Mutational analysis from circulating tumor cells using next-generation sequencing

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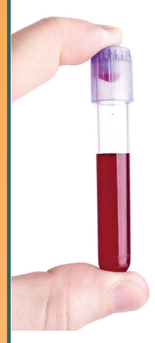
INTRODUCTION

Tumor mutational analysis provides insight into patient drug response, prognosis, and tumor biology. A key limitation to this process is the availability of tumor tissue that adequately represents the current disease status. This study presents a Next Generation Sequencing (NGS) workflow utilizing enriched circulating tumor cells (CTCs) as an input.

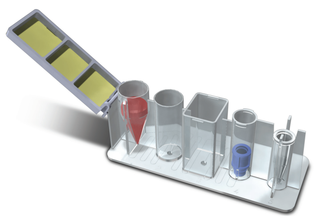
The CTC enrichment is performed using the IsoFlux System that accommodates multiple capture antibody cocktails and has been shown to be effective across multiple indications.

METHODS

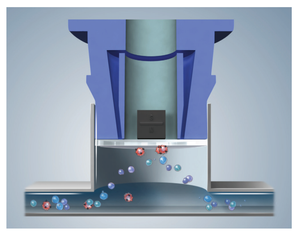


Sample Collection
- 2 x 10cc EDTA blood tubes were collected
- Shipped overnight at room temperature.

Bead Coupling
- RBCs removed with FICOLL
- EpCAM and EGFR magnetic beads added
- Sample loaded into microfluidic cartridge



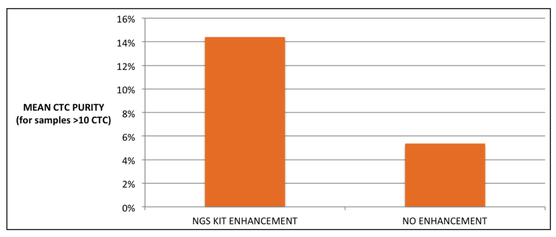
CTC Enrichment
- Cells pass through microfluidic isolation zone
- Target cells with beads captured on roof of channel
- Up to 4 samples/run, 12 samples/day, 4ºC controlled



NGS Analysis
- CTCs transfered in low volume (5µL)
- Purity enhancement with IsoFlux NGS Kit
- Illumina Workflow: enrichment with TruSight Tumor Panel (24 genes), NGS on MiSeq
- IonTorrent Workflow: enrichment with Ampliseq Cancer Hotspot v2 (50 genes), NGS on IonTorrent PGM
- Variant filtering and annotation using VarSeq

ANALYTICAL VALIDATION OF NGS WORKFLOW

Purity enhancement and NGS panel design



	ILLUMINA WORKFLOW	ION TORRENT WORKFLOW
Kit	MiSeq Reagent Kit V3	318 Chip Kit V2
Sequencer	MiSeq	Ion Torrent PGM
Total Reads	25M	5M
Targeted Panel	TruSight Tumor	Cancer Hotspot Panel v2
Oncogenes	26 genes	50 genes
Amplicons	174	207
Amplicon Size	165-195 bp	111-187 bp
Samples/run	9	6
Mean Depth	14,200x	3,600x

High purity enhancement: Bladder cancer samples (N=15) were processed using the IsoFlux NGS Kit that contains a purity-enhancement column. The mean purity in CTC-positive (>10) samples went from 5% to 15%, a level that is compatible with NGS analysis.

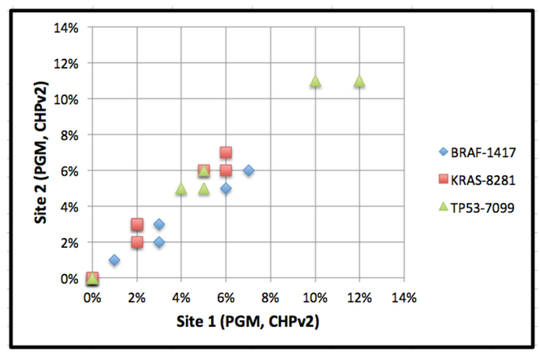
NGS design: Workflows were developed for Illumina and IonTorrent, the leading NGS platforms, using comparable tumor panels.

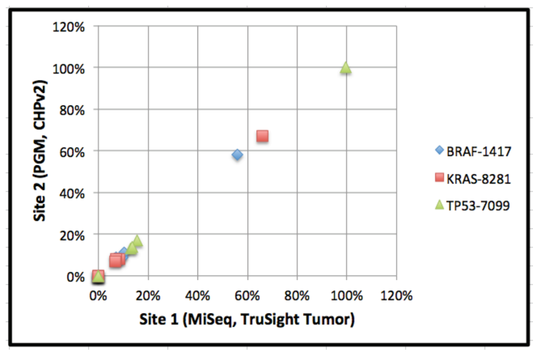
Variant detection in analytical samples

SAMPLES			MiSeq using the TruSight Tumor Panel				PGM using the CHPv2 Panel			
MDA-MB-231 Spike Level (cells/mL)	Recovery Rate	CTC Purity	Variant % BRAF-1417	Variant % KRAS-8281	Variant % TP53-7099	Other variants	Variant % BRAF-1417	Variant % KRAS-8281	Variant % TP53-7099	Other variants
Pure MDA	100%	N/A	56.0%	66.1%	99.5%	ND	58.0%	67.0%	100.0%	TP53 (3%, 31/143)
15	61%	73%	37.8%	38.9%	66.3%	ND	(not tested)			
7	44%	16%	9.6%	8.3%	15.7%	ND	9.0%	8.0%	17.0%	ND
7	44%	16%	7.0%	7.0%	13.0%	ND	9.0%	8.0%	13.0%	ND
3	60%	12%	10.3%	6.8%	13.5%	ND	11.0%	7.0%	14.0%	ND
2	71%	13%	(not tested)				4.0%	4.0%	11.0%	ND
0	0%	0%	ND	ND	ND	ND	ND	ND	ND	ND
0	0%	0%	ND	ND	ND	ND	ND	ND	ND	ND
0	0%	0%	ND	ND	ND	ND	ND	ND	ND	ND

Variant detection: Spiked samples (MDA-MB-231 cell line) were prepared at Fluxion and sent to multiple labs for analysis, using both IonTorrent and Illumina workflows. All of the 3 variants present in the cell line (KRAS, BRAF, TP53) were detected at all concentrations (range: 2-15 cells/mL spiked in).

Comparison across multiple sites and cancer panels

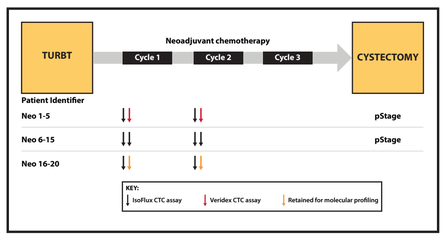


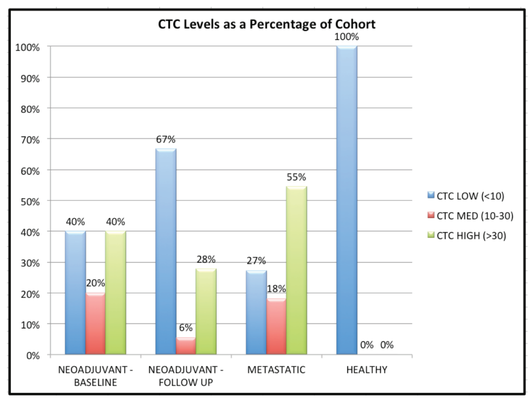


Site and panel comparisons: The same analytical samples were sent to two sites (UCLA, Thermo Fisher Scientific) for sequencing with the Ion Torrent workflow (left). Another set of duplicate samples were sent to two sites (UCLA, Moffitt) for comparison of the IonTorrent and Illumina workflows. Both comparisons showed detection of variants expected in the cell line with good alignment of allelic frequencies across all concentrations tested.

NGS ANALYSIS OF BLADDER CANCER SAMPLES

Study design and CTC recovery performance





Study design (left) : Twenty neoadjuvant bladder cancer patients were enrolled at time of transurethral resection of bladder tumor (TURBT). All patients had two tubes of blood drawn prior to the first cycle of neoadjuvant chemotherapy, and two tubes drawn after the first cycle (approximately one month later). One tube was used for enumeration, and the matched tube was used for molecular analysis (NGS).

CTC levels (right): 60% of neoadjuvant (early stage) patients and 73% of metastatic patients had >10 CTCs. This level went down to 34% following one cycle of chemo.

NGS analysis of bladder samples

PATIENT IDENTIFIER	CANCER STAGE	CTC COUNT	CTC PURITY	SOMATIC VARIANTS DETECTED
Healthy	Healthy	0	0%	N/D
Healthy	Healthy	0	0%	N/D
Healthy	Healthy	0	0%	N/D
Healthy	Healthy	0	0%	N/D
N17 - 1mo follow up	Neoadjuvant	40	10%	FGFR2
N18 - 1mo follow up	Neoadjuvant	100	19%	PDGFRA
N22*	Neoadjuvant	20	12%	N/D
N22 - 1 mo follow up*	Neoadjuvant	21	8%	EGFR
M9	Metastatic	154	25%	JAK2
M10	Metastatic	34	4%	N/D
M12*	Metastatic	11	15%	N/D
M13*	Metastatic	8	N/D	N/D
Positive control #1 (80 MDA-MB-231 spike in sample)	Positive Control	N/D	N/D	KRAS, BRAF, TP53
Positive control #2 (80 MDA-MB-231 spike in sample)	Positive Control	60	8%	KRAS, BRAF, TP53

Mean CTC Purity	**13%**
% Samples > 5% Purity	**86%**
% of Samples with Somatic Mutation Detected	**50%**

NGS on clinical samples: Bladder cancer samples (N=4 neoadjuvant, N=4 metastatic) were enriched for CTCs, lysed, amplified, and sent through the NGS analysis workflow (IonTorrent). Somatic variants were detected in 4/8 (50%) of samples. Mean CTC purity was 13%, and 86% of samples had >5% CTC purity (target threshold for NGS analysis).

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All patients who contributed samples to these studies

CONCLUSIONS

- **The IsoFlux System provides a platform for performing NGS analysis on blood biopsy samples in oncology**
- **Multiple capture antibodies enhances CTC recovery, and IsoFlux NGS Kit enhances CTC purity**
- **NGS workflow has been developed and validated to produce high-confidence somatic variants, with good alignment between multiple sites, NGS platforms, and cancer-specific NGS panels**
- **IsoFlux currently being used in numerous translational studies to monitor patients and profile tumor cells using NGS analysis**