APPLICATION NOTE

LiquidBiopsy Platform and Ion S5 XL System

Mutational analysis of cfDNA, CTC, and germline DNA from a single blood sample

Key findings

- From a single blood sample, variants in circulating tumor cells (CTCs), cell-free DNA (cfDNA), and genomic DNA (gDNA) from white blood cells (WBCs) can be analyzed simultaneously
- The LiquidBiopsy™ Platform enables recovery of as few as 3–10 tumor cells per 7.5 mL sample of blood; automated DNA recovery from plasma is equivalent to manual isolation methods
- Per sample, single nucleotide variants (SNVs) and insertions or deletions (indels) were detected with the Ion AmpliSeq™ Cancer Hotspot Panel v2 at ≥1% allele frequency with a sensitivity of >95% for ~2,800 mutational hotspots in 50 known oncogenes and tumor suppressor genes

Introduction

This application note describes a workflow for isolating CTCs, cfDNA, and gDNA from WBCs for use as germline controls from a single sample using the LiquidBiopsy Platform followed by variant analysis using the Ion AmpliSeq Cancer Hotspot Panel v2, the Ion S5™ XL System, and Ion Reporter™ Software (Figure 1).

Isolation and preparation of three DNA sample types from a single blood sample

Initially, a 10 mL blood sample was prepared with an equal volume of fixative that keeps the sample stable for 96 hours and facilitates easier sample collection, faster study accrual, and room temperature shipping.

LiquidBiopsy Platform workflow

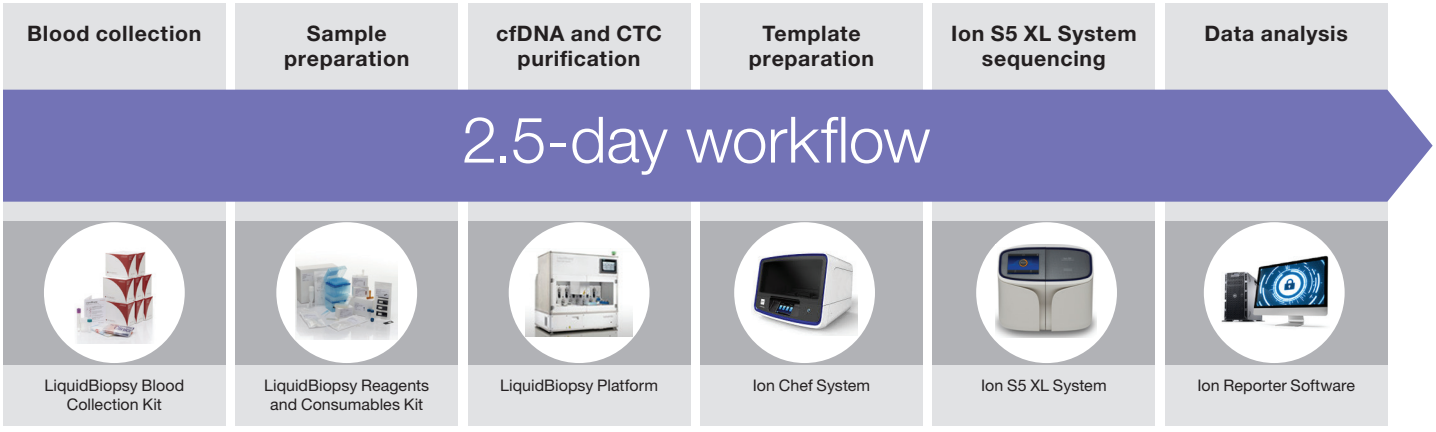

Figure 1. Comprehensive sample-to-results workflow for analysis of CTCs, cfDNA, and germline DNA collected from a single blood sample using the LiquidBiopsy Platform and Ion S5 XL System. The automated LiquidBiopsy Platform workflow is designed to efficiently recover: CTCs through isolation flow cell priming, blood sample loading, target cell isolation, and immunofluorescence labeling of captured cells, which allows high-content microscopic imaging; and cfDNA through manual or automated target nucleic acid isolation.

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DNA from white blood cells

To isolate genomic DNA from WBCs in the stabilized blood sample, the sample was centrifuged to isolate plasma (which contains any cfDNA) and pellet the cellular fraction (which contains any CTCs). A 200 µL fraction of WBCs from the washed cell pellet was removed and digested with Digestion Buffer included in the LiquidBiopsy™ Reagents and Consumables Kit. The resulting gDNA product was used as a germline control.

DNA from the cell-free component

To determine variants present in cfDNA, the plasma fraction was removed and automated cfDNA isolation was performed on the LiquidBiopsy Platform using the Applied Biosystems™ MagMAX™ Cell-Free DNA Isolation Kit. cfDNA was recovered from the isolation flow cell (IFC) in a single tube using a SpinElute™ tube.

DNA from CTCs

Following centrifugation, CTCs in the non-plasma fraction were labeled with a ferrofluid antibody conjugate. The antibody-labeled CTCs were isolated from a mixed population of nontarget cells using the IFC on the LiquidBiopsy Platform, which maximizes recovery while also minimizing sample transfer loss by integrating recovery and imaging in the same device (Figure 1).

The IFC has embedded ferromagnetic grids and 3D structures, which enable a three-layer laminar sheath flow that minimizes nonspecific binding of nontarget cells to IFC surfaces. Cells were permeabilized and labeled in-chamber under flow conditions with DAPI (nuclear DNA stain), anti–pan-cytokeratin (epithelial cell marker) antibody, and anti-CD45 (WBC marker) antibody. Following automated capture, labeled cells can be optionally imaged for internal quality control and enumeration. Cells were recovered from the IFC in a single tube using a SpinElute tube, and recovered cell pellets were digested with Digestion Buffer.

Library and template preparation

CTC lysate, WBC lysate, and purified cfDNA from plasma were amplified using the Ion AmpliSeq Cancer Hotspot Panel v2 following standard library construction and template preparation protocols on the Ion Chef™ System. The three libraries were barcoded using the Ion Xpress™ barcode Adapters 1–16 kit and sequenced on the Ion S5 XL System using the Ion 530™ Chip Kit.

Efficient and precise CTC recovery

To demonstrate the advantages of the LiquidBiopsy Platform, duplicate cell line spike-in experiments were performed in which a range of MCF-7 tumor cell concentrations (3, 9, 30, 90, 300, and 900 cells/mL) were added to 7.5 mL of normal blood samples (Figure 2A). Assessment of captured cell densities demonstrated a linear recovery of target cells between 1 and 1,000 cells/mL with a limit of detection of one target cell/mL.

Successful sequencing of CTCs depends not only on high recovery purity when the CTC load is high, but also on a recovery process that reproducibly controls for nontarget cell carryover, so that mutation detection is possible from a few CTCs per milliliter of blood. By limiting nontarget cell carryover, the signal-to-noise ratio of downstream next-generation sequencing (NGS) assays is improved so that mutational profiling of a few CTCs, or heterogeneous CTCs representing multiple tumor subclones, is possible.

To determine the limit of detection for variant sequencing, mixed cell line samples were created using four cell lines representing eight different Catalogue of Somatic Mutations in Cancer (COSMIC) SNVs. These mixed cell line samples were prepared in a wild type control cell line (GM12878) background and sequenced on the Ion S5 XL System. Averaging the results of 12 libraries, the incremental increase in the number of target cells and the percent mutant reads displayed a linear response with a limit of detection down to 1% variant frequency (Figure 2B). These results support the ability to detect as few as 3 to 10 target cells in a controlled background of nontarget cell carryover from a standard blood sample.

Uniform amplicon coverage for the Ion AmpliSeq Cancer Hotspot Panel v2

Coverage is the average number of reads that align to a reference base. As coverage increases, the trained variant caller detects putative variants with greater statistical confidence. Data from an Ion 530 Chip containing CTC, cfDNA, and WBC gDNA showed high uniformity of coverage across the 2,695 COSMIC mutational hotspots targeted by the Ion AmpliSeq Cancer Hotspot Panel v2, with >99% of amplicons achieving >2,000x coverage (Figure 3). The COSMIC mutational hotspots represented include 2,171 SNVs, 408 deletions, and 116 insertions.

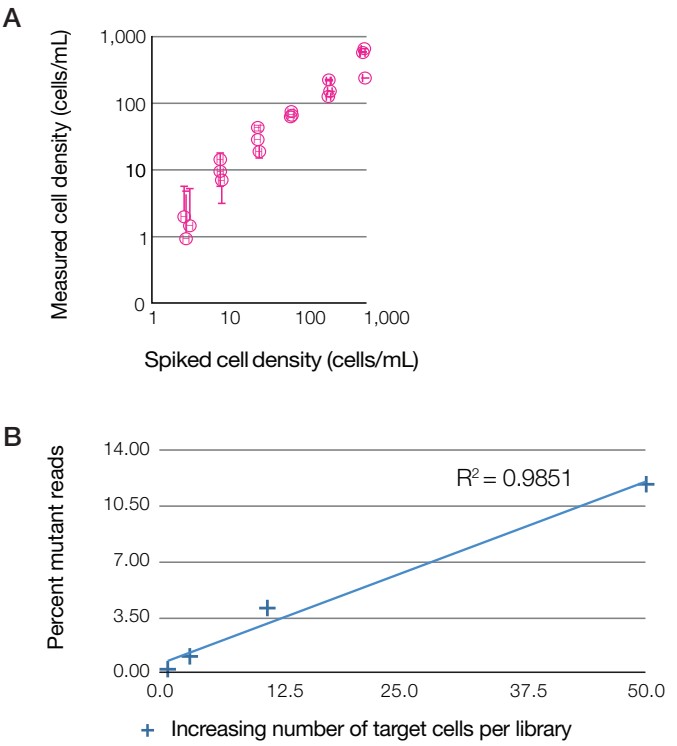

Figure 2. Efficiency and precision of the LiquidBiopsy Platform. **(A)** A linear recovery of target cells across a range of 1–1,000 cells/mL demonstrates a measured limit of detection of as low as 1%. **(B)** Averaging results from 12 mixed cell line libraries composed of four different cell lines (H195, HCC1419, MCF-7, and A549) and eight COSMIC single nucleotide variants (COSM517, COSM12925, COSM763, COSM10758, COSM13281, COSM12979, COSM21943, and COSM10660) across six chromosomal loci, the incremental increase in the number of target cells and the percent mutant reads displayed a linear response with a limit of detection down to 1% variant frequency.

Highly sensitive detection of ≥1% allele frequency for SNVs and indels from CTCs and cfDNA

A trained variant caller is part of a defined workflow for CTC and cfDNA analysis in Ion Reporter Software. To establish the detection of ≥1% allele frequency for SNVs and indels (that are <10 bp and are not part of homopolymers ≥5 bp) from CTCs and cfDNA, the variant caller was trained using the Thermo Scientific™ AcroMetrix™ Oncology Hotspot Control—a highly multiplexed quality control that covers >500 COSMIC mutations and tumor-associated mutations across 53 genes. The mutation spectrum covers >500 SNVs, 19 insertions, 29 deletions, and 3 complex mutations in a genomic background of characterized cell line GM24385, which is a National Institute of Standards and Technology (NIST) cell line that is part of the Genome in a Bottle public consortium.

The peripheral blood CTC and cfDNA workflow in Ion Reporter Software described above was verified on eight samples. Table 1 shows the results for three libraries

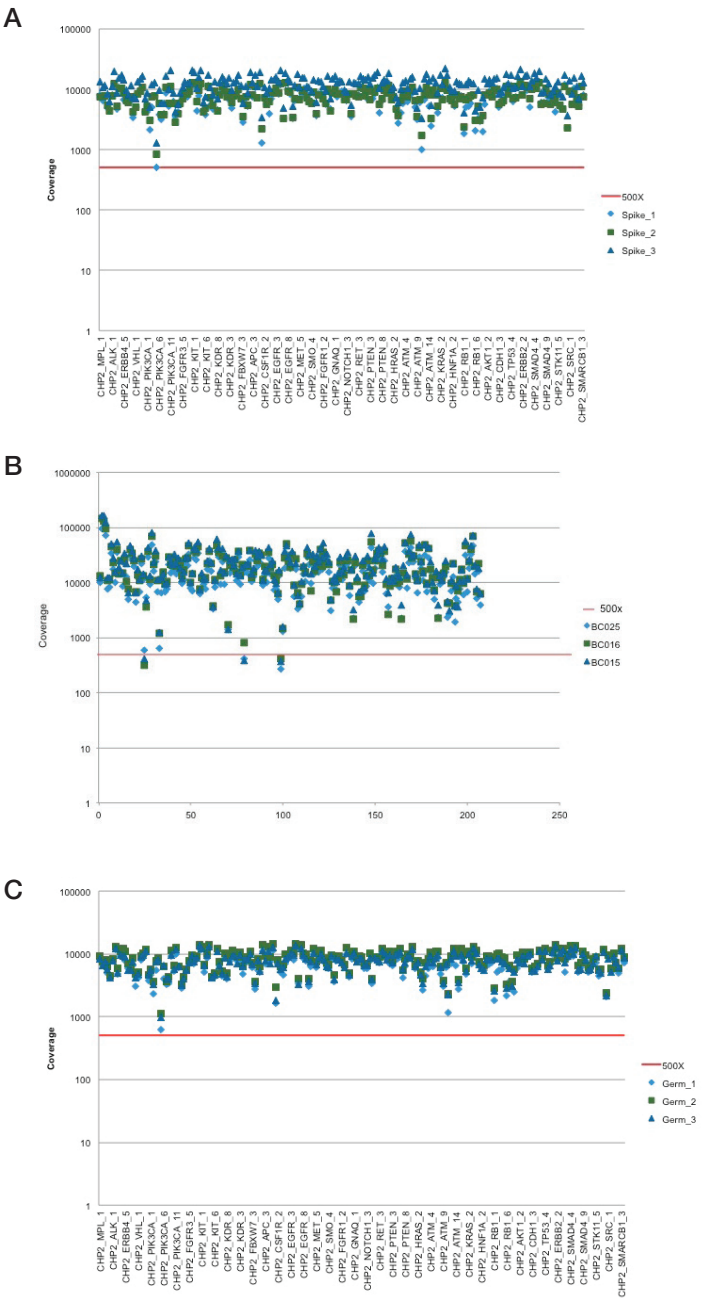

Figure 3. Amplicon coverage of libraries created with the Ion AmpliSeq Cancer Hotspot Panel v2 and sequenced on the Ion S5 XL System. After pooling libraries from **(A)** CTC DNA, **(B)** cfDNA, and **(C)** WBC DNA, the workflow demonstrates consistent target amplicon coverage of 90% at 2,000x.

(using gDNA from CTCs, cfDNA, and WBCs) from one of the eight samples sequenced on an Ion 530 Chip. One somatic variant was detected in cfDNA and CTCs that was not present in matched gDNA from WBCs in *FGFR3* for COSMIC variant COSM718. Based on verification work using the described workflow, three Ion AmpliSeq Cancer Hotspot Panel v2 libraries can be sequenced on a single Ion 530 Chip. The Ion Reporter workflow can then be used to consistently call variants at 1% limit of detection.

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Table 1. Variants and variant frequencies identified in gDNA from CTCs, cfDNA, and WBCs from a single blood sample using the LiquidBiopsy Platform workflow. Using the LiquidBiopsy Platform workflow, three libraries amplified using the Ion AmpliSeq Cancer Hotspot Panel v2 were multiplexed on a single Ion 530 Chip and sequenced on the Ion S5 XL System. A somatic variant not present in gDNA from WBCs is in bold typeface, indicating a mutation in *FGFR3* for COSMIC variant COSM718.

Variants				Variant frequency			
Position	**Gene**	**Reference**	**Variant**	**WBCs**	**CTCs**	**cfDNA**	**COSMIC ID**
chr3:178917005	*PIK3CA*	A	G	0.496	0.5	0.6378	
chr4:1806099	***FGFR3***	**A**	**G**	**Not present**	**0.0119**	**0.0156**	**COSM718**
chr4:1807894	*FGFR3*	G	A	0.996	1	0.9969	
chr4:55141055	*PDGFRA*	A	G	1	1	0.994	COSM1430082
chr4:55152040	*PDGFRA*	C	T	0.996	0.9819	0.996	COSM22413
chr4:55980239	*KDR*	C	T	0.339	0.36	0.339	
chr5:112175770	*APC*	G	A	1	1	1	
chr5:149433596	*CSF1R*	TG	GA	0.988	0.995	0.988	
chr7:55249063	*EGFR*	G	A	0.5089	NC	0.517	COSM1451600
chr11:534242	*HRAS*	A	G	0.507	0.498	0.5308	COSM249860
chr13:28610183	*FLT3*	A	G	1	1	1	
chr18:48586344	*SMAD4*	C	T	0.506	0.469	0.468	

Conclusions

We describe a research workflow to analyze cfDNA, CTCs, and germline DNA controls by NGS simultaneously on one chip. The LiquidBiopsy Platform workflow allows accurate profiling of mutations from rare cells in about 2.5 days:

- The LiquidBiopsy Blood Collection Kit stabilizes the sample, enabling easier sample collection, faster study accrual, and room temperature shipping
- The LiquidBiopsy Reagents and Consumables Kit helps prepare the three DNA types for analysis
- The LiquidBiopsy Platform enables streamlined, automated isolation of CTCs and automated or manual isolation of cfDNA, using genomic DNA from WBCs as germline controls
- The Ion AmpliSeq Cancer Hotspot Panel v2 is used for amplification of target genes and template preparation on the Ion Chef System
- The Ion S5 XL System enables sequencing of all three libraries, pooled as one on a single Ion 530 Chip

This versatile workflow enables analysis of SNVs and indels at 1% allele frequency for ~2,800 mutational hotspots in 50 known oncogenes and tumor suppressor genes with >95% sensitivity and 96% reproducibility.

Find out more at **thermofisher.com/liquidbiopsyplatform**

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