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 white blood cells (WBCs) can be analyzed simultaneously.
- The Ion Torrent™ LiquidBiopsy™ Platform enables recovery of 3–10 tumor cells per mL from whole blood.
- Single nucleotide variants (SNVs) and indels were detected with Ion AmpliSeq™ Cancer Hotspot Panel v2 at ≥1% allele frequency with a sensitivity of >95% for ~2,800 mutational hotspots in 50 oncogenes and tumor suppressor genes.

Introduction

This application note describes a workflow for isolating CTCs, cfDNA, and germline WBC controls from a single sample using the LiquidBiopsy Platform followed by variant analysis using the Ion AmpliSeq Cancer Hotspot Panel v2, the Ion PGM™ System and Ion Reporter™ Software v4.4 (Figure 1).

The LiquidBiopsy workflow

Initially, a 10mL 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.

From the stabilized blood sample, 200µL was removed and centrifuged to isolate and pellet the WBCs, which were then digested with Digestion Buffer included in the LiquidBiopsy™ Reagents and Consumables Kit. The resulting gDNA product was used as a germline control. The remaining stabilized blood sample was centrifuged to separate the cellular content from the plasma fraction, which contains cfDNA. To determine variants present in circulating DNA, the plasma fraction was removed and the cfDNA was isolated using QIAamp™ Circulating Nucleic Acid Kit.

Following centrifugation, CTCs in the non-plasma fraction were labeled with a ferrofluid anti–epithelial cell adhesion molecule (EpCAM) antibody conjugate. The antibody-labeled CTCs were isolated from a mixed population of nontarget cells using the isolation flow cell (IFC), 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 3-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.

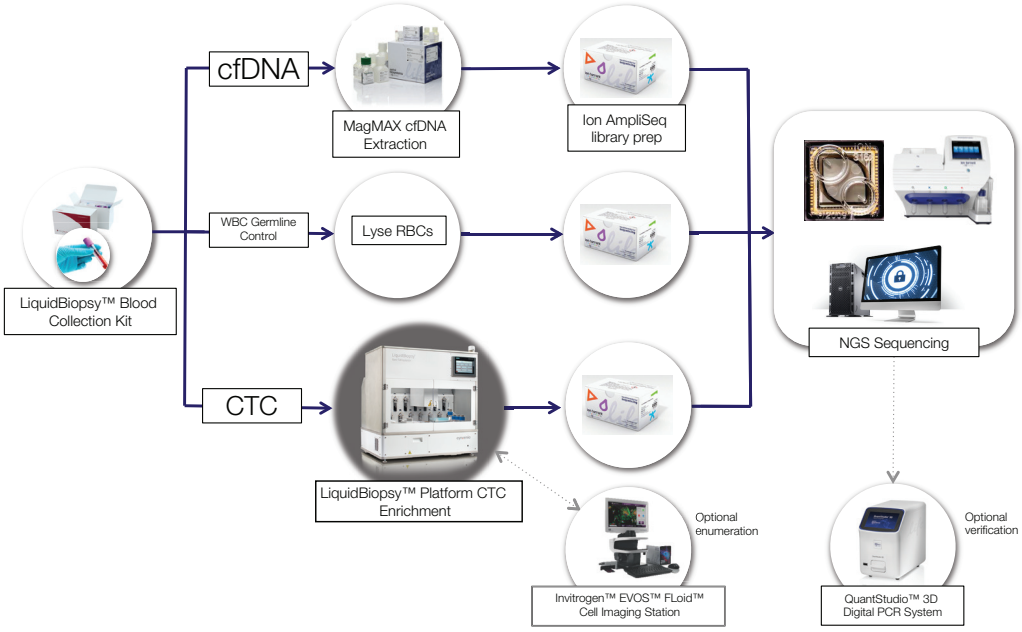

Figure 1. Comprehensive workflow for analysis of CTCs, cfDNA and germline DNA collected from a single blood sample. The automated LiquidBiopsy Platform is designed to efficiently recover CTCs through isolation flow cell priming, blood sample loading, target cell isolation and immunofluorescence labeling of captured cells, which allows both high-content microscopic imaging and downstream genomic analysis. Three libraries (CTCs, cfDNA and germline WBCs) were constructed using the Ion AmpliSeq Cancer Hotspot Panel v2, and multiplexed sequencing was performed on the Ion PGM System using a single Ion™ 318 Chip. Primary data analysis was performed using Torrent Suite™ Software followed by low frequency (≥1%) variant calling as part of a predefined workflow in Ion Reporter Software v4.4.

Cells were recovered from the IFC in a single tube using a SpinElute™ tube, and recovered cell pellets were digested with Digestion Buffer. The resulting gDNA was PCR-amplified using the Ion AmpliSeq Cancer Hotspot Panel v2. Amplified DNA from CTCs, cfDNA and WBCs were barcoded (Ion Xpress™ Barcode Adapters 1–16 Kit). Following standard library construction and template preparation protocols, all 3 samples were sequenced on the Ion PGM System (Ion PGM™ Sequencing 200 Kit v2 and Ion 318™ Chip Kit v2).

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 7.5mL 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 1 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 mL of blood. Using 198 samples, the LiquidBiopsy Platform demonstrated a mean nontarget cell carryover of 22 cells/mL (Figure 2B). The data indicate that a stable and controlled average background of 165 contaminant cells per recovery is present. By limiting the 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 4 cell lines representing 8 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. 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 2C). Taken together, 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

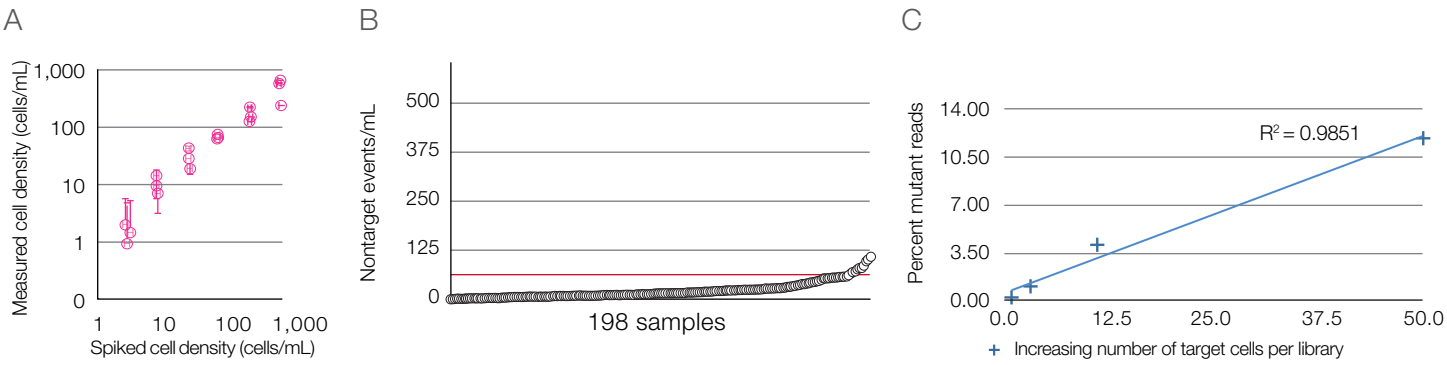

Figure 2. Efficiency and precision of the LiquidBiopsy Platform. (A) A linear recovery of target cells across a range of 1–1,000 cells/mL demonstrates a measured limit of detection of 1 target cell/mL. **(B)** Using 198 samples, the LiquidBiopsy Platform confirmed a stable and controlled nontarget cell background with mean nontarget cell carryover of 22 cells/mL (red line). **(C)** Averaging results from 12 mixed cell line libraries composed of 4 different cell lines (H195, HCC1419, MCF-7, A549) and 8 COSMIC single-nucleotide variants (COSM517, COSM12925, COSM763, COSM10758, COSM13281, COSM12979, COSM21943, COSM10660) across 6 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.

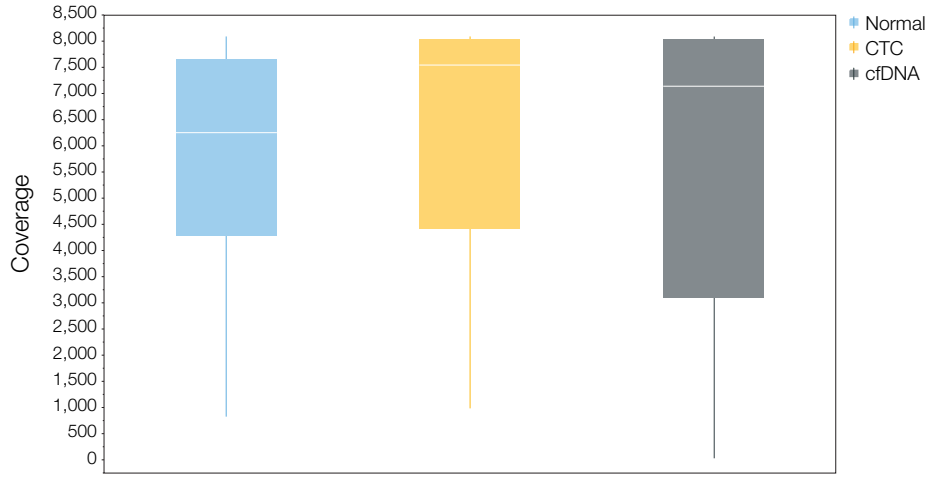

Figure 3. Uniform amplicon coverage. Multiplexed sequencing results from 3 libraries (CTCs, cfDNA and germline WBCs) amplified using the Ion AmpliSeq Cancer Hotspot Panel v2 and sequenced on a single Ion 318 Chip illustrates that 90% of amplicons have >2,000x coverage.

variant caller detects putative variants with greater statistical confidence. Genomic data from a single Ion 318 Chip containing CTC, cfDNA and germline templates showed high uniformity of coverage across the 2,695 COSMIC mutational hotspots targeted by the Ion AmpliSeq Cancer Hotspot Panel v2, with 90% of amplicons achieving >2,000x coverage (Figure 3). The COSMIC mutational hotspots represented include 2,171 SNVs, 408 deletions and 116 insertions.

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 v4.4. To establish the detection of ≥1% allele frequency for SNVs and indels (that are <10bp and are not part of homopolymers ≥5bp) 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 v4.4 was verified on 29 samples. Table 1 shows the results for three libraries (CTCs, cfDNA and germline WBCs) from a single sample sequenced on an Ion 318 Chip. Two somatic variants were detected in cfDNA and CTCs that were not present in matched germline WBCs, including COSMIC variant COSM760. Based on verification work using the described workflow, 3 Ion AmpliSeq Cancer Hotspot Panel v2 libraries (CTCs, cfDNA and germline WBCs) can be sequenced on a single Ion 318 Chip with a sensitivity of >95% for a ≥1% allele frequency (at 2,000x read depth) from a run with >1.7 million reads.

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Variants				Variant frequency		
Position	Gene	Reference	Variant	CTCs	cfDNA	WBCs
chr10:43613843	RET	G	T	1	1	1
chr13:28610183	FLT3	A	G	0.54	0.51	0.50
chr14:105241437	AKT	G	A	0.35	0.082	Not present
chr17:7579472	TP53	G	C	1	1	1
chr19:1220321	STK11	T	C	0.38	0.46	0.49
chr2:212812097	ERBB4	T	C	0.64	0.44	0.56
chr3:178917005	PIK3CA	A	G	0.50	0.52	0.50
chr3:178927410	PIK3CA	A	G	0.55	0.50	0.56
chr3:178936082	PIK3CA*	G	A	0.36	0.071	Not present
chr4:1807894	FGFR3	G	A	1	1	1
chr4:55152040	PDGFRA	C	T	0.49	0.45	0.49
chr4:55972974	KDR	T	A	0.50	0.53	0.51
chr7:55249063	EGFR	G	A	1	1	1

* COSMIC variant COSM760.

Table 1. Variants and variant frequencies identified from CTCs, cfDNA and germline WBCs from a single blood sample using the LiquidBiopsy Platform. Sequencing results from a single Ion 318 Chip multiplexed with 3 libraries amplified using the Ion AmpliSeq Cancer Hotspot Panel v2 defined as the LiquidBiopsy workflow. Somatic variants not present in matched germline WBCs are highlighted in red, including COSMIC variant COSM760.

Conclusions

From a single blood sample, variants in CTCs, cfDNA and germ-line WBC were analyzed simultaneously using the Ion Torrent LiquidBiopsy Platform. The described workflow enabled the recovery of ≥1 cell per mL from blood with detection of SNVs and indels at ≥1% allele frequency (with >95% sensitivity) for ~2,800 mutational hotspots in 50 oncogenes and tumor suppressor genes using the Ion AmpliSeq Cancer Hotspot Panel v2.

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

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