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Analysis of Cell-Free DNA with the TruSight® Tumor 15 Assay

Exploring the potential of using next-generation sequencing for variant detection in liquid biopsies.

Introduction

Molecular analysis of cell-free DNA (cfDNA), small fragments of DNA circulating in blood plasma, provide information on the genetic state of normal and tumor cells. Due to its noninvasive nature, molecular interrogation of cfDNA is becoming increasingly popular for tumor characterization and treatment monitoring. Indeed, it has been shown that the genetic variants detected in cfDNA are highly concordant with those found in tumor tissue biopsies.[1,2] To explore the possibility of analyzing cancer-related genes in cfDNA, targeted molecular analysis was performed with Illumina next-generation sequencing (NGS) technology using the TruSight Tumor 15 Assay. TruSight Tumor 15 assesses 15 genes that are frequently mutated in solid tumors. It was optimized to work with highly fragmented and degraded DNA, which often comes from formalin-fixed, paraffin-embedded (FFPE) samples. This white paper demonstrates variant analysis in cfDNA using the standard workflow of the TruSight Tumor 15 Assay.

DNA Samples

Sequencing libraries were generated according to instructions in the TruSight Tumor 15 Reference Guide.[3] The assay requires a minimum of 10 ng input DNA for each of 2 oligo sets (pool A and pool B) that are pooled and sequenced together after the library amplification step.[3] Input DNA was used from 4 DNA sources.

- Plasma cfDNA—Human plasma was purchased from BioreclamationIVT;[4] DNA extraction was performed using the QIAmp Circulating Nucleic Acid Kit.[5]
- FFPE DNA—The AllPrep DNA/RNA FFPE Kit[6] was used to extract DNA from an FFPE tissue block purchased from BioOptions.[7]
- OCT1 DNA—The positive control DNA sample is provided with the TruSight Tumor 15 Library Prep Kit.
- Simulated cfDNA—The Multiplex I cfDNA Reference Standard Set (Catalog No. HD780) was purchased from Horizon Discovery.[8] Standards are derived from genomic DNA of cell lines containing engineered variants. DNA was fragmented to an average size of 160 bp to simulate cfDNA. From the reference set, standard HD777 contains 7 single nucleotide variants (SNVs) that are included in the TruSight Tumor 15 Panel (Table 1); standard HD776 contains wild-type alleles for these genes.

Table 1: Known Somatic Variants in Simulated cfDNA

Gene	Variant	Expected Frequency
EGFR	L858R	5.0%
EGFR	ΔE746-A750	5.0%
EGFR	T790M	5.0%
KRAS	G12D	6.3%
NRAS	Q61K	6.3%
NRAS	A59T	6.3%
PIK3CA	E545K	6.3%

Expected allelic frequency is based upon dilution of DNA from different cell lines with known variants. Each variant is targeted by the TruSight Tumor 15 Panel.

Library Yield

Following the amplification and post-PCR purification steps, samples were analyzed with the AccuClear Ultra High Sensitivity dsDNA Quantitation Kit[9] to assess library yield. A minimal concentration of 20 ng/µl is recommended for sequencing. FFPE DNA was included as a positive control representing fragmented DNA and was used in the assay design to determine the minimal input guidelines. All 4 DNA samples surpassed the ≥ 20 ng/µl requirement (Figure 1). These data demonstrate the ability of the TruSight Tumor 15 Panel to generate sequence-quality libraries from cfDNA extracted from human plasma.

Sequencing

Libraries from plasma cfDNA, simulated cfDNA, and OCT1 DNA were sequenced on the MiSeq® Sequencing System with paired-end configuration (2 × 151 bp). Data were analyzed using the on-instrument MiSeq Reporter 2.6.2. Software (TruSight Tumor 15 Workflow).

Amplicon Coverage

Coverage is the number of times a region is sequenced (the number of reads) within a single run. In general, the deeper the coverage of a targeted region, the more sensitive and reliable the assay. For variant calling, 500× coverage is required for reliable detection of mutations occurring at frequencies as low as 5%. To pass quality control (QC) metrics for the TruSight Tumor 15 Assay, samples should yield > 500× coverage on > 93.5% of bases targeted by the assay. Libraries generated from analytical cfDNA and clinical cfDNA performed similarly to the OCT1 control DNA for this metric, with > 99.8% of amplicons yielding coverage ≥ 500× with both oligo sets (Figure 2).

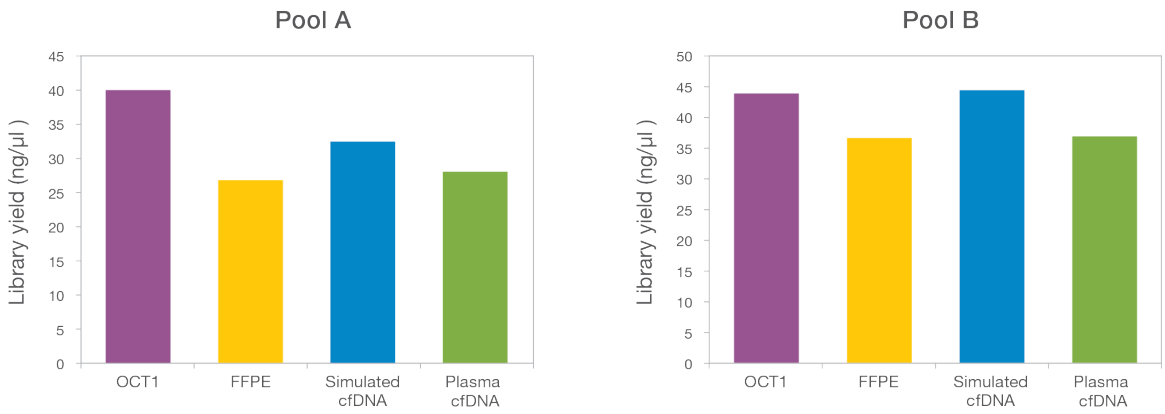

Figure 1: Library Yield from Different Sample Types—All 4 samples yielded library DNA at a concentration ≥ 20 ng/µl, which is the minimum amount recommended for sequencing.

Variant Detection

Analytical cfDNA from Horizon Discovery contains cancer-related variants that are also covered by the TruSight Tumor 15 Panel (Table 1). The expected variant frequencies in standard HD777 range from 5.0-6.3%, as determined by the supplier using droplet digital PCR (ddPCR). This range approximates the limit of detection of 5% allele frequency for the TruSight Tumor 15 Assay. Using the TruSight Tumor 15 Assay, all variants were detected, and quantitative analysis of undiluted standard HD777 showed high concordance between observed allele frequency, and the expected frequency based on ddPCR (Figure 3).

Standard DNA HD777 was then diluted with standard HD776 (DNA containing wild-type alleles) to lower the expected variant frequency to a range of 2.5-3.15%. Diluted standard HD777 was sequenced on the MiSeq System and on-instrument software was used for variant detection. Analysis of the 7 known variants in 4 replicates of diluted standard HD777 resulted in 93% sensitivity, with detection of 26/28 minor alleles (Table 2).

Table 2: Coverage Details

SNV	Expected Frequency	Replicate 1	Replicate 2	Replicate 3	Replicate 4
KRAS G12D	3.15%	Detected	Detected	Detected	Detected
NRAS Q61K	3.15%	Detected	Detected	Detected	Detected
NRAS A59T	3.15%	Detected	Detected	Detected	Detected
PIK3CA E545K	3.15%	Detected	Detected	Detected	Detected
EGFR L858	2.5%	Detected	Detected	NC	NC
EGFR dE746-A750	2.5%	Detected	Detected	Detected	Detected
EGFR T790M	2.5%	Detected	Detected	Detected	Detected

NC = not called. Software does not make calls with variants detected under 2.5% allele frequency.

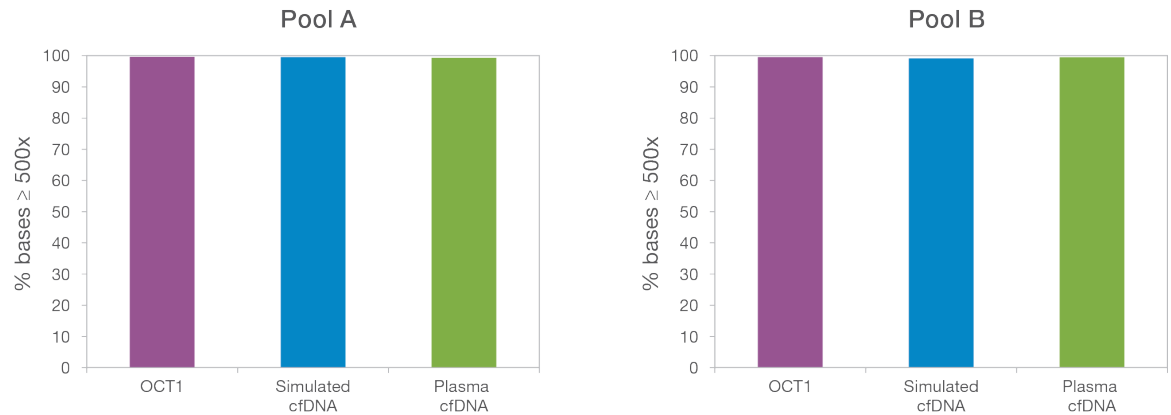

Figure 2: Percentage of Bases with Coverage ≥ 500x—All samples tested passed the QC specification metric of > 93.5% of bases with > 500× coverage.

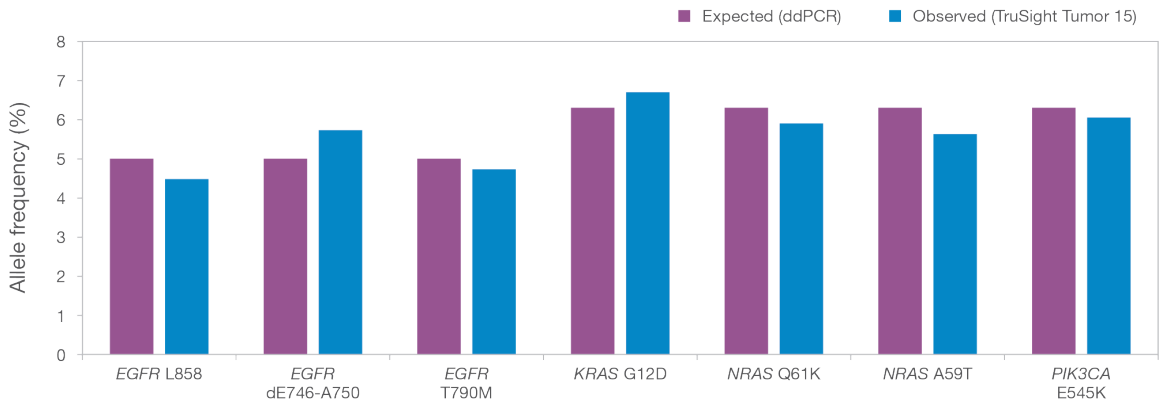

Figure 3: Concordance Between ddPCR and TruSight Tumor 15 Assay—Expected frequency of analyzed variants was determined by Horizon Discovery using ddPCR. For comparison, the allele frequency was measured with the TruSight Tumor 15 Assay using on-instrument software.

Summary

The discovery of cfDNA presents the potential to analyze and monitor tumors by the relatively noninvasive method of liquid biopsy. As proof-of-principle, detection of variants in plasma and simulated cfDNA samples was performed using the standard workflow of the TruSight Tumor 15 Assay, including library preparation and variant calling. HIgh-quality libraries from plasma cfDNA and simulated cfDNA with known variants were successfully prepared and sequenced on the MiSeq System. Both libraries passed the TruSight Tumor 15 QC metric of > 93.5% of bases with > 500× coverage. Observed allele frequencies of 7 known SNVs in simulated cfDNA, determined by the TruSight Tumor 15 Assay, were highly concordant with expected allele frequency as determined by ddPCR. The TruSight Tumor 15 Assay demonstrated high sensitivity for variant detection, with 100% detection of variants with expected allele frequency of 5-6.3%, and > 90% sensitivity at variant frequencies between 2.5-3.2%. Although standards for analysis of cfDNA are currently being developed in the field of liquid biopsy, this study demonstrates the potential to develop NGS applications for this goal.

Learn More

To learn more about the TruSight Tumor 15 Assay, visit www.illumina.com/products/trusight-tumor-15-gene.html

References

1. Spindler KL, Pallisgaard N, Andersen RF, Brandslund I, Jakobsen A. Circulating free DNA as biomarker and source for mutation detection in metastatic colorectal cancer. *PLoS One*. 2015;10(4):e0108247. doi: 10.1371/journal.pone.0108247.
2. Takai E, Totoki Y, Nakamura H, et al. Clinical utility of circulating tumor DNA for molecular assessment in pancreatic cancer. *Sci Rep*. 2015;5:18425. doi: 10.1038/srep18425.
3. Illumina (2015) TruSight Tumor 15 Reference Guide. (http://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/trusight/trusight-tumor-15-reference-guide-1000000001245-05.pdf).
4. BioreclamationIVT home page. www.bioreclamationivt.com/ Accessed August 23, 2016.
5. QIAamp Circulating Nucleic Acid Kit. www.qiagen.com/us/shop/epigenetics/qiaamp-circulating-nucleic-acid-kit#orderinginformation. Accessed August 23, 2016.
6. AllPrep DNA/RNA FFPE Kit. www.qiagen.com/fi/shop/sample-technologies/combined-sample-technologies/allprep-dnarna-ffpe-kit/#orderinginformation. Accessed August 23, 2016.
7. BioOptions Biospecimens for Discovery.biooptions.com/ Accessed August 23, 2016.
8. Multiplex I cfDNA Reference Standard Set. www.horizondiscovery.com/multiplex-i-cfdna-reference-standard-set-hd780. Accessed August 23, 2016.
9. AccuClear Ultra High Sensitivity dsDNA Quantitation Kit with 1 DNA Standard. biotium.com/product/accuclear-ultra-high-sensitivity-dsdna-quantitation-kit-with-1-dna-standard/ Accessed September 15, 2016.

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