Detecting primary tumor drivers and resistance mutations using cell-free total nucleic acid

Performance of the Oncomine Lung Cell-Free Total Nucleic Acid Research Assay

The analysis of circulating cell-free total nucleic acid (cfTNA) from blood samples has emerged as a vital new approach to cancer research, and may guide future diagnosis and therapy decisions. Such analyses of circulating nucleic acids, commonly referred to as "liquid biopsies", may be used in the future to monitor response to treatment, assess the emergence of drug resistance, and quantify minimal residual disease [1]. By enabling a noninvasive and cost-effective alternative to traditional biopsy samples, liquid biopsies are poised to redefine the future of cancer care [2].

In lung cancer, the most common variant tested in liquid biopsy samples is epidermal growth factor receptor (EGFR) T790M, a component of the molecular signaling pathway that controls the proliferation and growth of cells. EGFR-targeting therapies have become a key component of lung cancer therapy. In the future, the ability to analyze cfTNA from blood samples may overcome the challenges of obtaining lung tumor samples from biopsy, cytology specimens via bronchoscopy, computed tomography-guided biopsy, surgical resection, or drainage from malignant pleural effusions [3].

Obtaining insight into molecular tumor evolution and detection of primary driver and resistance mutations through a comprehensive view of lung tumor mutations is highly desirable as research continues for the integration of liquid biopsies into cancer management.

Oncomine Lung cfTNA Research Assay

The Ion Torrent™ Oncomine™ Lung cfTNA Research Assay is a highly sensitive, next-generation sequencing (NGS) assay that provides researchers insight into molecular tumor evolution and detection of primary driver and resistance mutations. The optimized content covers single nucleotide variants (SNVs), indels, copy number variations (CNVs), and fusions for multibiomarker analysis, with variant detection as low as 0.1%. Starting from a blood sample, variant data are generated in a streamlined two-day workflow.

Based on multiplex PCR with flexible input amounts from one tube of blood, our proprietary technology together with Ion Torrent™ NGS enables researchers to develop tests that may impact treatment selection, treatment monitoring, and recurrence monitoring in the future.

For researchers who plan to evaluate fusion biomarkers by running liquid biopsy samples, this paper provides guidance for the following:

- Blood sample storage conditions in K_2 EDTA and Streck DNA tubes for cfTNA
- Quality assessment of cfTNA samples
- Sensitivity and specificity for SNV detection

- Sensitivity, specificity, and limit of detection (LOD) for fusions
- Sensitivity, specificity, and input titration for CNV detection

Gene content

The gene content includes key targets selected and verified by the OncoNetwork consortium, which consists of leading clinical researchers worldwide. The assay queries genes that have been identified as frequently mutated in non-small cell lung cancer (NSCLC) and enables analysis of all types of key mutations: SNV, indels, CNVs, and fusions (Table 1). It allows the detection of somatic variants at low frequencies (as low as 0.1%) in cfTNA from plasma.

Assay overview and requirements

The Oncomine Lung cfTNA Research Assay requires cell-free total nucleic acid as input to enable variant calls from both DNA and RNA. For total nucleic acid extraction, the Applied Biosystems™ MagMAX™ Cell-Free Total Nucleic Acid Isolation Kit (Cat. No. A36716) is recommended. For quantification, the Invitrogen™ Qubit™ dsDNA HS Assay Kit (Cat. No. Q32851) is recommended. From a 10 mL blood sample, 5–50 ng of cfDNA is typically obtained.

The recommended input amount of 20 ng cfDNA into the amplification-based assay enables the detection of rare variants present at 0.1% frequency. Successful libraries can be generated from 1–50 ng of cfDNA (Figure 1).

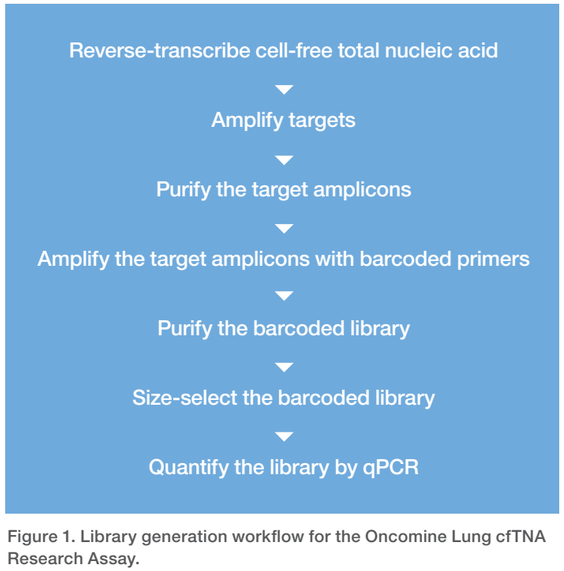

Figure 1. Library generation workflow for the Oncomine Lung cfTNA Research Assay.

Table 1. Oncomine Lung cfTNA assay targets. The full list of hotspots in this assay is provided in the Hotspot BED file, available at thermofisher.com/order/catalog/product/A35864, under "Product Literature".

Assay	Nucleic acids	Genes	Selected SNV hotspots	CNVs	Fusions	Extra
Oncomine Lung cfTNA Research Assay	DNA, RNA	*ALK, BRAF, EGFR, ERBB2, KRAS, MAP2K1, MET, NRAS, PIK3CA, RET, ROS1, TP53*	174 hotspots including: *EGFR:* T790M, C797S, L858R, exon 19 del *KRAS:* G12X, G13X, Q61X *BRAF:* V600E *ALK:* exon 21–25 *PIK3CA:* E545K, H1047R, E542K	*MET*	*ALK, RET, ROS1*	*MET* exon 14 skipping

Evaluation of blood storage conditions

Blood collection tubes

The tubes used for blood collection are an important variable. BD Vacutainer™ K_2 EDTA tubes and Streck Cell-Free DNA BCT™ tubes, two of the most commonly used tubes for blood collection, were compared for use with the Oncomine Lung cfTNA Research Assay.

Blood from six healthy donors was collected in each tube type and processed for SNV detection after extraction and quantification of cfTNA. Barcoded libraries were generated by following standard protocols found in the user guide. Six libraries were multiplexed for templating on the Ion Chef™ Instrument and subsequently sequenced on the Ion S5™ XL System using the Ion 530™ Chip Kit. The results are shown in Table 2.

Table 2. Sequencing results from 6 healthy donors using K_2 EDTA and Streck tubes.

Tube type, sample	Median read coverage	Median molecular coverage	LOD (%)	Variants	Hotspot variants
EDTA, donor 1	24,457	1,130	0.1327–0.1461	0	0
EDTA, donor 2	28,822	2,831	0.0530–0.0670	0	0
EDTA, donor 3	22,623	1,774	0.0845–0.1018	0	0
EDTA, donor 4	43,230	2,927	0.0512–0.0679	1	0
EDTA, donor 5	29,869	3,162	0.0474–0.0597	1	0
EDTA, donor 6	27,173	1,636	0.0917–0.1094	0	0
Streck, donor 1	14,615	1,205	0.1244–0.1553	1	1
Streck, donor 2	26,269	2,616	0.0573–0.0732	0	0
Streck, donor 3	18,253	1,158	0.1295–0.1661	0	0
Streck, donor 4	41,419	2,560	0.0586–0.0750	1	1
Streck, donor 5	18,413	1,382	0.1085–0.1278	1	1
Streck, donor 6	36,362	2,978	0.0504–0.0645	0	0

A higher false-positive rate was observed with the Streck tubes than with the EDTA tubes at 0.1% LOD. Hence, K_2 EDTA blood collection tubes are recommended for running the Oncomine Lung cfTNA Research Assay.

A critical consideration when using K_2 EDTA tubes is that the samples should be processed within 6 hours of collection in order to avoid lysis of white blood cells. This ensures that normal gDNA released from lysed white blood cells [4] does not dilute the cell-free DNA.

When blood processing within 6 hours of collection is not feasible, leukocyte stabilization tubes such as Streck tubes allow greater flexibility in processing time.

Blood storage times in Streck tubes

Two different post-collection processing times were evaluated with Streck tubes in order to assess the impact of storage on cfTNA quality. Additionally, results were compared between glass and plastic Streck Cell-Free DNA BCT tubes.

Blood samples from 10 healthy donors were collected in duplicate in either glass Streck tubes (for 4 donors) or plastic Streck tubes (for 6 donors) by a third-party vendor and shipped overnight on cold packs to the processing laboratory at Thermo Fisher Scientific.

Plasma preparation followed by cfTNA extraction and quantification was performed on the samples as listed below and as shown in Figure 2:

- Upon receipt of blood (day 0)
- After storage of blood at room temperature for 24 hours (day 1)

Barcoded libraries were generated by following standard protocols found in the user guide. Six libraries were multiplexed for templating on the Ion Chef Instrument and subsequently sequenced on the Ion S5 XL System using the Ion 530 Chip Kit. The sequencing results are shown in Table 3.

After storage at room temperature for a day, more false positives are observed at hotspot positions, suggesting more DNA damage. Hence, to minimize false-positive calls, plasma preparation and cfTNA extraction within 24 hours of blood collection is recommended when using Streck tubes.

Molecular counts of two RNA process control targets were also obtained and shown in Table 4. No significant change in the expression pattern can be observed between day 0 and day 1, confirming the stability of the RNA targets after storage at room temperature for 1 day. Furthermore, RNA targets can be detected in plasma samples in Streck tubes even after one week of incubation at room temperature, indicating the stability of RNA upon extended storage (data not shown).

When comparing plastic tubes to glass, lower molecular counts of RNA targets were observed in samples collected in Streck DNA plastic tubes. Hence, Streck DNA glass tubes are recommended for the Oncomine Lung cfTNA Research Assay.

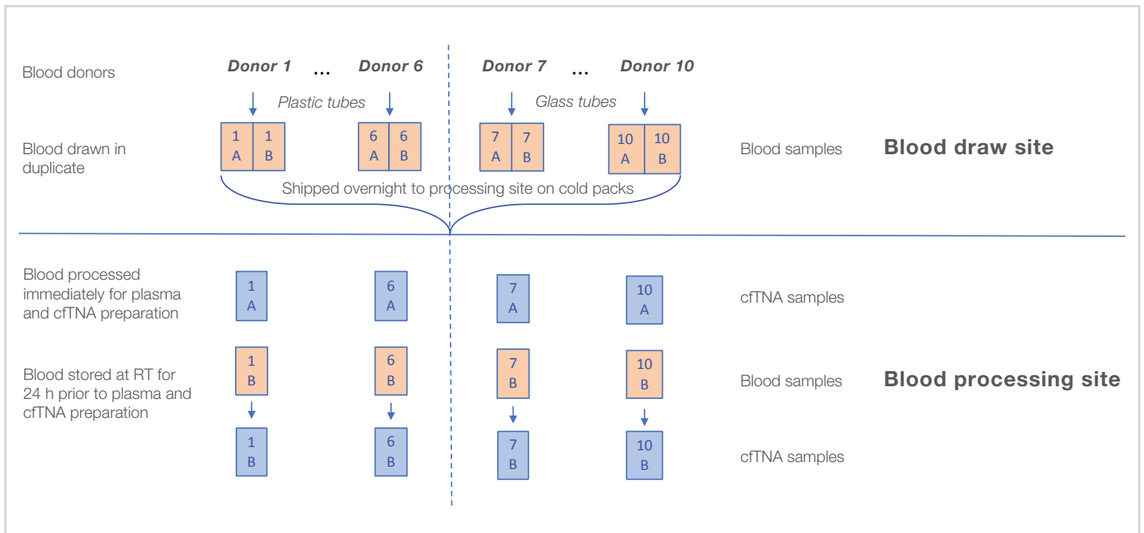

Figure 2. Blood collection and storage conditions.

Table 3. Sequencing results from 10 healthy donors comparing two different post-collection processing times.

		Day 0						Day 1					
Tube type	Donor	Input (ng)	Median read coverage	Median molecular coverage	LOD (%)*	Variants	Hotspot variants	Input (ng)	Median read coverage	Median molecular coverage	LOD (%)*	Variants	Hotspot variants
Streck DNA tubes, plastic	1	13.1	24,338	3,105	0.0483–0.0569	1	0	11.2	27,925	2,620	0.0572–0.0688	0	0
	2	8.7	59,848	2,229	0.0673–0.0783	0	0	7.9	22,727	1,778	0.0843–0.1097	0	0
	3	9.2	22,647	1,945	0.0771–0.0923	0	0	10.7	29,906	2,411	0.0622–0.0728	1	0
	4	22.7	35,288	5,125	0.0293–0.0322	0	0	26.8	36,812	6,087	0.0246–0.0284	1	0
	5	11.5	28,989	2,736	0.0548–0.0663	0	0	10.7	21,011	2,223	0.0675–0.0815	0	0
	6	11.8	36,320	2,777	0.0540–0.0609	0	0	12.9	35,197	3,389	0.0443–0.0503	0	0
Streck DNA tubes, glass	7	14.1	26,404	3,157	0.0475–0.0574	0	0	17.7	39,755	4,211	0.0356–0.0425	0	0
	8	9.6	24,862	2,134	0.0703–0.0886	1	0	9.3	23,770	2,126	0.0706–0.0891	2	2
	9	7.9	20,297	1,745	0.0860–0.1009	0	0	6.4	9,395	1,198	0.1252–0.1511	1	0
	10	12.5	28,777	2,685	0.0559–0.0650	0	0	10.0	22,515	2,271	0.0661–0.0770	1	0

*Numbers represent range from minimum to maximum

Table 4. Molecular counts of 2 RNA process controls included in the panel (HMBS, TBP).

		Day 0		Day 1	
Tube type	Donor	HMBS molecular coverage	TBP molecular coverage	HMBS molecular coverage	TBP molecular coverage
Streck DNA tubes, plastic	1	112	115	139	169
	2	97	98	61	91
	3	112	82	61	157
	4	238	194	143	162
	5	52	43	36	71
	6	39	60	60	152
Streck DNA tubes, glass	7	189	136	248	328
	8	1,842	309	609	265
	9	396	238	371	379
	10	191	79	256	290

Recommendations

To ensure cfTNA sample stability, minimal cellular gDNA/RNA contamination, and minimal DNA damage from fixative reagents in blood collection tubes, the following guidelines for plasma and cfTNA preparation should be followed.

For K_2 EDTA tubes, plasma preparation and cfTNA extraction is recommended within 6 hours of blood collection. If Streck DNA tubes are used, glass tubes are recommended, with plasma preparation and cfTNA extraction within 24 hours of blood collection.

Quality assessment of starting cfTNA sample

The quality of both DNA and RNA in the cfTNA samples was assessed, for input into the Oncomine Lung cfTNA Research Assay.

cfDNA size distribution and yield

The Agilent Bioanalyzer™ system and Invitrogen Qubit™ Fluorometer are recommended to evaluate the size distribution and yield of cfDNA from a cfTNA sample.

As shown in Figure 3, the cfDNA size distribution is highly similar when the cfDNA is isolated from blood collected in Streck DNA tubes or K_2 EDTA tubes. A typical yield of cfDNA from 4 mL of presumably healthy donor plasma (from 10 mL of blood) is between 5 and 50 ng. Higher cfDNA yields have been observed from patient plasma samples.

Estimation of the number of RNA transcripts

Due to the extremely low amount of cfRNA in the cfTNA sample, traditional RNA QC methods using the Agilent RNA Pico chip and Invitrogen™ Qubit™ RNA Assay are no longer applicable. We developed a method based on digital PCR (dPCR) to generate absolute quantitation of RNA transcripts in the samples.

Study design

An Applied Biosystems™ TaqMan® Gene Expression Assay targeting one of the RNA process controls in the panel, HMBS (Hs00609296_g1), was performed using the Applied Biosystems™ QuantStudio™ 3D Digital PCR System to estimate the number of HMBS transcripts from cfTNA samples prepared from healthy donors. Correlation of dPCR copy number and molecular counts of HMBS target from sequencing runs was evaluated.

Results

A high correlation is observed between dPCR copy numbers and molecular counts from sequencing runs,

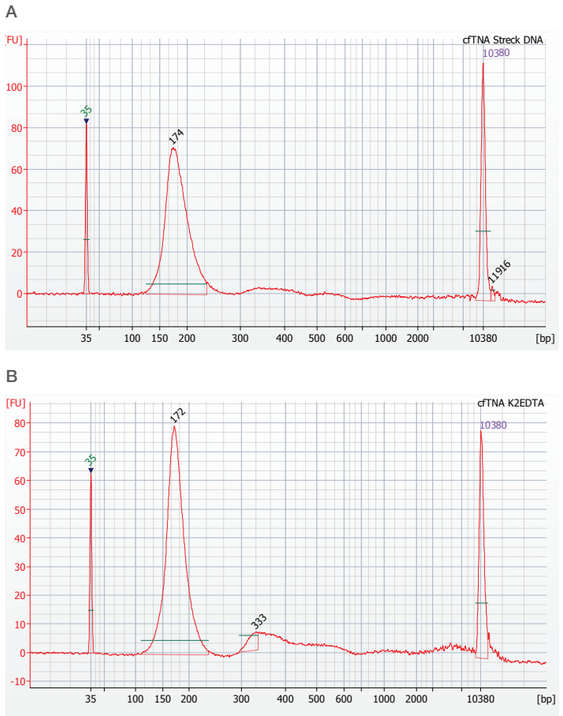

Figure 3. Typical size distribution of cfDNA from blood collected in (A) Streck DNA and (B) K_2 EDTA collection tubes.

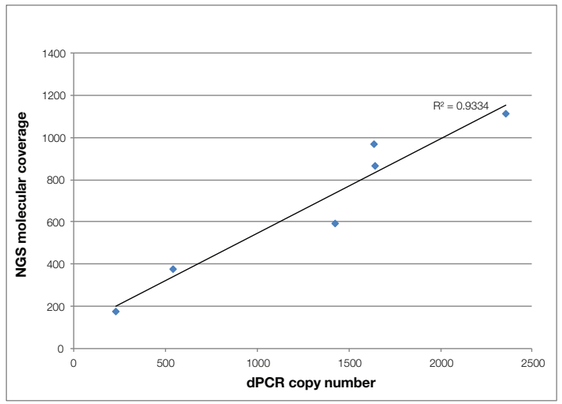

Figure 4. Transcript copy number from dPCR (x-axis) vs. molecular counts from sequencing runs (y-axis).

indicating that dPCR data can be used to estimate the molecular coverage of the RNA process control HMBS. We typically observe more than 200 molecular counts of HMBS (~230 copies by dPCR) from 4 mL of healthy donor plasma samples, and the number varies between donors. The digital PCR method described here is recommended for estimating the number of RNA transcripts.

Sensitivity and specificity for SNV detection

The fragmented Thermo Scientific™ AcroMetrix™ Oncology Hotspot Control was used to evaluate sensitivity and specificity of the Oncomine Lung cfTNA Research Assay for SNV detection.

The 0.5% and 0.1% control mixes were verified by dPCR for allele frequencies, and were used to measure the actual number of DNA copies/µL of material. We observed similar high performance for SNV detection in terms of sensitivity and specificity using 0.1 and 0.5% controls when compared to the Oncomine Lung cfDNA assay kit [5], as shown in Table 5.

Sensitivity, specificity, and LOD assessment on fusions

Sensitivity was measured using a trifusion mix containing RNA extracted from cell lines harboring *ALK, RET,* and *ROS1* fusions and *MET* exon skipping. Specificity was evaluated using cfTNA from healthy donor samples. Limit of detection (LOD) experiments were performed using Seraseq™ Fusion RNA Mix v2 (SeraCare, Milford, MA).

Sensitivity and specificity for fusion detection

A trifusion mix was prepared by spiking total RNA extracted from positive cell lines harboring *ALK, RET,* and *ROS1* fusions and *MET* exon skipping into a normal cfTNA background at 1% frequency by mass.

To calculate sensitivity, 30 replicates in total were assessed, across 4 users at two sites and with 2 different manufactured lots of the assay panel (Table 6). Specificity was evaluated using cfTNA isolated from six individual donor samples using the two most commonly used blood collection tubes, BD Vacutainer K_2 EDTA tubes and Streck Cell-Free DNA BCT tubes, as shown in Table 7.

Thus, fusion and *MET* exon skipping detection can be achieved at 1% frequency with mean sensitivity of 98% and mean specificity of ≥99.9%.

Table 5. Comparision of SNV detection between the Oncomine Lung cfDNA and cfTNA assays.

Product	Sample	Sensitivity	Specificity
Oncomine Lung cfDNA kit	0.1% SNV control	92.2%	99.70%
	0.5% SNV control	>99.9%	99.60%
Oncomine Lung cfTNA kit	0.1% SNV control	92.1%	>99.9%
	0.5% SNV control	>99.9%	>99.9%

Table 6. Sensitivity measurement for structural variants (fusions and *MET* exon skipping).

Fusion results (n = 30)	
User	**Average sensitivity**
User 1	94.44%
User 2	100%
User 3	100%
User 4	100%
Mean sensitivity	**98.88%**
***MET* exon skipping results (n = 30)**	
User	**Average sensitivity**
User 1	100%
User 2	100%
User 3	100%
User 4	100%
Mean sensitivity	**100%**

Table 7. Specificity measurement for structural variants (fusions and *MET* exon skipping).

Donor	Tube	Specificity
Donor 1	EDTA	100%
Donor 2	EDTA	100%
Donor 3	EDTA	100%
Donor 4	EDTA	100%
Donor 5	EDTA	100%
Donor 6	EDTA	100%
Donor 1	Streck	100%
Donor 2	Streck	100%
Donor 3	Streck	100%
Donor 4	Streck	100%
Donor 5	Streck	100%
Donor 6	Streck	100%
Mean specificity		**100%**

LOD assessment

The reference material Seraseq Fusion RNA mix v2, containing purified RNA from cell lines harboring 14 clinically relevant gene fusions, a *MET* exon skipping variant, and a multi-exon deletion and with previously determined transcript levels, was used to establish the LOD for fusions. The targets of the Oncomine Lung cfTNA Research Assay present in the reference material are shown in Table 8.

Libraries were prepared using serial dilutions of Seraseq RNA Fusion Mix v2 reference material into 20 ng of cfTNA from healthy donor blood research samples. Quantification of transcript levels by digital PCR was used to establish the number of copies present in each serial dilution.

Digital PCR results were obtained using QuantStudio 3D Digital PCR Systems with assays recommended by SeraCare. Sequencing data were correlated with available dPCR results to establish LOD for detecting fusions in cell-free nucleic acid from blood.

Data for all 5 structural variants present in Seraseq RNA Fusion Mix v2 reference material covered by the Oncomine Lung cfTNA Research Assay are shown in Table 9.

Table 8. Targets in the Seraseq RNA Fusion mix v2 reference material that can be detected using the Oncomine Lung cfTNA Research Assay.

Fusion RNA	5′ partner	3′ partner	Primary cancer tissue
EML4-*ALK*	*EML4* exon 13	*ALK* exon 20	Lung
KIF5B-*RET*	*KIF5B* exon 24	*RET* exon 11	Lung
CD74-*ROS1*	*CD74* exon 6	*ROS1* exon 34	Lung
SLC34A2-*ROS1*	*SLC34A2* exon 4	*ROS1* exon 34	Lung, stomach
MET exon 14 skipping	*MET* exon 13	*MET* exon 15	Lung

For all 5 structural variants present in the Seraseq reference control and included in the panel, a true positive call (≥3 molecular counts) was confirmed using sequencing data with 10 or more copies of fusion transcript (as measured by dPCR) present in the sample. This is consistent with library conversion rates (40–50%) for SNV detection at 0.1% from 20 ng of input DNA (6,000 copies). It is possible that other cfTNA samples may contain a larger number of degraded RNA transcripts; hence, the minimal input copy number requirement might be higher.

Sensitivity, specificity, and input titration for copy number variant detection

Cell-free DNA (cfDNA) isolated from cell culture medium of the *MET* CNV-positive cell line NCI-H1573 was spiked into the cfTNA background from healthy donor research samples to generate control CNV samples with different fold changes. The conversions between fold change and CNV in a liquid biopsy sample are shown in Table 10.

Table 9. Fusion titration data.

Fusion ID	Copy number by dPCR	Molecular counts by sequencing rep 1	Molecular counts by sequencing rep 2
CD74-ROS1.C6R34	17	12	26
	9	13	3
	4	1	1
	2	6	0
EML4-ALK.E13A20	14	31	27
	7	20	15
	3	9	5
	2	1	0
MET-MET.M13M15	38	55	51
	19	26	10
	10	10	9
	5	5	1
KIF5B-RET.K24R11	19	9	22
	10	4	8
	5	11	2
	2	4	0
SLC34A2-ROS1.S4R34	16	4	8
	8	1	2
	4	2	0
	2	0	0

Table 10. Conversion between fold change and CNV status of positive cell line. The conversion between fold change and CNV status of positive cell line with different titration levels (tumor fraction in cfTNA background). For example, spiking 10% cfDNA from a positive cell line with CNV status of 5 (10 copies) into 90% normal cfTNA background will yield a sample with 1.4-fold amplification.

		Cancer cell line CNV status (fold amplification)					
		2	4	5	6	8	10
Titration level approximating tumor fraction	20.0%	1.20	1.60	1.80	2.00	2.40	2.80
	10.0%	1.10	1.30	1.40	1.50	1.70	1.90
	5.0%	1.05	1.15	1.20	1.25	1.35	1.45
	1.0%	1.01	1.03	1.04	1.05	1.07	1.09

Sensitivity and specificity for CNV detection

For sensitivity evaluation, a control sample was prepared at 1.34-fold amplification using the method described above, and the fold change was confirmed with dPCR. Thirty libraries were prepared by 4 users across 2 sites and using 2 different manufactured lots of the assay panel (Table 11). Specificity was evaluated using cfTNA isolated from six individual donor research samples, using the two most commonly used blood collection tubes, BD Vacutainer K_2 EDTA tubes and Streck Cell-Free DNA BCT tubes (Table 12). *MET* CNV at 1.4-fold amplification can be detected with mean sensitivity and specificity of >99.9%.

Table 11. Detection of *MET* CNV target with fold change, *P* value, and MAPD information.

Sample ID	Panel lot	CNV ratio	*P* value	MAPD	CNV call
User 1, rep 1	Lot 1	1.38	0.00E+00	0.127	Gain
User 1, rep 2	Lot 1	1.40	0.00E+00	0.152	Gain
User 1, rep 3	Lot 1	1.41	0.00E+00	0.160	Gain
User 1, rep 4	Lot 1	1.36	0.00E+00	0.113	Gain
User 1, rep 5	Lot 1	1.39	0.00E+00	0.153	Gain
User 1, rep 6	Lot 1	1.45	0.00E+00	0.182	Gain
User 2, rep 1	Lot 1	1.38	0.00E+00	0.172	Gain
User 2, rep 2	Lot 1	1.41	0.00E+00	0.163	Gain
User 2, rep 3	Lot 1	1.41	0.00E+00	0.134	Gain
User 2, rep 4	Lot 1	1.41	0.00E+00	0.124	Gain
User 2, rep 5	Lot 1	1.40	0.00E+00	0.130	Gain
User 2, rep 6	Lot 1	1.35	0.00E+00	0.172	Gain
User 2, rep 7	Lot 2	1.35	0.00E+00	0.166	Gain
User 2, rep 8	Lot 2	1.34	0.00E+00	0.160	Gain
User 2, rep 9	Lot 2	1.29	0.00E+00	0.164	Gain
User 2, rep 10	Lot 2	1.37	0.00E+00	0.191	Gain
User 2, rep 11	Lot 2	1.34	0.00E+00	0.181	Gain
User 2, rep 12	Lot 2	1.26	0.00E+00	0.160	Gain
User 3, rep 1	Lot 1	1.42	0.00E+00	0.168	Gain
User 3, rep 2	Lot 1	1.38	0.00E+00	0.163	Gain
User 3, rep 3	Lot 1	1.38	0.00E+00	0.171	Gain
User 3, rep 4	Lot 1	1.41	0.00E+00	0.141	Gain
User 3, rep 5	Lot 1	1.45	0.00E+00	0.159	Gain
User 3, rep 6	Lot 1	1.42	0.00E+00	0.164	Gain
User 4, rep 1	Lot 1	1.38	0.00E+00	0.167	Gain
User 4, rep 2	Lot 1	1.35	0.00E+00	0.148	Gain
User 4, rep 3	Lot 1	1.35	0.00E+00	0.167	Gain
User 4, rep 4	Lot 1	1.36	0.00E+00	0.142	Gain
User 4, rep 5	Lot 1	1.42	0.00E+00	0.184	Gain
User 4, rep 6	Lot 1	1.39	0.00E+00	0.166	Gain
				Average sensitivity	100%

Table 12. Specificity for CNV detection.

Donor	Tube type	CNV ratio	*P* value	MAPD	CNV call
Donor 1	EDTA	1.04	1.00E+00	0.126	Pass
Donor 2	EDTA	1.00	1.00E+00	0.101	Pass
Donor 3	EDTA	0.98	1.00E+00	0.115	Pass
Donor 4	EDTA	1.01	1.00E+00	0.107	Pass
Donor 5	EDTA	1.00	1.00E+00	0.096	Pass
Donor 6	EDTA	1.00	1.00E+00	0.077	Pass
Donor 1	Streck	0.98	1.00E+00	0.103	Pass
Donor 2	Streck	0.98	1.00E+00	0.096	Pass
Donor 3	Streck	0.99	1.00E+00	0.132	Pass
Donor 4	Streck	1.00	1.00E+00	0.139	Pass
Donor 5	Streck	0.98	1.00E+00	0.083	Pass
Donor 6	Streck	1.00	1.00E+00	0.099	Pass
			Mean specificity (n = 30)		100%

Input amount titration for CNV detection

To evaluate the LOD for CNV using a broad input range, the following samples were prepared as detailed in Table 9, where cfDNA from positive cell lines was titrated into normal cfTNA background. Samples were prepared with a broad cfDNA input range of 1–20 ng and 2 different fold changes (Table 13). Fold change was also calculated using dPCR for cfDNA isolated from the *MET* CNV-positive cell line to confirm expected fold changes for these sample titrations. The Oncomine Lung cfTNA Research Assay enables detection of *MET* CNV at 1.29-fold amplification with input amounts as low as 1 ng.

Libraries were prepared using standard protocols as outlined in the user guide to produce barcoded libraries. Six libraries were multiplexed for templating on the Ion Chef System and subsequently sequenced on the Ion S5 XL System using the Ion 530 Chip Kit.

Conclusion

The Oncomine Lung cfTNA Research Assay is a highly sensitive NGS assay that covers SNVs, indels, CNVs, and fusions for multibiomarker analysis, with variant detection as low as 0.1%. Starting from a single tube of blood, variant data are generated in a streamlined two-day workflow.

K_2 EDTA blood collection tubes are recommended for running the assay, with plasma preparation and cfTNA extraction within 6 hours of blood collection. If storage of blood is an important consideration, glass Streck DNA tubes may be used, with processing within 24 hours of blood collection.

For cfTNA extraction, the MagMAX Cell-Free Total Nucleic Acid Isolation Kit is recommended, and for quantification, the Invitrogen™ Qubit™ dsDNA HS Assay Kit. DNA quality may be assessed with the Agilent Bioanalyzer system, and for RNA quality, a new digital PCR method described here is recommended.

Table 13. Correlation between expected fold change (from dPCR) and observed fold change (from sequencing results) across an input range of 1–20 ng.

Input amount	Expected fold change (dPCR)	Sequencing fold change replicate 1	Sequencing fold change replicate 2
20 ng	1.29x	1.22x	1.26x
	1.58x	1.45x	1.50x
10 ng	1.29x	1.29x	1.31x
	1.58x	1.57x	1.58x
2 ng	1.29x	1.27x	ND
	1.58x	1.47x	1.56x
1 ng	1.29x	1.20x	1.24x
	1.58x	1.54x	1.39x

ion torrent

High sensitivity and specificity were observed for SNV detection, fusion and *MET* exon skipping detection, and CNV detection.

The Oncomine Lung cfTNA Research Assay is a reliable tool for liquid biopsy clinical research and enables researchers to develop tests that may impact treatment selection, treatment monitoring, and recurrence monitoring in the future.

References

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Ordering information

Product	Cat. No.
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Ion GeneStudio S5 System	A38194
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