasma per method. Qualitative detection of either methylation marker was deemed positive for ctDNA.

Table 1 I PCR Assay Conditions

Both PCR methods used oligonucleotides previously described in Symonds et al[1].

qPCR Conditions:

Assay conditions: Quantitect Multiplex PCR NoROX mastermix (QIAGEN; 2X) with 200nM each primer, 100nM each probe. 8uL template into 20uL final PCR volume.

Cycling conditions (LC480 II): 1 cycle 95°C/15 min; 50 cycles 95°C/15sec, 62°C/40sec with acquisition; 1 cycle 40°C, 10 sec.

ddPCR Conditions:

Assay conditions: ddPCR Supermix for Probes (No dUTP) (BioRad; 2X) with 450nM each primer, 250nM each probe. 8uL template into 20uL final PCR volume.

Cycling conditions (C1000): 1 cycle 95°C/10 min; 45 cycles 94°C/30sec, 58°C/60sec; 1 cycle 98°C/10 min; 12°C, hold. Ramp rate of 2°C/sec; volume 40µl (including oil).

Figure 1 I Analytical Testing: PCR Result Comparisons.

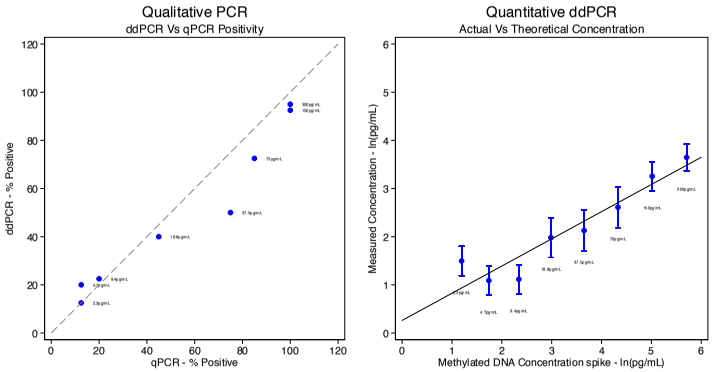
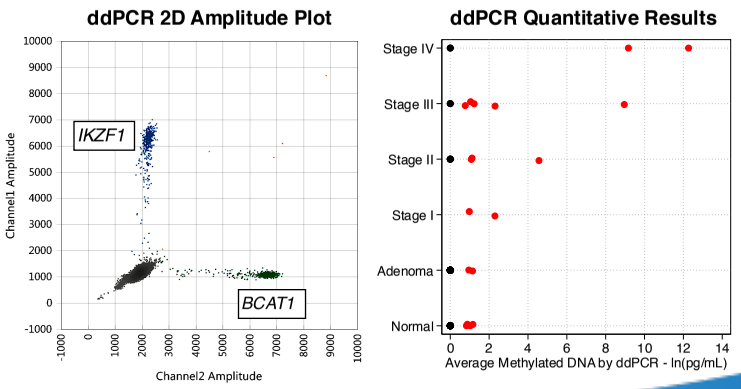


Table 2 I Contingency tables for Clinical Data Method comparison

	qPCR					
	+	-	+	-	+	-
ddPCR	Non-neoplastic		Adenoma		CRC	
+	3	1	1	1	9	3
-	1	15	0	18	4	4
P value[1]:	1.000		0.3173		0.7055	

[1]P-value generated using χ^2 test

Figure 2 I Clinical Testing: ddPCR Results.



Results

Analytical testing: Analytical testing determined an equivalent Limit of Detection for both assays when assuming an input of 4mL per sample or 5 PCR replicates (qPCR, 14.8 pg/mL; ddPCR, 15.9 pg/mL; Probit regression analysis). Figure 1 compares the qualitative results for both methods on each individual sample replicate and results correlate well (rho=0.97). The quantitative results for ddPCR are also shown by plotting the mean of the 40 spiked sample ddPCR replicates with a 95% confidence interval against the theoretical concentration of the sample. According to the ddPCR quantitative results there is an approximate loss of 90% of DNA during the DNA extraction and bisulphite conversion processes.

The ddPCR assay was estimated to be quantitative down to 75pg/mL, which equates to 90 copies of methylated DNA per 4mL of plasma. In contrast, the qPCR assay was not quantitative in the concentration range tested (i.e. the qPCR limit of quantification was >300pg/mL, data not shown).

Clinical testing: The two assays showed 82% concordance in 60 clinical samples, including 20 with no evidence of disease (NED), 20 adenoma and 20 CRC. Samples that were above the median concentration determined for the 75pg/mL of plasma spike (8pg/mL, *BCAT1* and *IKZF1* combined) were positive for both ddPCR and qPCR.

As per table 2 there was no significant difference in the qualitative positivity rates between the two PCR methods. Six of the 12 CRC positive cases were within the quantitative range of the ddPCR assay.

Conclusion

Detection of methylated *BCAT1* and *IKZF1* using ddPCR is comparable to the qPCR method used in this study. In the clinical cohort 50% of the ddPCR-positive CRC cases were within the quantitative range.

The ddPCR assay was further successfully developed as a triplex, using ACTB as a positive control gene, which maintained the distinction for all 3 markers using only 2 fluorophores. Further clinical evaluation of the triplex assay is required to appraise the utility of the quantitative assay.

[1]Symonds et al. Clinical and Translational Gastroenterology. 2016;7(1):e137-. doi:10.1038/ctg.2015.67.