Fluxion Biosciences and Swift Biosciences

Somatic variant detection from liquid biopsy samples using targeted NGS

OVERVIEW

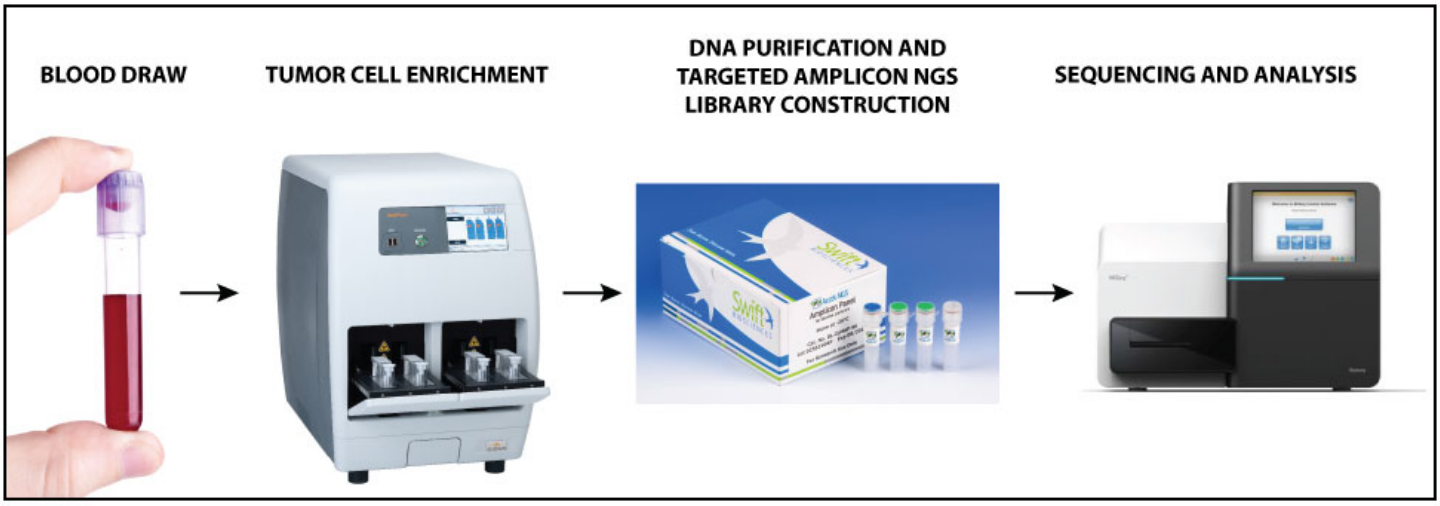
This application note describes a robust method for detecting somatic mutations from liquid biopsy samples by combining circulating tumor cell (CTC) enrichment with a high sensitivity targeted next-generation sequencing (NGS) panel. The combined workflow (Figure 1) has broad implications for how cancer can be monitored non-invasively using a routine blood draw without the need for tissue biopsies. Genomic aberrations detected with this approach can help elucidate the genomic signature of the cancer unique to each patient, and ultimately, can help guide therapeutic decisions.

- The **IsoFlux™ System** (Fluxion Biosciences) enriches rare CTCs from peripheral blood samples and prepares them at high purity for molecular analysis, including NGS. Multiple capture antibodies are employed, including both epithelial and mesenchymal markers, to capture the widest spectrum of CTCs in the circulation. The captured CTCs are lysed and DNA is recovered for genomic analysis.

- The **Accel-Amplicon™ 56G Oncology Panel** (Swift Biosciences) covers 56 clinically-relevant cancer genes in a targeted amplicon format for use with Illumina® sequencers. The panel accommodates low DNA input levels making it ideally suited for liquid biopsy applications.

The data presented includes analytical validation of the combined workflow using spiked tumor cell samples. A range of clinically-relevant tumor cell concentrations going down as low as 2 cells / mL of blood was tested. The overall detection sensitivity was 94-100% with very low false positive rates. Low DNA inputs (1ng total DNA) from unamplified CTC samples were compared against whole-genome amplified (WGA) CTC samples (1µg total DNA) and the results were equivalent, confirming the ability of the workflow to accommodate low DNA input levels. The combined workflow is readily deployable in the clinical research environment using widely available tools and reagents.

Figure 1. Overview of complete workflow for somatic mutation detection from liquid biopsy samples.



METHODS

Analytical sample preparation

Twelve analytical samples, each consisting of 14mL of healthy donor blood, were spiked with the adenocarcinoma breast cancer cell line, MDA-MB-231, at different concentrations in triplicate (Table 1). This cell line was selected for its mesenchymal-like properties and low EpCAM expression to more closely approximate clinical samples. The MDA-MB-231 cell line harbors the following known mutations:

- BRAF chr7:140481417 heterozygous c.1391G>T, p.G464V
- KRAS chr12:25398281 heterozygous c.38G>A, p.G13D, and
- TP53 chr17:7577099 homozygous c.839G>A p.R280K

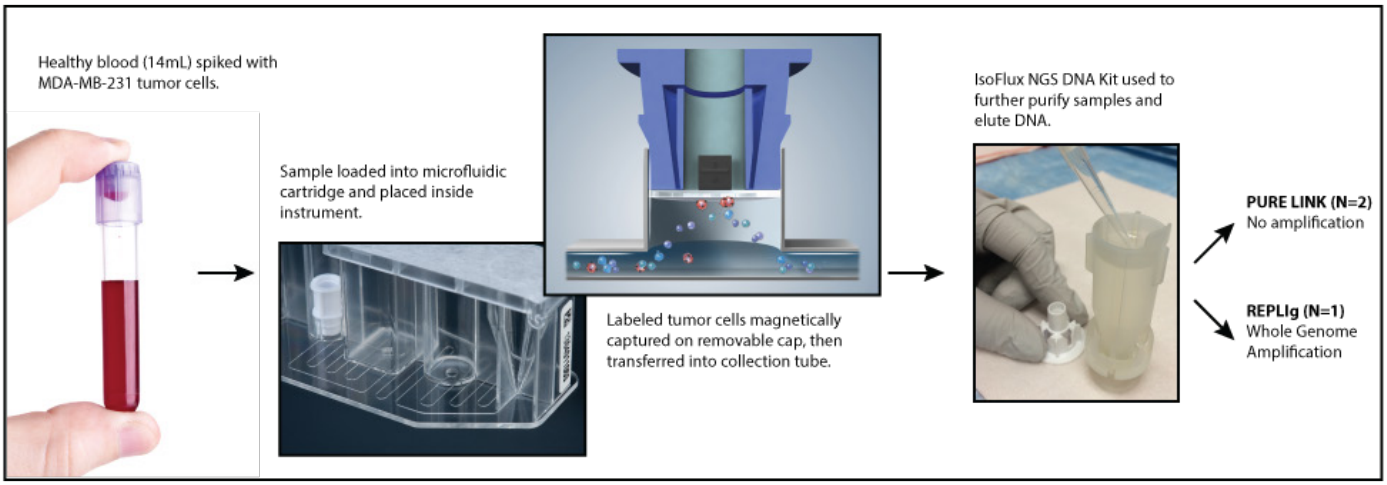
Table 1: Samples used in this study.

Sample	MDA-MB-231 (cells/mL)	MDA-MB-231 (total cells)	Expected Allele Frequency for Heterozygous BRAF & KRAS Mutations	Expected Allele Frequency for Homozygous TP53 Mutation
Negative Control (N=3)	0	0	0%	0%
Low Spike (N=3)	2	28	2.5%	5%
Medium Spike (N=3)	4	56	5%	10%
High Spike (N=3)	8	112	10%	20%

CTC Enrichment with the IsoFlux System

The IsoFlux System was used to enrich the cell admixtures following standard procedures. Anti-EpCAM and anti-EGFR antibodies were used to enrich for CTCs. Enriched CTCs transfer directly into the IsoFlux NGS DNA Kit that brings the overall CTC purity >10% and collects the lysate for DNA recovery. Genomic DNA (gDNA) was extracted from two of each of the three admixtures using the PureLink® Genomic DNA kit (Life Technologies) and yielded approximately 1ng of purified gDNA, while the third replicate was directly processed with the QIAGEN REPLI-g® Whole Genome Amplification (WGA) kit and yielded approximately 1µg of amplified material.

Figure 2. CTC enrichment workflow using the IsoFlux System.



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NGS Workflow with the Accel-Amplicon 56G Oncology Panel

Illumina-compatible multiplexed amplicon libraries were prepared from the samples according to the Accel-Amplicon 56G Oncology Panel workflow using 0.5ng DNA for Non-WGA and 10ng DNA for WGA samples. The number of cycles for the Multiplex PCR Step was increased to 4+23 for Non-WGA samples. Libraries were quantified by qPCR and loaded on the MiSeq® using v2 reagents. Sequencing data were aligned with BWA (Li and Durbin, 2010) and variant calling was performed using GATK Best Practices (Broad Institute), LoFreq haplotypecaller (Wilm et al., 2012), and SAMTOOLS mpileup (Li et al., 2009). Final variant filtering was performed using VarSeq (Golden Helix) using a combination of sequencing, statistical, and functional metrics.

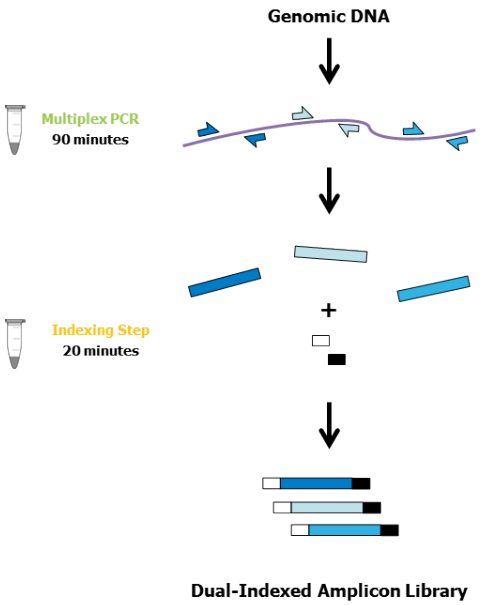
Table 2. Genes represented in the 56G Oncology Panel and number of amplicons.

ABL1	5	CSF1R	2	FBXW7	6	GNAS	2	KIT	14	NPM1	1	SKT11	5
AKT1	2	CTNNB1	1	FGFR1	2	HNF1A	4	KRAS	3	NRAS	3	SMAD4	10
ALK	2	DDR2	1	FGFR2	4	HRAS	2	MAP2K1	5	PDGFRA	4	SMARCB1	14
APC	9	DNMT3A	1	FGFR3	6	IDH1	1	MET	6	PIK3CA	11	SMO	5
ATM	19	EGFR	9	FLT3	4	IDH2	2	MLH1	1	PTEN	14	SRC	1
BRAF	2	ERBB2	4	FOXL2	1	JAK2	2	MPL	1	PTPN11	2	TP53	21
CHD1	3	ERBB4	8	GNA11	2	JAK3	3	MSH6	4	RB1	1	TSC1	1
CDK-N2A	2	EZH2	1	GNAQ	2	KDR	9	NOTCH1	3	RET	6	VHL	3

The Accel-Amplicon 56G Oncology Panel includes both clinically relevant hotspot loci and regions of contiguous coverage, depending on the allele distribution across each target gene. The table depicts the genes represented, followed by the number of amplicons for each gene.

- Contiguous, overlapping coverage is included for APC, ATM, EGFR, FBXW7, FGFR3, HNF1A, KIT, MSH6, PIK3CA, PTEN, SMAD4, and TP53.
- Comprehensive coding exon coverage is included for TP53.

Figure 3. NGS workflow using the Accel-Amplicon 56G Oncology Panel.

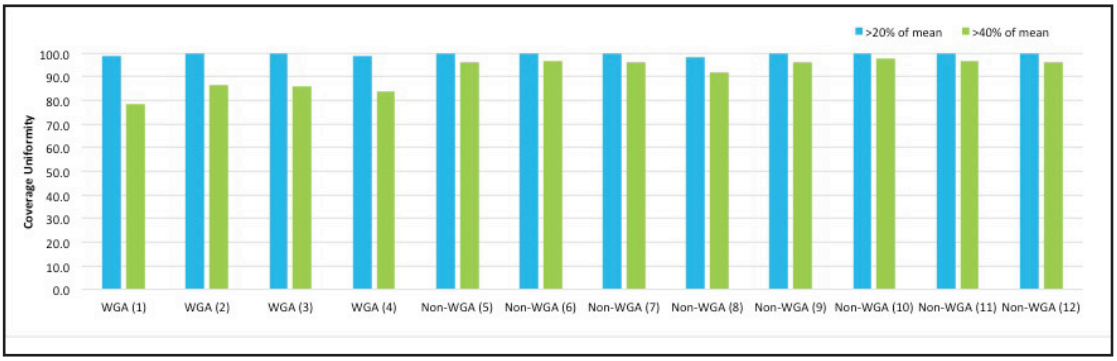


RESULTS

Sequencing performance

The mean read depth for amplicon targets across all the non-WGA gDNA samples was 7900x, versus 12,300x for the amplified (WGA) samples. Coverage uniformity, defined as the percentage of loci having read depth >20% of the mean, was >98% for all samples. Figure 4 illustrates the coverage uniformity for all samples at levels of >20% and >40% of the mean coverage.

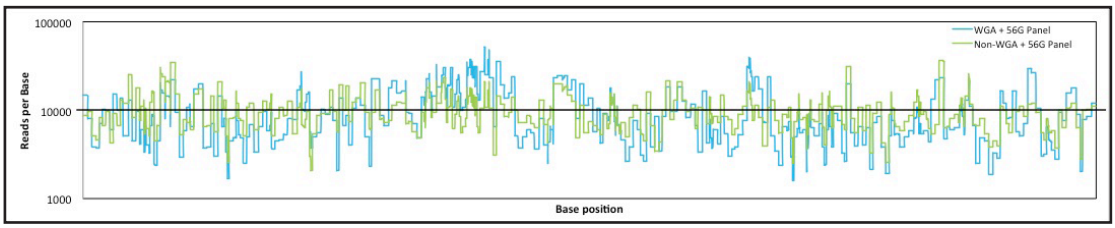
Figure 4. Coverage uniformity across all reference sites on the panel.



(A) Coverage uniformity is shown for WGA-amplified and non-WGA samples processed with the Accel-Amplicon 56G Oncology Panel at levels >20% of the mean coverage and >40% of the mean coverage.

	Input DNA	Total Reads (M)	Average Coverage	Coverage Uniformity (> 20% avg)	% On-Target
WGA + 56G Panel (1)	10ng	3.9	15476	98.6	97.0%
WGA + 56G Panel (2)	10ng	3.2	12669	99.2	96.4%
WGA + 56G Panel (3)	10ng	3	11960	99.7	96.6%
WGA + 56G Panel (4)	10ng	2.8	11365	99.0	96.5%
No WGA + 56G Panel (5)	0.5ng	2	7952	100	94.9%
No WGA + 56G Panel (6)	0.5ng	2.1	8363	100	95.0%
No WGA + 56G Panel (7)	0.5ng	2	7862	100	94.6%
No WGA + 56G Panel (8)	0.5ng	2.2	8965	98.3	96.9%
No WGA + 56G Panel (9)	0.5ng	2.2	8629	100	95.0%
No WGA + 56G Panel (10)	0.5ng	1.8	6988	100	94.7%
No WGA + 56G Panel (11)	0.5ng	1.7	6477	99.8	94.8%
No WGA + 56G Panel (12)	0.5ng	2	7777	100	93.8%

(B) The number of reads, resulting average coverage per sample, coverage uniformity values, and on-target percentage are consistent across samples and WGA vs. non-WGA workflows.



(C) The normalized reads per base across the 56G Oncology Panel targets is compared between a WGA sample and a non-WGA sample (horizontal line represents mean coverage).

Detection sensitivity

The detection sensitivity for each type of DNA preparation (non-WGA and WGA-amplified DNA) is shown in Table 3. The total number of true positive mutations is calculated as the 3 known mutations in the cell line (KRAS, BRAF, TP53) times the number of samples in each group, yielding 9 (3x3) and 18 (3x6) true positives available for the WGA and No-WGA (PureLink) groups, respectively. From the final variant list emerging from the bioinformatics pipeline, the 3 known mutations were considered true positives and anything else was considered a false positive. The overall detection sensitivity was 100% for the WGA group and 94% from the Unamplified (PureLink) group (one BRAF mutation was undetected from the mid-spike group). The total number of false positives was 2 for the WGA group (0.5 per sample) and 0 for the Unamplified (PureLink) group (0 per sample).

Table 3: Known mutation detection levels and sensitivity across all samples.

Samples Grouped by DNA Prep Type	Total True Positives Available	True Positives Detected	Sensitivity	Total False Positives (all samples)	False Positives / Sample
WGA	9	9	100%	2.0	0.6
Non-WGA	18	17	94%	0.0	0.0

CONCLUSIONS

The application described here provides a method to characterize genomic mutations from liquid biopsy samples using a sensitive CTC collection system and targeted NGS panel. CTCs can be collected from most solid tumor indications and the capture antibodies can be tailored to specific applications. The NGS panel has been shown to work with low starting DNA inputs and to be capable of detecting low frequency variants. The complete workflow can be established in any lab using the tools and reagents mentioned within.

For more information on the IsoFlux System, please visit: www.fluxionbio.com/isoflux
For more information on the Accel-Amplicon 56G Oncology Panel, please visit: www.amplicons.com

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