Mutational analysis of circulating tumor cells using the IsoFlux™ System and QX100™ Droplet Digital™ PCR Platform

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ABSTRACT

Circulating tumor cells (CTCs) are rare cells found in the blood of cancer patients with solid tumors and play a key role in cancer dissemination. There has been considerable interest in analyzing these cells as a potential source of clinically-actionable information relating to molecular profile of the patient's disease. Numerous approaches have been employed to isolate and utilize CTCs for diagnostic and discovery applications. One of the current challenges in the field is high recovery and purity of CTCs followed by reliable detection of rare cell populations in a background of leukocytes. The IsoFlux System (Fluxion Biosciences) provides high recovery of CTCs in a format optimized for downstream analysis. The QX100 Droplet Digital PCR (ddPCR) System (Bio-Rad Laboratories) provides sensitive biomarker detection in a digital format. When combined, these two platforms provide a robust solution for analyzing rare mutations in CTCs obtained from a simple blood draw.

Here we developed a start to finish assay for quantitative mutational analysis of CTCs. We ran analytical validation of both the CTC isolation process and the digital mutation detection using a model CTC system (MDA-MB-231, heterozygous for KRAS G13D mutation, spiked into healthy donor blood). The IsoFlux System was shown to be effective in recovering CTCs from the model system. The digital mutation detection assay was characterized for sensitivity and linearity using titration studies with purified G13D mutant gDNA in a wild-type gDNA background. The assay was shown to be linear across the range tested and sensitive down to 0.06% mutant with wild-type background. Model CTC samples were tested in the range of 10-100 CTCs per 7mL blood. Mutations were detected with high probability across this entire range.

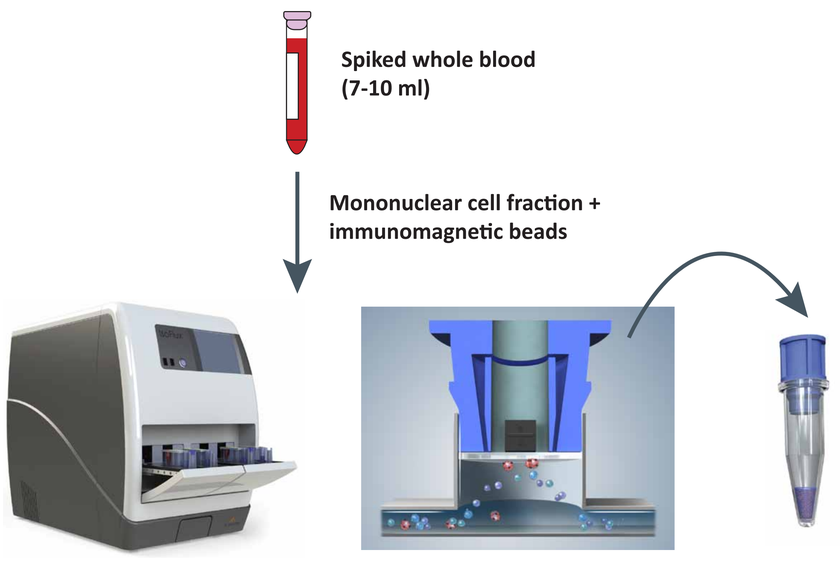
The results of these experiments indicate that the IsoFlux System can be combined with the QX100 ddPCR System to provide quantitative mutational analysis of CTCs captured from a simple blood draw. This assay workflow is also amenable to other mutations and biomarkers relevant to oncology.

METHODS AND WORKFLOW

Model CTC System - Spiked cancer cells (MDA-MB-231, heterozygous for G13D mutation) were spiked into fresh healthy human blood tubes (7mL, EDTA). Ficoll gradient was used to separate the mononuclear fraction.

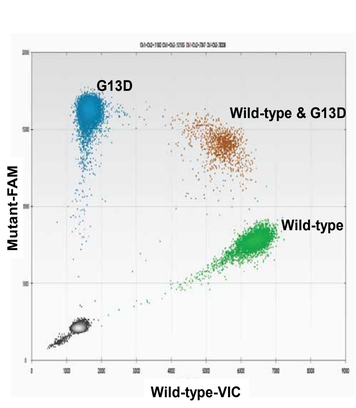
IsoFlux CTC Isolation - Samples were processed on the IsoFlux System using anti-EpCAM immunomagnetic beads. Enriched CTCs were eluted in less than 10µL and saved for either enumeration or mutation detection. Enumeration samples were counted using fluorescence microscopy.

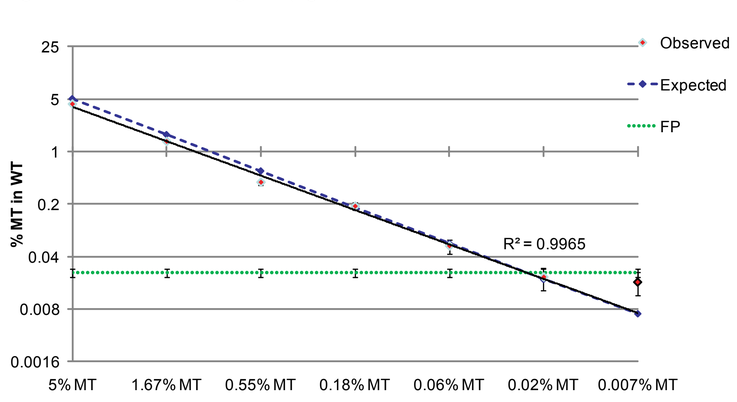
Droplet Digital PCR detection - gDNA was isolated from enriched CTC samples and processed using the QX100 Droplet Digital PCR System. Readout was given in terms of absolute number of mutant and wild-type copies detected.



RESULTS

ddPCR analytical validation
ddPCR assay for KRAS G13D mutation detection was optimized with purified gDNA from KRAS wild type human peripheral blood mononuclear cells and KRAS mutant MDA-MB-231 cells (heterozygous G13D KRAS mutation). A typical 2-D image of MDA-MB-231 gDNA in ddPCR was shown for the detection of both wild-type and G13D mutant KRAS (left). Assay sensitivity and linearity of KRAS G13D ddPCR was analyzed by titration studies with purified gDNA. ddPCR showed good linearity of detecting G13D mutation in a background of wild-type gDNA ranging from 0.007%-5%. The lower limit of detection as determined by false positive rate with wild-type gDNA was 0.06% mutant gDNA (right).

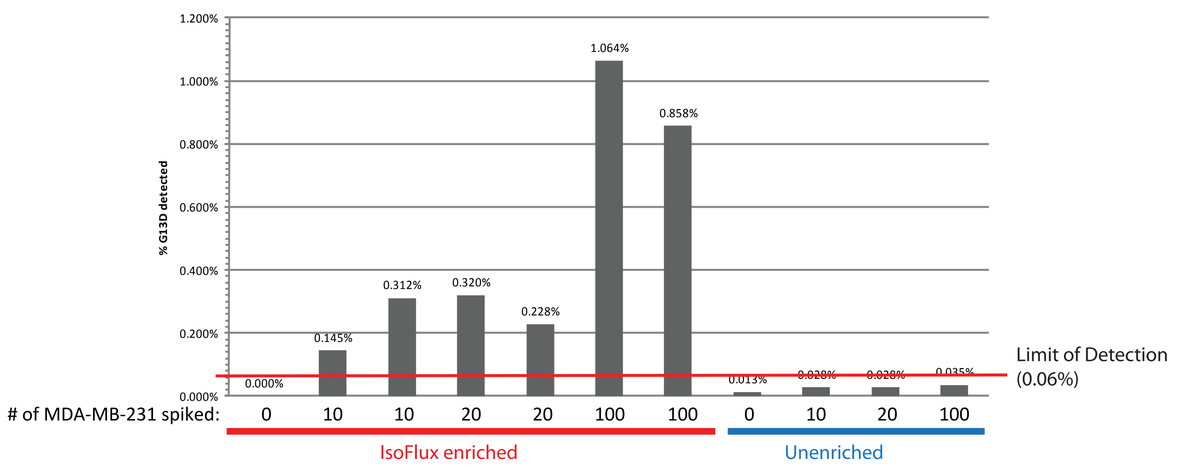




IsoFlux CTC Isolation
MDA-MB-231 cells were pre-labeled with fluorescent dye and spiked into 7 ml of fresh healthy human donor whole blood (with EDTA). Anti-EpCAM immunomagnetic beads were used to isolate and recover spiked MDA-MB-231 cells with the IsoFlux System. One set of the sample (4, 7, 10) were enumerated to determine total number of CTCs and nucleated background cells.

	Sample#	# MDA-MB-231 spiked	# MDA-MB-231 recovered	# Total nucleated cell recovered (background)	Theoretical G13D% in sample
IsoFlux processed cancer cell spiked blood samples (samples 4, 7, 10 were enumerated)	1	0			0%
	2	10	4	4508	0.044%
	3				
	4				
	5	21	15	4156	0.180%
	6				
	7				
	8	100	51	5716	0.446%
	9				
	10				
Pure MDA-MB-231 cells	11				50%
Non-IsoFlux processed cancer cell spiked blood samples*(estimated total cell # of 10,000,000)	12	0		10,000,000	0%
	13	10		10,000,000	0.0001%
	14	20		10,000,000	0.0002%
	15	100		10,000,000	0.0010%

QX100 ddPCR Detection of CTC mutations
gDNA isolated from both IsoFlux-enriched and un-enriched samples were tested by ddPCR for G13D mutation detection. IsoFlux-enriched spiked samples were positively detected by ddPCR to contain G13D mutations. In contrast, G13D mutations were not detected in unenriched matched spiked blood samples. The higher number cancer cell spiked samples could be discriminated from lower number cancer cell spiked samples.



Statistical analysis
Poisson counting statistics were used to determine the likelihood that the positives (G13D mutant) are true positives. IsoFlux-enriched samples and pure MDA-MB-231 samples positively detected for G13D mutation with high statistical confidence. IsoFlux-enriched samples have relatively low amount of gDNA, and thus, using the probability from low concentrations of wild-type gDNA as control for false positive rate is more appropriate.

Sample #	G13D copy detected	Wild-type copy detected	Prob. that this occurs by chance (use high WT gDNA as control for FP rate)	Prob. that this occurs by chance (use low WT gDNA as control for FP	% G13D detected	Theoretical G13D %
Wild type gDNA (high concentration)	16	88405	0.000002%			
Wild type gDNA (low concentration)	0	12429		0		
1	0	550	9.5000%	0	0.000%	0.000%
2	1	690	11.7000%	0	0.145%	
3	2	640	0.6200%	0	0.312%	0.044%
5	2	623	0.5900%	0	0.320%	
6	1	437	7.6044%	0	0.228%	0.180%
8	9	837	0%	0	1.064%	
9	5	578	0.00001%	0	0.858%	0.446%
11	46	24	0%	0	65.71%	50%
12	5	39937	84.7%	84.7%	0.013%	0%
13	9	32668	14.4%	14.4%	0.028%	0.0001%
14	10	36049	12.5%	12.5%	0.028%	0.0002%
15	12	34358	2.5%	2.5%	0.035%	0.0010%

CONCLUSIONS

- The IsoFlux System delivers high recovery of circulating tumor cells in an optimal format for downstream molecular analysis (high viability, low elution volume, low background)
- The QX100 ddPCR System demonstrated high linearity and sensitivity in detecting rare mutations in the presence of wild-type background
- Capturing CTCs with the IsoFlux System followed by ddPCR for DNA mutation detection provides a complete 'sample-to-answer' workflow for real-time information on tumor mutation status