TECHNICAL NOTE

Using lower input amounts with TruSight™ Oncology ctDNA v2

A study of results achieved using 5–30 ng ctDNA

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Introduction

Comprehensive genomic profiling (CGP) takes advantage of next-generation sequencing (NGS) to assess a wide range of biomarkers in a single assay, using less sample and returning results faster than multiple, iterative testing strategies.[1,2] CGP is often performed using tissue biopsies; however, sufficient tissue sample may not always be available (can occur up to 25% of the time[3]), the tumor may be unaccessible, or results from tissue biospsy may be delayed. Performing CGP using circulating tumor DNA (ctDNA) in the blood, often called a liquid biopsy, delivers multiple advantages, including:

- A minimally invasive method for obtaining sample[4]
- More information about cancer heterogeneity within a sample (temporal and spatial)[4]
- Accessible repeat sampling (eg, when monitoring disease progression or response to potential therapies)[4]
- Faster, less expensive sample preparation method compared to tissue biopsies[4]

Levels of ctDNA can vary depending on several factors, including cancer type, disease stage, plasma amount, total blood volume, etc.[5] To enable sensitive, fast CGP from liquid biopsy, Illumina offers TruSight Oncology 500 ctDNA v2 with a recommended input amount of 20 ng ctDNA for optimal performance. For situations where 20 ng ctDNA is not available, reliable results are still achievable. This technical note demonstrates results generated using TruSight Oncology 500 ctDNA v2 with input amounts ranging from 5–30 ng with varying allele frequencies and sequencing depths.

Methods

Samples

To ensure accurate, repeatable results, two next-generation sequencing (NGS) reference samples with known variants at 0.2% and 0.5% VAF (Table 1) were tested at 5, 10, 20, and 30 ng input. Library preparation included at least two replicates per VAF level and input level. Samples included 19 small variants, 3 CNVs, and 3 gene rearrangements.

Table 1: NGS reference samples obtained from SeraCare[a]

Sample	Catalog no.
Seraseq ctDNA Complete Mutation Mix AF 0.5%	0710-0531
Seraseq ctDNA Complete Mutation Mix AF 0.2% (diluted to targeted VAF in cfDNA from a pool of healthy donor samples)	0710-0531

a. SeraCare is part of LGC Clinical Diagnostics.

Library preparation and sequencing

Seraseq samples are provided as extracted nucleic acids and were tested directly with TruSight Oncology 500 ctDNA v2 for library preparation. Libraries were sequenced on the NovaSeq™ 6000 Sequencing System using an S4 flow cell (Table 2).

Table 2: Sequencing parameter specifications

Parameter	Specification
System	NovaSeq 6000 Sequencing System
Read length	2 × 151 bp
No. of cycles	300
No. of reads per sample	~400M paired-end reads
No. samples per S4 flow cell	24

Error rate reduction with UMIs

To enable ultralow frequency variant identification, library preparation takes advantage of unique molecular identifiers (UMIs) that reduce error rates inherent in NGS to ≤ 0.007% (Figure 1).[6] Noise reduction is achieved by filtering out false variants based on alignment of the UMI barcodes and subsequent read collapsing.

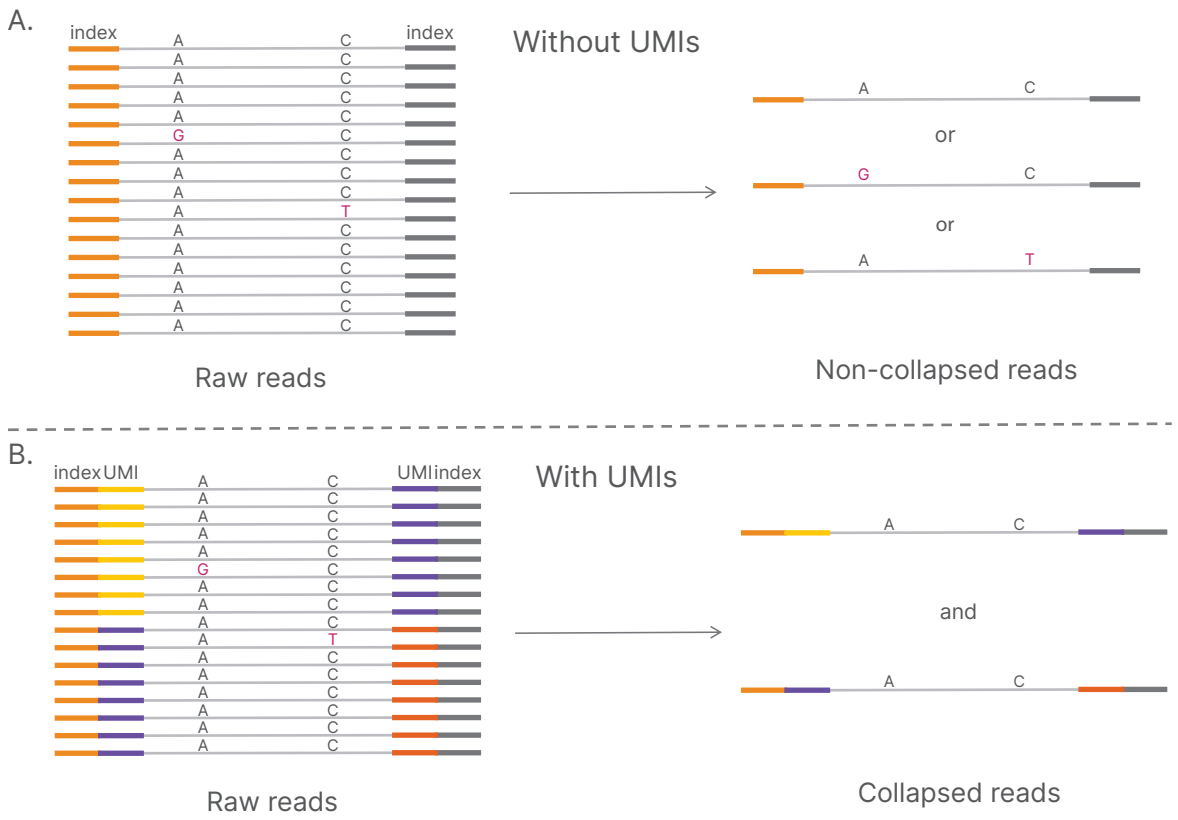

Figure 1: Error correction using UMIs and DRAGEN TruSight Oncology 500 ctDNA v2.1 analysis software for accurate detection of variants—UMIs are integrated with error correction software to enable true mutations to be distinguished from background noise. (A) 16 reads with two variants that could be true rare variants or introduced errors. Without error correction, it is impossible to distinguish between true variants and false positives. (B) Integration of UMIs enables recognition of multiple reads from the same starting molecule and collapses them into a single read. Each set of reads contains one error. After error correction, only one correct sequence remains.

Analysis

Secondary analysis was performed locally using the DRAGEN™ TruSight Oncology 500 ctDNA v2.1 pipeline. Additional analyses of variant calls and sample QC metrics were performed using JMP and Excel. Sequencing reads were downsampled* bioinformatically to simulate recommended (35,000×) and reduced (25,000× and 15,000×) raw sequencing depth. Variant calling sensitivity at each sequencing depth and input was then evaluated.

* Downsampling decreases the number of reads in a sequencing run that are analyzed.

Results

Sequencing depth

When looking for low-frequency variants, increasing the sequencing depth maximizes the probability of their detection. To ensure accurate variant calling, Illumina recommends sequencing to 35,000× minimum raw sequencing depth. Median exon coverage (MEC) is the median number of read families spanning the exon region (Figure 2). Using 20 ng DNA input and 400M reads results in a MEC of 3500× with raw sequencing, and > 2500× at 35,000×. This means that after read collapsing, the

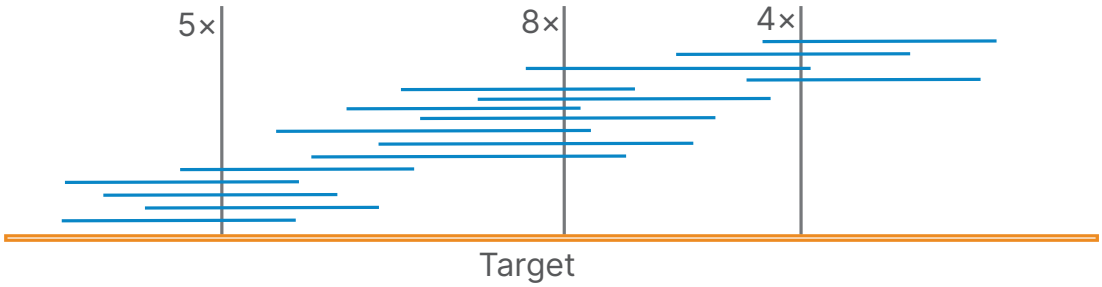

Figure 2: Median exon coverage—Horizontal lines represent sequencing reads for the targeted region. Vertical lines indicate which reads cover three specific bases. Sequencing depths and positions shown are for illustration only. Every base across a target is considered. The MEC for the region shown is 5×. The recommended MEC for 0.5% limit of detection (LoD) following error correction is ≥ 1000×. To achieve an equivalent number of supporting reads for a 0.2% VAF, a MEC of 2500× is recommended.

median collapsed fragment coverage for exonic bases in the panel is 3500×, and typically > 95% of exonic reads have coverage of ≥ 1000× and > 90% of exonic reads will have coverage ≥ 1500×. At 1000× coverage, a variant at 0.5% VAF will have approximately five supporting reads and a variant at 0.2% VAF will have approximately three supporting reads. At 1500× coverage, a variant at 0.5% VAF will have approximately seven supporting reads. Libraries can be sequenced to a lower sequencing depth and still achieve low-level variant detection, but there will be an observed reduction in coverage and variant sensitivity (Table 3 and Table 4).

When using TruSight Oncology 500 ctDNA v2, Illumina recommends a sequencing depth of 35,000× and a median exon coverage of 2500× to generate a sufficient number of supporting reads to call 0.2% VAF in small variants.

Analytical performance

Assay performance is directly related to the analytical sensitivity and specificity across different limits of detection. Analytical sensitivity is defined as the ability to identify a variant correctly that is present (the true positive rate). Analytical specificity is defined as the ability not to call a variant when it is absent (the true negative). Using TruSight Oncology 500 ctDNA v2, the stated specification for detecting small nucleotide variants (SNVs) is ≥ 90% at 0.2% VAF and ≥ 95% at 0.4% VAF when using 20 ng input ctDNA and sequencing at 35,000× raw coverage depth. For hotspot regions, where variants have a > 50 recurrence in the COSMIC database, the analytical sensitivity for SNVs at 0.2% VAF rises to ≥ 95% (Figure 3).

Table 3: Median exon coverage within known Seraseq samples for small variants

Input	Sequencing depth	n	0.2% VAF	0.5% VAF
5 ng	15K×	4	886	1234
	25K×	4	1060	1654
	35K×	4	1132	1854
10 ng	15K×	2	1067	1210
	25K×	2	1570	1550
	35K×	2	1849	1697
20 ng	15K×	4	1122	1357
	25K×	4	1949	2139
	35K×	**4**	**2517**	**2599**
30 ng	15K×	2	1120	1335
	25K×	2	2124	2410
	35K×	2	2919	3181

Input of 20 ng at 35,000× coverage is the standard recommendation for use with TruSight Oncology 500 ctDNA v2. n, number of replicates at each sequencing depth; VAF, variant allele frequency.

Table 4: Recall rates for multiple variant types at different sequencing depths

Input	Sequencing depth	n	Small variants		CNVs		Gene rearrangements	
			0.2% VAF	0.5% VAF	0.2% VAF	0.5% VAF	0.2% VAF	0.5% VAF
5 ng	15K×	4	68%	91%	25%	67%	50%	75%
	25K×	4	76%	96%	33%	92%	67%	92%
	35K×	4	75%	97%	42%	100%	75%	100%
10 ng	15K×	2	58%	95%	67%	100%	67%	83%
	25K×	2	79%	97%	67%	100%	67%	83%
	35K×	2	84%	97%	67%	100%	83%	100%
20 ng	15K×	4	66%	89%	67%	100%	67%	67%
	25K×	4	87%	97%	67%	100%	58%	83%
	35K×	4	**92%**	97%	67%	**100%**	75%	**100%**
30 ng	15K×	2	66%	89%	67%	100%	33%	67%
	25K×	2	84%	95%	67%	100%	83%	100%
	35K×	2	89%	97%	67%	100%	83%	100%

Grey box indicates claimed LoD values. n, number of replicates at each sequencing depth; VAF, variant allele frequency.

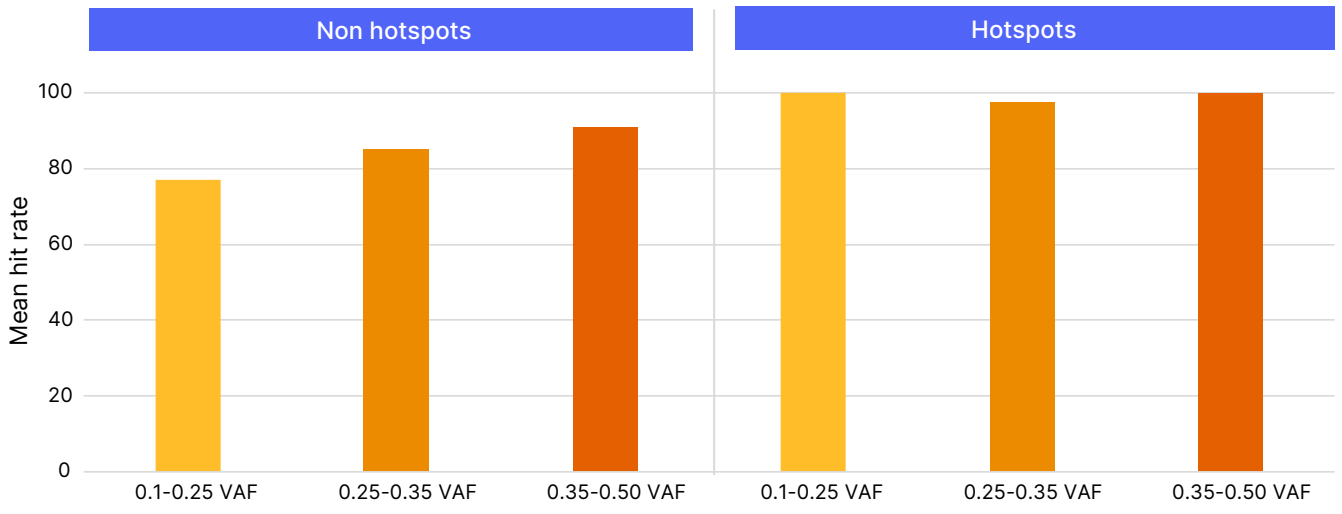

Figure 3: High analytical performance for key SNV hotspots at 0.2% VAF—A nucleosome prep of a mixture of three cell lines and Seraseq ctDNA control samples with known VAF for each single nucleotide was used with TruSight Oncology 500 ctDNA v2 to generate libraries that were sequenced on the NovaSeq 6000 Sequencing System using an S4 flow cell. Sequencing depth was at least 35,000×, the coverage recommended by Illumina. Analysis was performed using the DRAGEN TruSight Oncology 500 ctDNA pipeline. Hit rates were measured for both non-hotspot and hotspot regions at 0.1–0.5% VAF. The overall sensitivity observed for SNVs in hotspot regions is ≥ 95%.

Discussion

TruSight Oncology 500 ctDNA v2 employs enhanced chemistry, powerful technology, and advanced bioinformatics to enable CGP from liquid biopsy samples. The inclusion of UMIs in library preparation and the error correction capabilities of DRAGEN TruSight Oncology ctDNA software (≥ v2.1) create an accurate, sensitive workflow that reliably detects variants from sample inputs as low as 5 ng ctDNA and at 0.2% VAF.

Learn more

TruSight Oncology ctDNA v2

Unique molecular identifiers (UMIs)

DRAGEN TruSight Oncology ctDNA secondary analysis (≥ v2.1)

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