Detection of somatic mutations at 0.1% frequency from cfDNA in peripheral blood with a multiplex next-generation sequencing assay

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ABSTRACT

Effective blood screening for the purpose of tracking tumor recurrence and resistance of tumors may improve outcomes in the future. Research studies suggest that virtually all tumors carry somatic DNA mutations, and these may serve as biomarkers that also can be tracked in blood. One of the sources containing tumor DNA in blood is circulating cell-free DNA (cfDNA). Tumor DNA comes from different tumor clines, and its abundance in plasma can be very low at critical stages such as early recurrence or development of resistance. Hence, there is great interest in being able to detect mutation biomarkers at very low frequency from cfDNA for detection and characterization of tumor clines.

We present a research use only analysis workflow for peripheral monitoring that enables detection of low frequency DNA variants. We developed an analysis algorithm that models errors accumulated during amplification and sequencing, and accurately reconstructs sequence of original DNA molecules based on multiple next generation sequencing reads. The reads contain genomic sequence and an adapter that allows identification of reads originated from the same DNA molecule. We then developed a variant calling method that uses accurately reconstructed sequences to enable sensitive and specific detection of somatic mutations to 0.1% allele ratio. We demonstrate the analysis in control and archived cfDNA and FFPE research samples.

RESULTS

We achieved >95% sensitivity with >20ng input DNA and >85% sensitivity with 20ng input DNA and 1 false call per sample for variants in hotspot positions present at frequency 0.1% in the sample (Table 1). Due to sampling variability the detected frequency of the variants ranges between 0.05% and 0.15% (Figure 2). The workflow delivers 100% sensitivity and 100% specificity with 10ng input for variants at frequency above 0.5% Control samples were used for sensitivity calculations, cfDNA samples were used for specificity and FP rate calculations.

Library preparation and Sequencing

We used centrifugation at 1600 x g for 10 min at 4°C to extract plasma from blood; cfDNA was extracted from the plasma fraction using MagMAX™ cfDNA isolation protocol.

We used next generation sequencing cfDNA Lung Assay that allows interrogation of 171 biomarkers relevant in lung from COSMIC and Oncomine databases, and de novo variant detection at ~1,700 genomic positions in 11 genes implicated in non-small cell lung cancer (NSCLC). The assay delivers >95% on target reads and highly uniform amplification across targeted cfDNA molecules (Figure 6).

We barcoded 8/32 samples and ran them on a single Ion S5™ 530/540 sequencing chip, that enables very deep (>50,000x coverage) and accurate sequencing. We also tested 4 samples on a single Ion PGM™ 318 chip that delivers >25,000x coverage. The research assay uses a small amount of input DNA (~20ng for 0.1% LOD. Figure 3), and has a fast turnaround time from extracted DNA to variants of less than 24 hr.

Verification Data

We tested the limits of variant detection in controlled dilution series, in cfDNA, and in FFPE cell lines.

1. First, we diluted engineered plasmid controls (AcroMetric™ Oncology Hotspot Controls) in background GM42485 genomic DNA down to 0.1% or 0.5% frequency, and then fragmented the DNA mix into fragments with average size of 170bp. AcroMetric sample contains 40 common tumor mutations intermingled by our assay. The distribution of fragment size looks similar to Horizon’s built to eliminate errors and represent the original molecules.

2. Next, we used 0.1%, 1%, 5% Horizon’s (HD780) cfDNA reference sample that contains 8 low frequency mutations at our hotspot positions including two large insertion and deletion variants of size longer than 110bp.

3. Finally, we performed analytical verification of variant detection performance in normal cfDNA samples and FF/FFPE tumor samples.

Sample-to-variant 2 day cfDNA Lung Assay Workflow

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CONCLUSIONS

The cfDNA Lung Assay Workflow with the Ion Torrent™ platform is a comprehensive 2 days sample-to-variant solution that facilitates researchers to study relevant biomarkers at 0.1% frequency in cfDNA/FFPE DNA. Analysis is compatible with lower frequency variant detection, but will require higher input DNA amount and higher sequencing coverage (Figure 3).

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