Next-generation sequencing analysis of low-frequency mutations from cell-free DNA

Key findings

- Perform targeted next-generation sequencing (NGS) analysis from cell-free DNA (cfDNA) in blood samples
- Detect single-nucleotide variant (SNV) hotspots and insertions or deletions (indels) at 0.1% allele frequency with the Ion Torrent™ Oncomine™ Lung cfDNA Assay
- Obtain results from more samples using flexible DNA input amounts, from 1 ng to 50 ng
- Investigate tumor heterogeneity and identify primary tumor drivers and resistance mutations with an assay targeting more than 150 SNV hotspots across 11 genes in non-small cell lung cancer (NSCLC) samples
- Enable easy access to vital information with a comprehensive 2-day workflow (blood sample to refined variant data) that uses the Ion Torrent™ Oncomine™ Knowledgebase Reporter to contextualize sample-specific variants in a clinical research report

Introduction

Advanced molecular profiling methods using NGS are enabling clinical researchers to accurately profile mutations of interest in blood samples. These methods may potentially impact the approach to initial diagnosis, therapy monitoring, and reoccurrence monitoring in the future.

Cancer researchers know every tumor is different. While sampling tissue remains standard practice, there are several advantages to liquid biopsy samples: a) they enable serial analysis of multiple samples from the same subject; b) they potentially enable better analysis of tumor heterogeneity; c) they can be collected in cases where tissue samples cannot be collected, as is frequently the case for lung cancer samples; and d) they are less invasive and less expensive to obtain than tissue samples.

cfDNA is unencapsulated DNA in the bloodstream and other body fluids. Of interest is the cfDNA that originates from a tumor and is called circulating tumor DNA (ctDNA). ctDNA is rare and represents a low percentage of mutant DNA (0.1–1.0%) in typical samples.

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To unlock the potential in liquid biopsy samples, translational and clinical researchers need an NGS assay that enables multibiomarker analysis at low mutation frequencies to identify primary tumor drivers and resistance mutations. This application note describes a workflow for the isolation of cfDNA from a single blood sample followed by variant analysis using the Oncomine Lung cfDNA Assay, the Ion S5™ XL System, and a trained variant caller plug-in in Torrent Suite™ Software version 5.2 or higher (Figure 1).

Methods

Isolation of cell-free DNA

To isolate cfDNA from a 10 mL blood sample, the sample was centrifuged to isolate the plasma fraction. Using the protocol in the Applied Biosystems™ MagMAX™ Cell-Free DNA Isolation Kit manual, cfDNA was recovered from the plasma. From the starting sample, 5–20 ng cfDNA was obtained from healthy donors and 5–100 ng cfDNA was obtained from late-stage lung cancer samples.

Preparation of library and template

Purified cfDNA from plasma was amplified using the Oncomine Lung cfDNA Assay following standard library construction to produce barcoded libraries as outlined in the user guide. Eight libraries were multiplexed for templating using standard protocols on the Ion Chef™ System and sequenced on the Ion S5 XL System using the Ion 530™ Chip Kit.

This verified analysis was conducted using the Ion 530 Chip that enables up to 8-sample multiplexed sequencing. Additional analyses were demonstrated using the Ion PGM™ System with Ion OneTouch™ 2 templating, and the Ion Proton™ System with Ion Chef System templating.

Technology

The Oncomine Lung cfDNA Assay has a detection limit of 0.1% frequency (with a sensitivity of >90% and specificity of >98%), or 1 mutant copy in a background of 1,000 wild-type (WT) copies. To achieve a 0.1% limit of detection (LOD), 20 ng of input cfDNA is required. Lower amounts of cfDNA can be used, but the limit of detection may be higher depending on the input amount (Figure 2).

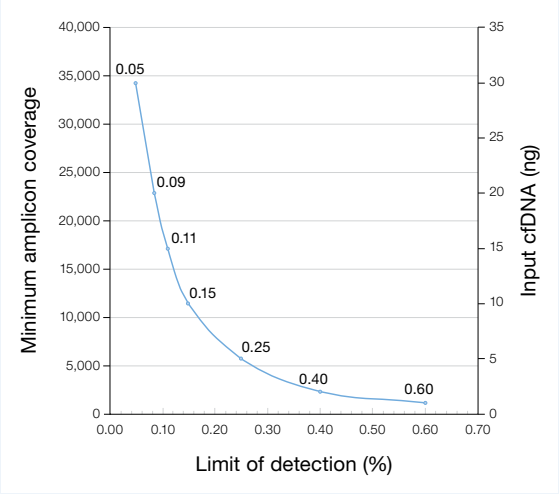

Figure 2. Oncomine Lung cfDNA Assay sequencing and input requirements for limit of detection (LOD) levels ranging from 0.05% to 1%.

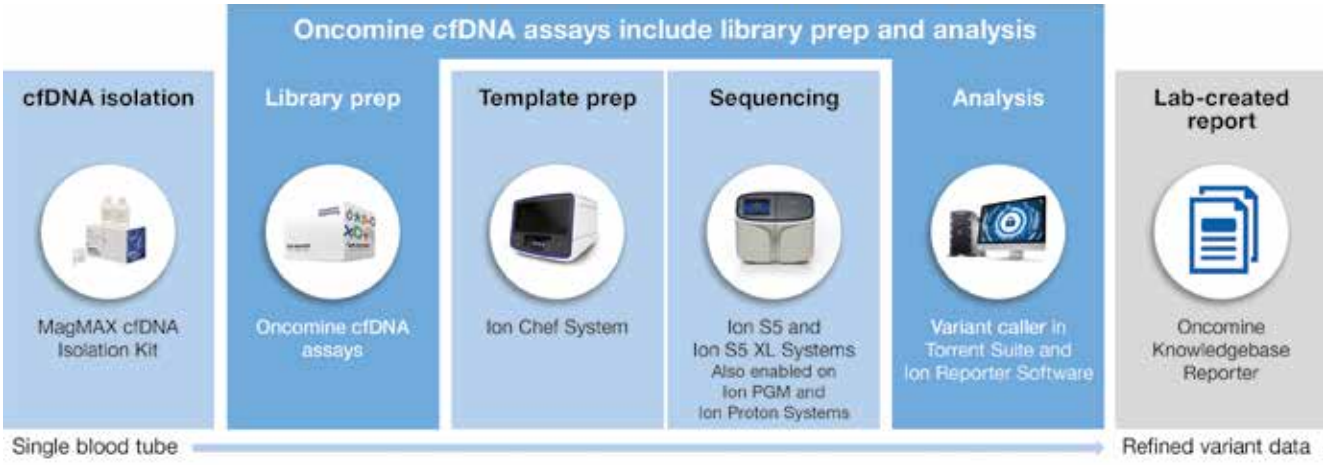

Figure 1. Comprehensive Oncomine cfDNA assay workflow. Oncomine Knowledgebase Reporter is enabled in Ion Reporter Software version 5.2 or higher.

Analysis

Sequencing data were analyzed with Torrent Suite Software version 5.2 or higher, using the cfDNA variant caller plug-in with parameters optimized for the "Oncology-Liquid Biopsy" application.

Results

The Oncomine Lung cfDNA Assay targets key genes involved in NSCLC: *ALK, BRAF, EGFR, ERBB2, KRAS, MAP2K1, MET, NRAS, PIK3CA, ROS1,* and *TP53*. Using matched tissue and blood from late-stage NSCLC samples, high correlations were measured between the variants called in formalin-fixed, paraffin-embedded (FFPE) samples and those called in cfDNA from plasma (Table 1). As expected, there is a higher allelic fraction in the FFPE tumor samples compared to that measured in plasma when the variant is derived from cancer cells. As well, germline variants in *MET*-T1010I are seen at close to 50% in both FFPE and plasma in two cases.

With mean sensitivity at 90% and mean specificity at 98%, the observed performance of the Oncomine Lung cfDNA Assay at an LOD of 0.1% enables amplification of more difficult research samples (Table 2). In addition to these measurements of expected variants in control samples, the actual cfDNA samples tested had less than one false positive in more than 8 libraries, indicating very high specificity for calling low-frequency variants in cfDNA.

To verify this performance, Oncomine lung cfDNA libraries were generated with engineered controls from Horizon Discovery as input. Data in Table 3 show the variant frequencies called by the variant caller analysis plug-in for the 8 somatic variants engineered into the Horizon Discovery™ Multiplex cfDNA Reference Standard Set when analyzed using the Oncomine Lung cfDNA Assay.

Table 1. Allelic fractions for six FFPE and cfDNA matched samples from late-stage NSCLC samples. As expected, the allelic fraction in an FFPE tumor samples is greater than that measured in plasma when the variant is derived from cancer cells.[1]

Samples	Variant	FFPE samples	cfDNA
1	*EGFR*-L858R	**71.42%**	2.62%
2	*TP53*-R158L	**51.89%**	4.32%
3	*MET*-T1010I	43.87%	51.75%
	KRAS-G12C	**34.62%**	0.28%
4	NA	No detection	No detection
5	*EGFR*-L858R	**58.44%**	7.28%
	MET-T1010I	41.93%	48.72%
	TP53-Y220C	**35.54%**	1.93%
6	*TP53*-R158L	**10.19%**	1.26%

[1] Bold: somatic mutations; normal: germline mutations.

Table 2. Sensitivity and specificity at 0.1% frequency and 0.5% frequency with engineered control samples.

	0.1% frequency[1]	0.5% frequency[2]
Mean sensitivity	90%	100%
Mean specificity	98%	98%

[1] Tested with fragmented 0.1% engineered oncology hotspot control sample.
[2] Tested with fragmented 0.5% engineered oncology hotpsot control sample.

Table 3. Variants called from the Multiplex cfDNA Reference Standard Set.

Sample	*EGFR* E746_A750delELRA	*EGFR* L858R	*EGFR* T790M	*EGFR* V769_D770insAV	*KRAS* G12D	*NRAS* A59T	*NRAS* Q61K	*PIK3CA* E545K
0.1% HDX[1]	0.06	0.17	0.06	0.10	0.22	0.17	0.15	0.10
1% HDX[2]	0.72	1.07	0.75	0.74	1.14	1.15	1.15	2.29
5% HDX[3]	4.52	4.86	6.32	3.97	6.34	6.11	6.94	5.29
100% WT[4]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

1. For 0.1% the data shown here come from a library using 50 ng of the Horizon Discovery™ 0.1% LOD control mix.
2. For 1% the data shown here come from a library using 10 ng of the Horizon Discovery™ 1.0% LOD control mix.
3. For 5% the data shown here come from a library using 5 ng of the Horizon Discovery™ 5% LOD control mix.
4. The 100% WT is the negative control material that comes with the Multiplex cfDNA Reference Standard Set. The data shown here come from a library using 20 ng of the 100% WT control mix. No false positives were observed with the 100% WT control.

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Conclusion

The Oncomine Lung cfDNA Assay contains 35 amplicons covering >150 SNV hotspots across 11 genes. Coupled with the Ion S5 System, this assay enables efficient multibiomarker analysis of cfDNA in blood. With flexible limits of detection as low as 0.1% with high specificity and sensitivity, clinical cancer researchers may detect ctDNA sooner to better study resistance mutations as they emerge.

Ordering information

Product	Cat. No.
Oncomine Lung cfDNA Assay	A31149
Tag Sequencing Barcode Set 1-24	A31830
Tag Sequencing Barcode Set 25-48	A31847
MagMAX Cell-Free DNA Isolation Kit	A29319
Ion Chef Instrument	4484177
Ion 520 and Ion 530 Kit-Chef	A30010
Ion 530 Chip Kit	A27764
Ion S5 XL System	A27214

Find out more at **thermofisher.com/cfdna-assays**

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