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Illumina TruSight Oncology 500 ctDNA

Technical Specification & Performance Evaluation

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The Illumina TruSight Oncology 500 circulating tumour DNA (ctDNA) assay is an **NGS-based assay** that enables **comprehensive genomic profiling to assess multiple variant types in 523 cancer-related genes from cell-free DNA (cfDNA)**. This assay features the same DNA content as the TruSight Oncology 500 solid tumour assay for detection of single nucleotide variants (SNVs), indels, copy number variants (CNVs), DNA fusions, tumour mutational burden (TMB), and microsatellite instability (MSI) from plasma samples.

In addition, the **DRAGEN Bio-IT Platform** offers speed and accuracy to detect variants detections through its ultra-rapid variant calling algorithm.

Technical specification:

Metric	TSO-500 ctDNA Solution
Chemistry	Illumina TruSight Oncology 500 ctDNA
System	NovaSeq 6000™ System
Genes	523 genes. See Illumina Website under 'Product Literature' for *(Full Gene List)*
Panel Size	1.94 Mb DNA
Sample type	Plasma
DNA input requirement	30ng
Standard Sequencing Specification	Median Exon Coverage ≥1300X ≥80% of target exons covered at ≥1000X
Total assay turnaround time	For batch retrospective testing - turnaround time agreed on a per project basis
Run time	44 hrs Sequencing run time (S4 flowcell)
Sample throughput	24 Libraries per run (S4 flowcell)
Variants	SNVs and indels, DNA fusions, CNVs, MSI, TMB
Limit of detection (VAF)	0.5% VAF for small variants (as per Illumina's manufacturing guidelines)
Data Analysis	DRAGEN Server pipeline
Deliverables/ Output files	BCL FASTQ gVCF Annotated Small Variant Report Copy Number Variants Report Fusion Report Combined Variant Report Metrics Report

Benefits:

- Enables comprehensive tumour cell profiling when tissue biopsy is not an option.
- Targets multiple biomarkers and somatic variant types in a single assay with limited sample input requirements.
- Allows monitoring of disease progression, response to treatment and acquired resistance.

Features:

- Coverage of current and emerging biomarkers including comprehensive coverage of key cancer-associated genes across multiple tumour types.
- Immuno-oncology relevant biomarker readouts including blood TMB (bTMB) and blood MSI (bMSI).

- Unique molecular barcode (UMI) technology, high raw coverage enabled by the NovaSeq 6000 system, and *in silico* error correction models improve variant calling and reduce artefacts.
- Ultra-rapid variant calling powered and accelerated by DRAGEN Bio-IT Platform.
- Robust variant calling performance demonstrated for low frequency (<0.5%) variants.
- Hybrid capture enrichment library preparation coupled with comprehensive content enables novel variant and rearrangement detection without a *priori* knowledge.
- Upstream cfDNA QC enables assessment of gDNA contamination in sample prior to library preparation and sequencing, enabling contextualisation of observed results.
- Comprehensive variant annotation allows effective triage of clinically actionable variants.

Performance Evaluation of Illumina TruSight Oncology 500 ctDNA Assay

Disclaimer: The data demonstrated herein does not reflect the analytical performance in clinical samples.

The overall study objectives were to perform a preliminary evaluation of the platform and to help guide input requirements for acceptable coverage and robust variant detection.

1. Assess the technical performance (sensitivity and repeatability) of the TSO-500 ctDNA.
2. Assess the concordance of genomic aberrations reported in liquid samples by the TSO-500 ctDNA assay with those detected in the matched FFPE samples using the TSO-500 solid tumor assay.
3. Determine the effect of variance in library prep template input with respect to the above two factors.
4. Finalise a protocol to be used going forward for routine delivery of the RUO assay.

Assessment of Assay Performance with SeraCare Control Samples:

SeraCare control samples with known small variant (SNVs and indels), CNV and fusion status were selected to assess assay performance (Table 1).

Table 1: SeraCare control sample with known mutational status and variant allele frequency (VAF) (n=4).

Control Type	Variant Allele Frequency (%)	Mutational Status
Seraseq® ctDNA Ref Material v2	0.25%	SNV, Fusions
Seraseq® ctDNA Ref Material v2	0.5%	SNV, Fusions
Seraseq® ctDNA Complete™ Ref Material AF	0.5%	SNV, CNV, Fusions
Seraseq® ctDNA Complete™ Ref Material AF	1.0%	SNV, CNV, Fusions

1. Sensitivity of Small Variant, Fusion and CNV Calling

Sensitivity of small variants, fusions and CNV detection was determined by assessing agreement between variant calls from TSO-500 ctDNA assay and known mutations across the SeraCare controls. All SeraCare controls were processed in duplicate at two input levels (30ng and 100ng).

Table 2: Sensitivity estimates of SeraCare controls by variant allele frequency (VAF) and input (ng).

Input (ng)	Variant Allele Frequency (%)	Estimate	(95%) Confidence Intervals
100	0.25	97.37	90.90-99.28
100	0.5	99.12	95.20-99.84
100	1.0	100.00	90.82-100.00
30	0.25	88.16	79.00-93.64
30	0.5	98.25	93.83-99.52
30	1.0	100.00	90.82-100.00

Conclusion - Template input at 100ng improved sensitivity, but only in variants with extremely low variant allele frequency (VAF) (0.25%). The analytical limit of detection for small variants at either input is ~0.5% (VAF). *(N.B. Requires verification with clinical samples)*.

While sensitivity was improved at 100ng for variants in the <0.5% VAF SeraCare control samples, this input also resulted in a dramatic reduction in molecular bin depth (family size). This reduction would cause less accurate consensus reads to be generated and in turn is likely to affect the specificity of the assay. Also, due to the limited nature of cfDNA in plasma, obtaining cfDNA at a concentration high enough to achieve 100ng input for library preparation during routine sample processing is highly challenging. For these reasons an optimum input of 30ng was selected for further assessments of the assay performance.

Table 3: Sensitivity of fusion detection estimate as a function of allele frequency in SeraCare controls at 30ng input.

Variant Allele Frequency (%)	Estimate	(95%) Confidence Intervals
0.25	75.00	30.06-95.44
0.5	80.00	49.02-94.33
1	100.00	60.97-100.00

Conclusion – High level of fusion calls detected in all samples at 30ng despite low number of fusion events. *(N.B Contrived samples are not representative of fusions encountered in clinical samples therefore variance in performance could be observed. The low number of samples limits the statistical power of this study)*.

Table 4: Sensitivity of CNV detection in SeraCare controls at 30ng input. Selected SeraCare controls (n=2) were used for the sensitivity assessment that had confirmed amplification calls, harbouring three CNV events, namely amplifications in MET, ERBB2 and MYC.

Seraseq® ctDNA Complete™ Ref Material AF (0.5%)				Seraseq® ctDNA Complete™ Ref Material AF (1.0%)		
Gene ID	Average CNV in ctDNA compared to normal CN of 2	Approximate CNV in tumour cell based on 2% ctDNA fraction (Absolute Copies)	Reproducibility of Call	Average CNV in ctDNA compared to normal CN of 2	Approximate CNV in tumour cell based on 2% ctDNA fraction (Absolute Copies)	Reproducibility of Call
ERBB2	2.56	30	100%	2.87	45.50	100%
MET	2.41	22.5	100%	2.68	36.00	100%
MYC	2.37	20.5	100%	3.07	55.50	100%

Conclusion – All expected CNV events were detected at 30ng input. *(N.B. Due to the contrived nature of these controls, CNV calling is hampered by the fact that baseline coverage profiles are not reflective of normal samples).*

2. Repeatability of Small Variant, Fusion and CNV Calling

Repeatability of small variants, fusions and CNV calls in SeraCare controls processed at 30ng input were assessed by estimating the proportion of expected events that were detected across the two sample replicates.

Table 5: Repeatability of small variant detection in SeraCare controls at 30ng Input.

Sample	Variant Allele Frequency (%)	Agreement (%)	(95%) Confidence Intervals
Seraseq® ctDNA Ref Material v2	0.25%	99.986	99.984-99.987
Seraseq® ctDNA Ref Material v2	0.5%	99.986	99.984-99.987
Seraseq® ctDNA Complete™ Ref Material AF	0.5.%	99.987	99.986-99.989
Seraseq® ctDNA Complete™ Ref Material AF	1.0%	99.988	99.987-99.989
ALL	-	**99.987**	**99.986-99.987**

Conclusion – High levels of repeatability was observed for all samples, >99%.

Table 6: Repeatability of fusion events in SeraCare controls at 30ng Input.

Sample	Variant Allele Frequency (%)	Agreement (%)	(95%) Confidence Intervals
Seraseq® ctDNA Ref Material v2	0.25%	75	30.064-95.441
Seraseq® ctDNA Ref Material v2	0.5%	100	51.011-100
Seraseq® ctDNA Complete™ Ref Material AF	0.5%	66.667	29.999-90.323
Seraseq® ctDNA Complete™ Ref Material AF	1.0%	100	60.967-100
ALL	-	**85.417**	**43.01-96.441**

Conclusion – Fusion repeatability decreased in some samples but overall good agreement was maintained across all SeraCare controls. *(N.B. A limited number of fusion events were detected. These are contrived Seracare samples which may not adequately reflect the performance in clinical samples which the pipeline has been optimised for).*

Table 7: Repeatability of CNV detection in SeraCare controls at 30ng Input. Selected SeraCare controls (n=2) were used for the repeatability assessment that had confirmed amplification calls, harbouring three CNV events, namely amplifications in MET, ERBB2 and MYC.

Sample	Variant Allele Frequency (%)	Observed CNV	Agreement (%)	(95%) Confidence Intervals
Seraseq® ctDNA Complete™ Ref Material AF	0.5%	ERBB2, MET, MYC	100.00	60.97-100.00
Seraseq® ctDNA Complete™ Ref Material AF	1.0%	ERBB2, MET, MYC	100.00	60.97-100.00

Conclusion – Good repeatability of CNV calling observed across samples at clinically relevant amplification levels.

Assessment of Assay Performance in Clinical Samples

Matched FFPE and plasma clinical samples (n=3, disease indication: breast – Clinical Sample 1 and colorectal cancer – Clinical Sample 2 and 3) were selected to assess assay repeatability, and concordance between the TSO-500 solid tumor and ctDNA assays. Repeatability estimates of cfDNA samples were taken from two replicates run at 30ng and for matched FFPE at 100ng. All samples met the required alignment statistics for variant calling.

Table 10: Repeatability of small variant calling in cfDNA clinical samples (n=3).

Sample	Agreement (%)	(95%) Confidence Intervals
Clinical Sample 1	100	100-100
Clinical Sample 2	100	100-100
Clinical Sample 3	99.99	99.99-100
ALL	**100**	**100-100**

Conclusion – High small variant call repeatability across all samples.

In the samples assessed in this study, no fusion events or CNV events were present in either the FFPE samples or matched cfDNA samples when 30ng input was utilised for cfDNA library preparation. As such, assessments of concordance of fusion calls and CNV calls could not be made.

Table 11: Agreement of cfDNA and FFPE variant calls in clinical samples. The analytical accuracy of the assay for small variants was assessed in plasma samples against matched FFPE samples considered as the 'truth'. OPA= overall percent agreement, PPA= positive percent agreement, NPA= negative percent agreement.

Variant	Index	Mean	(95%) Confidence Intervals
SNV	OPA	99.99	99.99-99.99
	PPA	92.03	91.13-92.84
	NPA	100.00	100.00-100.00
Insertion	OPA	100.00	100.00-100.00
	PPA	86.25	77.03-92.15
	NPA	100.00	100.00-100.00
Deletion	OPA	100.00	100.00-100.00
	PPA	67.43	60.18-73.93
	NPA	100.00	100.00-100.00
Complex Variant	OPA	100.00	100.00-100.00
	PPA	100.00	78.47-100.00
	NPA	100.00	100.00-100.00

Table 12: Agreement of cfDNA and FFPE variant calls in clinical samples aggregated across all samples and variant bin classes.

	Mean	(95%) Confidence Intervals
Overall	99.99	99.99-99.99
Positive (+ agreement)	90.92	90.00-91.75
Negative (- agreement)	100	100.00-100.00

Conclusion – This small study demonstrated good concordance between TSO500 solid tumor and cfDNA assays, broadly in agreement with previously published assessments (Jahangiri and Hurst., 2019). However, concordance across different sample cohorts is likely to be impacted by various biological and technical factors such as temporal differences in sample collection, the transient nature of cfDNA, disease stage and tumour burden, metastatic stage and the degree of vasculature of the tumour(s) (Jahangiri and Hurst., 2019).

Summary:

- An initial assessment of the Illumina TruSight Oncology 500 ctDNA assay has provided an insight into the analytical performance. This study does not however represent a full analytical validation of the assay.
- The assay demonstrates high sensitivity and performs well at mutation frequencies representative of somatic events in cfDNA samples.
- From this limited study, 30ng input produced good coverage across the target regions with a significant loss of sensitivity observed only for variants <0.5% VAF.
- Our performance evaluation provides confidence in the suitability of the protocols employed with Almac Diagnostic Service's laboratory and that high-quality datasets are being delivered.
- The assay offers a unique ability to comprehensively profile cfDNA samples across a range of biomarker classes enabling a global picture of the underlying biology.

More Information:
For more information on Almac's range of Liquid Biopsy solutions visit our website here.

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