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Evaluation and Comparison of Liquid Biopsy Reference Materials from Commercial Sources using Oncomine™ Pan-Cancer Cell-free Assay

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INSTRODUCTION

Over the last few years, analysis of liquid biopsies has grown rapidly to interrogate cell-free nucleic acid and characterize tumor mutation profiles from plasma. To date, a number of liquid biopsy research assays utilizing next generation sequencing technologies have been made commercially available to detect various types of variants at low allele frequency with minimal sample input. To ensure each assay is performed consistently and adopted successfully by researchers, reference materials are essential in assay validation and can be used as standards to compare technologies and standardize protocols across different laboratories.

MATERIALS AND METHODS

Reference material

Multiplex cfDNA reference standard set: this standard set (Horizon Discovery, Cat# HD780) contains 4 contrived DNA samples which mimic cfDNA type of material. Each sample covers 8 engineered single nucleotide variants (SNVs/SNPs) with allele frequency at 5%, 1%, 0.1%, and 0% (100% wild type), respectively.

Structural Multiplex cfDNA Reference Standard: this standard (Horizon Discovery, Cat# HD786) contains RET and ROS1 fusion variants, MYC and MET amplifications.

Seraseq® cfDNA complete™ mutation mix: four ctDNA mutation mix samples were provided by Seracare including wild type (Cat# 0710-0533), 1% (Cat# 0710-0530), 0.5% (Cat# 0710-0531), and 0.1% (Cat# 0710-0532). Each sample contains 25 unique multiplexed variants in 16 genes, covering 12 SNVs, 7 INDELs, 3 CNVs, and 3 SVs at different allele frequencies.

Seraseq® fusion RNA mix: this contrived fusion standard (Cat# 0710-0431) contains 16 clinically relevant RNA fusions mixed in total RNA from GM24385 cell line as background. In this study, it was further diluted to 10%, 5%, and 2% using cfTNA from healthy donor as background to generate a serial of standards with different copy numbers of fusion variants for limit of detection (LOD) evaluation.

Library preparation and sequencing:

Targeted libraries were prepared using Oncomine™ Pan-Cancer Cell-free assay reagents (Thermo Fisher Scientific, Cat# A37664).

For Horizon multiplex cfDNA reference standard, 25 ng was used as input in library preparation. In addition, input amount for 0.1% standard was increased to 50 ng to mimic 6000 copies of DNA allowing for improved sensitivity at lower LOD.

For Seraseq® cfDNA complete™ mutation mix, 25 ng was used as input in library preparation. For the serial dilutions from Seraseq® fusion RNA mix in cfTNA background, 20 ng was used as input in library preparation.

The Ion 540™ Kit-Chef was used for template preparation on Ion Chef™ (Thermo Fisher Scientific, Cat# 4484177) and followed by sequencing on Ion GeneStudio S5 XL system (Thermo Fisher Scientific, Cat# A27214) using the Ion 540™ Chip (Thermo Fisher Scientific, Cat# A27766).

Data analysis:

Data analysis was performed using Torrent Suite™ Software v5.6 and Ion Reporter™ v5.6 for simultaneous SNV/Indel, CNV, and fusion variant calls .

Table 1. Oncomine™ Pan-Cancer Cell-free Assay Content

Assay	Configuration	Unique Genes	DNA	RNA
Pan Cancer	TNA (DNA + RNA)	52	50	12

Hotspot Genes		Tumor Suppressor Genes	Copy Number Genes	Gene Fusions
AKT1	*HRAS*	*APC*	*CCND1*	*ALK*
ALK	*IDH1*	*FBXW7*	*CCND2*	*BRAF*
AR	*IDH2*	*PTEN*	*CCND3*	*ERG*
ARAF	*KIT*	*TP53*	*CDK4*	*ETV1*
BRAF	*KRAS*		*CDK6*	*FGFR1*
CHEK2	*MAP2K1*		*EGFR*	*FGFR2*
CTNNB1	*MAP2K2*		*ERBB2*	*FGFR3*
DDR2	*MET*		*FGFR1*	*MET*
EGFR	*MTOR*		*FGFR2*	*NTRK1*
ERBB2	*NRAS*		*FGFR3*	*NTRK3*
ERBB3	*NTRK1*		*MET*	*RET*
ESR1	*NTRK3*		*MYC*	*ROS1*
FGFR1	*PDGFRA*			
FGFR2	*PIK3CA*			
FGFR3	*RAF1*			
FGFR4	*RET*			
FLT3	*ROS1*			
GNA11	*SF3B1*			
GNAQ	*SMAD4*			
GNAS	*SMO*			

Variant Type	Total Variants
SNV	> 900
CNV	12
Fusion/MET Exon Skipping	99

Single Pool design (DNA & RNA)

Performance Specs:

Hotspot SNV/Indel
- 0.1% AF LOD with 20 ng input

Whole target SNV/Indel
- 0.5% AF

CNV detection
- 1.4x fold change

Fusion detection
- 1% RNA fusions in cfTNA

Sample Plexy
- 1 library on a 530 chip
- 4 libraries on a 540 chip
- 8 libraries on a 550 chip

Table 1. Oncomine™ Pan-Cancer cell-free assay provides broad coverage across 52 unique genes that detects DNA and RNA variants across multiple cancer types. The workflow also offers multiplexing flexibility utilizing different chip configurations available to GeneStudio S5 systems to accommodate the needs for desired throughput..

RESULTS

Figure 1. Oncomine™ Pan-Cancer Cell-free Assay Workflow

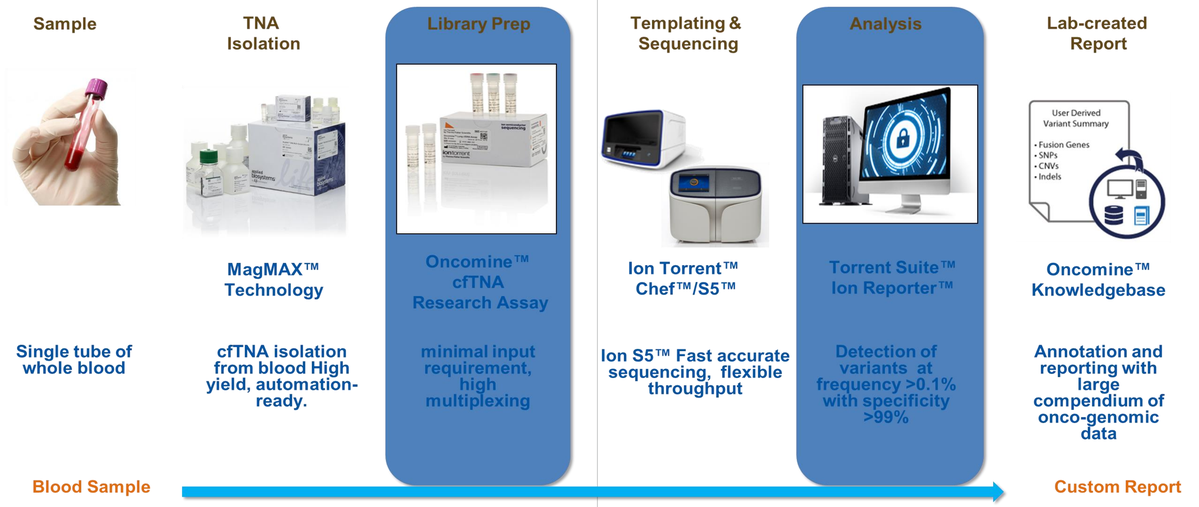

Figure 1. The OncomineTM Pan-Cancer cell-free assay enables a fast 3-day workflow that starts with cell-free nucleic acid purification from a single tube of blood and library preparation to interrogate both DNA and RNA in a single reaction. The amplified library will undergo high-throughput semiconductor sequencing, with results analyzed and reported through an integrated bioinformatics solution.

Table 2. Result Summary for Horizon Multiplex cfDNA Reference Standards

Sample			SNV / Indel			
	Gene	AA Chg	AF % (R1)	AF % (R2)	AF % (R3)	AF % (R4)
Horizon cfDNA RS WT	CTNNB1	p.S33Y	24.0	24.3	26.7	24.2
	PIK3CA	p.H1047R	24.9	24.0	26.5	24.7
	EGFR	p.G719S	20.6	23.5	22.9	22.2
	BRAF	p.V600E	29.9	29.3	30.7	33.5
	MAP2K1	p.Q56P	22.4	23.7	23.7	22.4
Sample			**SNV / Indel**			
	Gene	AA Chg	AF % (R1)	AF % (R2)	AF % (R3)	AF % (R4)
Horizon cfDNA RS 5% (25ng)	NRAS	p.Q61K	7.37	6.86	6.46	6.22
	NRAS	p.A59T	7.20	7.22	6.34	6.96
	PIK3CA	p.E545K	6.94	6.55	6.33	5.96
	EGFR	p.Q746_A750del	5.48	6.98	6.70	5.80
	EGFR	p.M766_A767insASV	3.60	3.78	3.37	3.12
	EGFR	p.T790M	3.72	3.53	3.01	3.98
	EGFR	p.L858R	6.05	4.74	4.20	5.09
	KRAS	p.G12D	6.33	5.28	6.22	6.27
Sample			**SNV / Indel**			
	Gene	AA Chg	AF % (R1)	AF % (R2)	AF % (R3)	AF % (R4)
Horizon cfDNA RS 1.0% (25ng)	NRAS	p.Q61K	1.12	1.47	1.15	1.46
	NRAS	p.A59T	1.46	0.78	1.77	1.19
	PIK3CA	p.E545K	1.10	1.13	1.52	1.12
	EGFR	p.Q746_A750del	1.12	1.53	1.45	1.71
	EGFR	p.M766_A767insASV	0.74	0.62	0.69	0.68
	EGFR	p.T790M	0.81	0.80	0.69	0.58
	EGFR	p.L858R	0.57	0.86	0.80	0.94
	KRAS	p.G12D	0.90	1.43	1.24	1.49
Sample			**SNV / Indel**			
	Gene	AA Chg	AF % (R1)	AF % (R2)	AF % (R3)	AF % (R4)
Horizon cfDNA RS 0.1% (25ng)	NRAS	p.Q61K	ND	0.21	0.17	
	NRAS	p.A59T	ND	0.13	0.17	0.13
	PIK3CA	p.E545K	ND	0.11	0.19	0.15
	EGFR	p.Q746_A750del	0.13	0.08	ND	ND
	EGFR	p.M766_A767insASV	0.11	ND	0.08	0.21
	EGFR	p.T790M	0.20	0.16	0.11	0.15
	EGFR	p.L858R	ND	ND	ND	ND
	KRAS	p.G12D	ND	ND	0.09	ND
Sample			**SNV / Indel**			
	Gene	AA Chg	AF % (R1)	AF % (R2)		
Horizon cfDNA RS 0.1% (50ng)	NRAS	p.Q61K	ND	0.098		
	NRAS	p.A59T	0.096	0.117		
	PIK3CA	p.E545K	0.079	0.212		
	EGFR	p.Q746_A750del	0.101	0.120		
	EGFR	p.M766_A767insASV	0.073	ND		
	EGFR	p.T790M	0.193	0.117		
	EGFR	p.L858R	0.136	0.117		
	KRAS	p.G12D	ND	ND		

Table 2. All 13 variants were successfully detected in Horizon Multiplex I cfDNA RS standards above 1% AF (25 ng input) using Pan-Cancer assay, including 8 SNV/Indels at several important gene loci and 5 additional SNVs from parental cell lines as shown in RS wild-type. For 0.1% RS, high DNA input at 50 ng that is equivalent 6000 copies of DNA molecules significantly improved variant detection sensitivity from ~73% to ~85% comparing to 25 ng input. No FPs were observed in any samples.

Table 3. Result Summary for Seraseq® cfDNA Complete™ Mutation Mix

Sample			SNV / Indel		
Seraseq ctDNA complete mix WT	Gene	AA Chg	R1 (%AF)	R2 (%AF)	R3 (%AF)
	FP		1	2	2
Seraseq ctDNA complete mix 1.0%	Gene	AA Chg	R1 (%AF)	R2 (%AF)	R3 (%AF)
	NRAS	p.Q61R	1.09	1.33	0.92
	ALK	p.G1202R	1.14	1.20	1.34
	ALK	p.F1174L	0.74	1.26	1.05
	PIK3CA	p.H1047R	1.18	1.50	1.13
	KIT	p.D816V	1.45	0.88	1.26
	EGFR	p.E746_A750del	1.31	1.48	0.95
	EGFR	p.T790M	1.29	1.43	0.91
	EGFR	p.L858R	1.02	1.29	0.77
	BRAF	p.V600E	0.70	0.47	1.08
	KRAS	p.Q61H	1.02	1.04	1.04
	KRAS	p.G12D	0.93	0.81	1.06
	KRAS	p.G12C	1.00	1.07	1.03
	EGFR	p.L747_P753>S	1.77	1.24	1.68
	EGFR	p.S752_I759del	1.16	1.03	1.11
	ERBB2	p.E770_A771insAYVM	0.94	1.00	0.87
	AKT1	p.E17K	0.75	0.76	0.69
	TP53	p.G245D	0.37	0.66	0.44
	FP		1	2	2
Seraseq ctDNA complete mix 0.5%	Gene	AA Chg	R1 (%AF)	R2 (%AF)	R3 (%AF)
	NRAS	p.Q61R	0.70	0.68	0.54
	ALK	p.G1202R	0.50	0.79	0.50
	ALK	p.F1174L	0.50	0.60	0.46
	PIK3CA	p.H1047R	0.51	0.58	0.52
	KIT	p.D816V	0.88	0.82	0.91
	EGFR	p.E746_A750del	0.56	0.59	0.68
	EGFR	p.T790M	0.20	0.42	0.45
	EGFR	p.L858R	0.55	0.64	0.72
	BRAF	p.V600E	0.28	0.64	0.55
	KRAS	p.Q61H	0.63	0.55	0.77
	KRAS	p.G12D	0.36	0.59	0.50
	KRAS	p.G12C	0.72	0.59	0.61
	EGFR	p.L747_P753>S	0.88	0.59	0.74
	EGFR	p.S752_I759del	0.88	0.59	0.59
	ERBB2	p.E770_A771insAYVM	0.18	0.28	0.20
	AKT1	p.E17K	0.53	0.65	0.52
	TP53	p.G245D	0.41	0.39	0.33
	FP		1	1	2
Seraseq ctDNA complete mix 0.1%	Gene	AA Chg	R1 (%AF)	R2 (%AF)	R3 (%AF)
	NRAS	p.Q61R	0.24	0.13	0.21
	ALK	p.G1202R	0.10	0.15	0.12
	ALK	p.F1174L	0.09	ND	ND
	PIK3CA	p.H1047R	ND	ND	0.24
	KIT	p.D816V	0.09	0.09	ND
	EGFR	p.E746_A750del	ND	0.31	0.12
	EGFR	p.T790M	ND	0.15	ND
	EGFR	p.L858R	0.21	0.13	ND
	BRAF	p.V600E	0.21	ND	ND
	KRAS	p.Q61H	0.43	ND	ND
	KRAS	p.G12D	ND	ND	0.14
	KRAS	p.G12C	ND	0.08	0.20
	EGFR	p.L747_P753>S	0.09	0.15	ND
	EGFR	p.S752_I759del	0.09	0.15	ND
	ERBB2	p.E770_A771insAYVM	0.13	ND	ND
	AKT1	p.E17K	0.15	ND	0.14
	TP53	p.G245D	0.65	0.29	0.41
	FP		3	1	3

Table 3. All 16 variants were successfully detected in Seraseq™ cfDNA Complete Mutation Mix with higher allele frequency ≥ 0.5%. As observed previously (data not shown) additional FP calls were made in these libraries.

Table 4. Result Summary for Seraseq® Fusion RNA Dilutions

Fusion Variants	Copies (20%)*	Molecule Count$^{\Delta}$			
	ddPCR_SeraCare	20%	10%	5%	2%
TPM3-NTRK1	95	77	66	22	11
LMNA-NTRK1	97	123	30	16	15
SLC45A3-BRAF	132	110	35	6	2
EML4-ALK	184	78	36	15	19
FGFR3-BAIAP2L1	259	38	16	16	3
FGFR3-TACC3	147	35	20	14	3
SLC34A2-ROS1	101	24	4	3	ND
CD74-ROS1	153	40	18	4	8
MET Exon14 Skipping	166	68	45	15	9
KIF5B-RET	83	76	45	15	5
NCOA4-RET	144	127	30	13	14
ETV6-NTRK3	128	87	51	15	8
TMPRSS2-ERG	83	8	ND	ND	ND
PAX8-PPARG1	259	Not covered			
EGFR-SEPT14	133				
EGFR variant III	124				

* Estimated copy number in 20% dilution based on ddPCR measurement from SeraCare

$^{\Delta}$ Measured copy number using Pan-Cancer assay

Table 4. All 13 fusion variants covered by Pan-Cancer assay were detected in Seraseq fusion standards diluted down to 5% in cfTNA background. Based on the molecule counts, LODs for each fusion target were able to achieve at 2% standard for Pan-Cancer assay.

Table 5. Result Summary for Horizon Structural Multiplex cfDNA Reference Standard

Sample	SNV / Indel			CNV		Fusion	
	Gene	AA Chg	AF % (R1)	Gene	CNV Ratio	Variant Exon	Molecule Counts
	CTNNB1	p.S33Y	5.4	FGFR3*	1.7	SLC34A2-ROS1.S4R32	50
	PIK3CA	p.E545K	5.8	CDK6*	1.4	SLC34A2-ROS1.S4R34	6
	PIK3CA	p.H1047R	15.7	MET	1.8	CCDC6-RET.C1R12	26
Structural	EGFR	p.G719S	5.8	MYC	3.1		
Multiplex	EGFR	p.Q746_A750del	5.7				
cfDNA	EGFR	p.M766_A767insASV	4.9				
Reference	BRAF	p.V600E	16.4				
Standard	KRAS	p.G13D	4.7				
	AKT1	p.E17K	5.0				
	MAP2K1	p.Q56P	4.7				
	TP53	p.S241F	6.9				
	TP53	p.R175C	0.2				
	GNA11	p.Q209L	5.8				

*FGFR3 and CDK6 gene amplification false positives due to baseline not compatible with contrived samples using genomic DNA material.

Table 5. All the expected variants in Horizon structural multiplex cfDNA standard were detected including 13 SNC/Indel, ROS1 and RET RNA fusion, MET and MYC gene amplifications.

CONCLUSIONS

- This study established overall performance of commercial cfDNA control materials using Oncomine™ Pan-Cancer cell-free assay and provided reference information for laboratories when selecting appropriate standards during assay evaluation and validation.
- Horizon Multiplex I cfDNA reference standard set contains 8 SNV/Indels at several important gene loci and 5 additional SNVs from parental cell lines using Pan-Cancer assay. All 13 variants were successfully detected from standards > 1%; for 0.1% standard, majority of variants were detected consistently at 50 ng DNA input with sensitivity of 85% and specificity of 100%.
- In Seraseq™ cfDNA Complete Mutation Mix, Pan-Cancer assay covered 16 out of 19 claimed SNV/Indels. In high AF samples above 0.5%, all 16 SNVs were successfully detected using Pan-Cancer assay. In 0.1% standard, quite a few variants were missed with sensitivity < 60%. In addition. More false positives were reported in this set of control samples.
- Similarly, all variant types in Horizon Structural Multiplex standard were successfully detected including SNV/Indel, CNV and fusion with 100% sensitivity. In addition, gene amplification of FGFR3 and CDK6 was detected possibly due to the CNV baseline in Pan-Cancer assay which were constructed mainly using cell free type of material and not including this type of contrived sample using genomic DNA material.

TRADEMARKS/LICENSING

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