ion torrent

Detect 0.1% Low Frequency Somatic Variants in Cell-Free DNA Using Oncomine™ cfDNA Assays and Ion Torrent Sequencing

Jeff Schageman, Jian Gu *Dima Brinza, Yanchun Li, *Richard Chen, *Dalia Dhingra, *Kunal Banjara,*Ruchi Chaudhary, Varun Bagai, Kris Lea, Priyanka Kshatriya, Efren Ballesteros-Villagrana, and Kelli Bramlett.

Thermo Fisher Scientific, 2130 Woodward St. Austin, TX 78744 and *180 Oyster Point Blvd. South San Francisco, CA 94080

ABSTRACT

We demonstrate a research use only workflow that aims to detect variants at low allelic frequencies in cell free DNA (cfDNA) extracted from liquid biopsy samples. The described workflow includes blood collection, cfDNA isolation, library preparation, sequencing, and data analysis to enable detection of rare DNA variants in blood plasma samples using the Oncomine™ cfDNA assays.

We achieved ~85% detection sensitivity at 0.1% frequency using engineered control samples with >99% sensitivity at these low limits of detection using the Oncomine™ Lung cfDNA Assay. We verified the workflow on a set of research samples from matched tumor FFPE and blood plasma collected from research subjects with NSCLC. Results indicate high sensitivity of the workflow and high concordance between variants detected in the two types of research samples. This workflow provides a non-invasive approach to monitor cancer status and evaluate cancer evolution in the future.

MATERIALS AND METHODS

Plasma preparation: Blood samples were collected into EDTA tubes following manufacturer's instructions. Plasma was obtained by centrifugation at 1600 x g for 10 min at 4°C, followed by another spin at 6,000 x g for 30 min at 4 °C to remove any residual blood cells.

cfDNA/FFPE DNA isolation: cfDNA was isolated from ~4mL of plasma using MagMAX™ Cell-Free DNA Isolation Kit following alternative protocol in User Guide appendix B. RecoverAll™ Multi-Sample RNA/DNA Isolation Workflow was used to isolate DNA from FFPE samples following standard protocol.

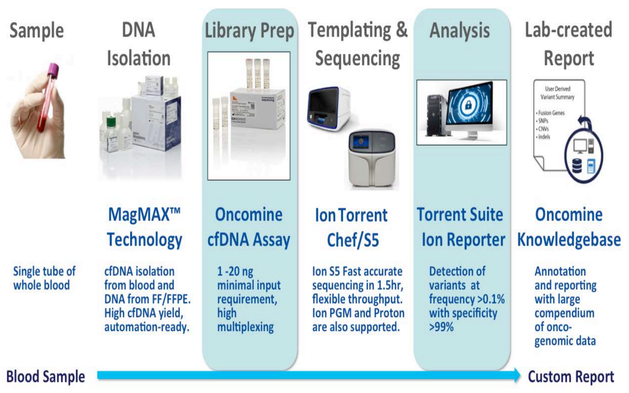
Library preparation: Targeted libraries were prepared using the Oncomine™ Lung, Breast or Colon cfDNA Assay reagents and user guide instructions for both cfDNA and FFPE DNA.

Sequencing: The Ion 520™ & Ion 530™ Kit-Chef were used for template preparation on the Ion Chef™, followed by sequencing on Ion S5™XL system using the Ion 530™ Chip. Lung cfDNA libraries were sequenced as an 8-plex, breast cfDNA libraries were run as a 12-plex, and colon cfDNA libraries were run as a 6-plex all on the Ion 530™ Chip.

Data analysis: Data analysis was performed in Torrent Suite™ using the variantCaller plugin.

RESULTS

Figure 1. Oncomine™ cfDNA Assays 2-day Research Workflow



A. The Oncomine™ cfDNA assay is a content-optimized solution for NGS cfDNA liquid biopsy research. It includes both library prep and analysis solutions that enable limit of detection down to 0.1% allelic frequency (with 20 ng input) for SNV and INDEL hotspots with the flexibility of using as little as 1ng input DNA. A blood sample to report workflow can be completed in 2 days.

Figure 2. Oncomine™ cfDNA Assay Methodology

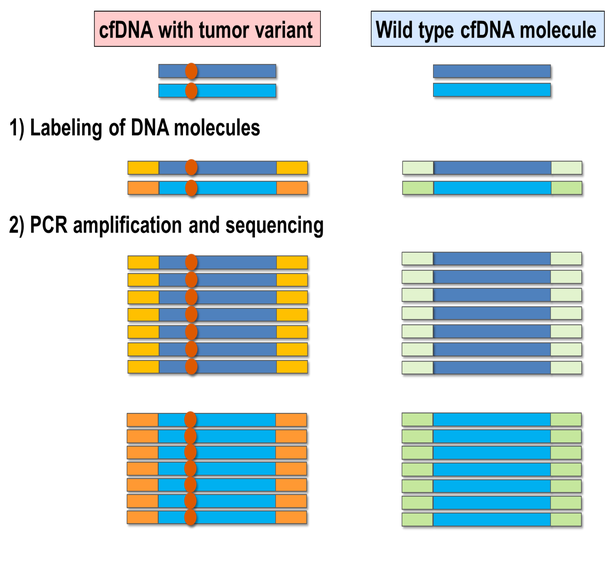


Table 1. Detection Sensitivity and Specificity – Horizon Controls

control	input	sensitivity	specificity
0.0%	20ng	100.0%	100.0%
0.0%	20ng	100.0%	100.0%
0.1%	50ng	91.7%	100.0%
0.1%	50ng	83.3%	100.0%
0.1%	50ng	100.0%	100.0%
0.1%	50ng	91.7%	100.0%
1.0%	10ng	100.0%	100.0%
1.0%	10ng	100.0%	100.0%
5.0%	5ng	100.0%	100.0%
5.0%	5ng	100.0%	100.0%

Horizon Multiplex I cfDNA Reference Standard Set (Horizon, PN HD780) was used to demonstrate detection sensitivity and specificity of the Oncomine™ Lung cfDNA Assay. Table shows the control sample (left most column) and sensitivity and specificity calculated for multiple input amounts of these control materials. Sensitivity was calculated based on 8 expected variants in the reference standard and specificity was based on 149 expected true negative hotspot positions in the cfDNA lung panel.

Table 2. Detection Sensitivity and Specificity – Oncology Hotspot Controls

	Sample	Sensitivity	Specificity
Lung	0.1% MM	92.2%	99.7%
	0.5% MM	>99.9%	99.6%
Colon	0.1% MM	85.9%	>99.9%
	0.5% MM	>99.9%	>99.9%
Breast	0.1% MM	81.3%	>99.9%
	0.5% MM	>99.9%	>99.9%

The Oncomine™ Lung, Breast and Colon cfDNA Assay performance was evaluated using engineered controls (AcroMetrix™ Oncology Hotspot Control) in background GM24385 genomic DNA diluted to 0.1% or 0.5% allelic frequencies. The control and background DNA was fragmented to mimic the size of cfDNA. Mean sensitivity and specificity values were obtained across the expected true positives and true negatives at oncology hotspot positions using the following equations:

SENSITIVITY = TP/(TP+FN)
SPECIFICITY = TN/(TN+FP)

Table 3. Validation of 0.1% Detection Sensitivity with Digital PCR

Gene	Variant	Oncomine™ cfDNA Lung assay	Oncomine™ cfDNA Breast assay	Oncomine™ cfDNA Colon assay	Digital PCR
KRAS	p.G12D	0.20%	0.20%	0.15%	0.27%
		0.17%	0.14%	0.14%	0.24%
PIK3CA	p.E545K	0.15%	0.14%	0.26%	0.02%
		0.11%	0.06%	0.16%	0.19%
EGFR	p.E746_A750 delELREA	0.12%	*N/A	*N/A	0.10%
		0.11%	*N/A	*N/A	0.12%
EGFR	p.L858R	0.07%	0.14%	*N/A	0.05%
		0.06%	0.06%	*N/A	0.83%

Data shown is from the Horizon 0.1% control material when used as input for the Oncomine™ lung, breast and colon cfDNA assays as well as dPCR assays for the indicated variants. Allelic frequency of variants was measured using the targeted NGS library method in the indicated columns. Allelic frequency was validated with digital PCR as an orthogonal method to measure low frequency variants. Measurements were made in duplicate. *N/A indicates variants in the Horizon 0.1% control material that are not in the Oncomine Breast and/or Colon cfDNA targeted hotspot file.

Table 4. Input Study for Variant Detection from cfDNA Research Sample

sample	variant	cfDNA input						
		20ng	10ng	5ng	2ng	1ng	0.5 ng	0.25ng
S1	EGFR p.L747_E749delLRE	9.42%	-	-	10.44%	14.66%	16.54%	7.92%
S2	KRAS p.G12D	0.22%	0.33%	0.10%	0.23%	N.D.	-	-
	MET p.T1010I	47.44%	49.81%	45.50%	47.60%	51.15%	-	-

Research sample S1 contains a fairly high frequency variant detected in cfDNA at 9.42% using recommended input of 20ng in the Oncomine™ Lung cfDNA assay. The variant could still be detected when input was decreased to 0.25ng of cfDNA. In research sample S2, a variant was identified using 20ng of cfDNA at low allelic frequency of 0.22%. This variant was successfully detected using input amounts down to 2ng. A germline variant present in sample S2 was consistently detected at levels ~50% in all input titration libraries.

Table 5. Observed Frequencies of Variants Detected from Matched FFPE and Plasma Late Stage NSCLC Research Samples

Samples	Variant	FFPE	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	N/A	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%**

Late stage NSCLC research samples were purchased from BioChain. Sample sets included solid tumor sample embedded in FFPE and 2-4 mls plasma from blood collected in EDTA tubes. Bold numbers indicate allelic frequencies as determined using the Oncomine™ Lung cfDNA assay for the indicated somatic variants. Non-bold frequencies show germline variants that were also detected in the targeted libraries. For these 6 independent research samples, there was very high concordance between variants detected in FFPE and cfDNA from matched plasma from the same research subjects. As expected, higher variant frequencies were observed in the FFPE solid tumor samples with significantly lower frequencies measured in the matched cfDNA samples.

CONCLUSIONS

- Described the Oncomine™ cfDNA Assay capability to measure low frequency variants from cfDNA in control and late stage NSCLC research samples
- High sensitivity (>80%) and specificity (>99%) for detecting variants at levels of 0.1% frequency in cfDNA standard material with all three Oncomine™ cfDNA Assays
- Simple workflow that has been verified from blood collection to variant detection in 32 hours, or two working days
- Measured allelic frequencies confirmed by orthogonal measurement system – dPCR
- Optimized variant calling parameters available in Torrent Suite software
- Oncomine™ cfDNA Assays perform over a wide range of input amounts (1-50ng cfDNA) depending on frequency of variant in liquid biopsy sample
- Oncomine™ cfDNA Assays are compatible with DNA extracted from FFPE blocks

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