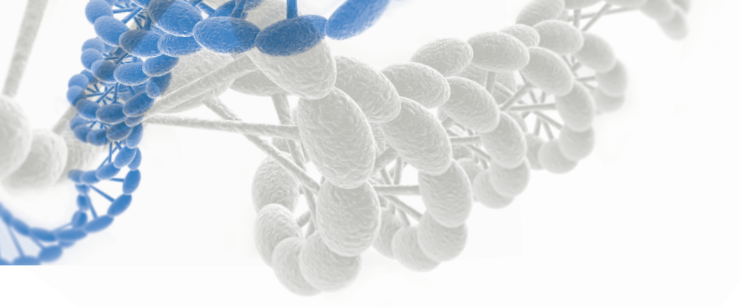


AVENIO ctDNA Analysis Kits: Performance Across Illumina Sequencing Platforms

Introduction

The AVENIO ctDNA Analysis Kits are verified to produce enriched circulating tumor DNA (ctDNA) libraries ready to be sequenced on Illumina NextSeq 500 and 550 sequencing platforms. The analysis kits enable the detection of four mutation classes: single nucleotide variants (SNVs), copy number variants (CNVs), insertions and deletions (Indels), and fusions. Three kits—AVENIO Targeted Kit, Expanded Kit, and Surveillance Kit—all demonstrate high sensitivity and positive predictive value (PPV) for the selected genes of interest.

In this study we compare the performance of the AVENIO ctDNA Analysis Kits across three widely available Illumina sequencing platforms: Illumina NextSeq 500, HiSeq 4000, and HiSeq 2500 systems. Ninety sequencing libraries containing variants with low allele frequencies were sequenced using all three sequencing platforms and then analyzed with the AVENIO Oncology Analysis Software version 1.1.0. For each AVENIO ctDNA Analysis Kit, assay performance was evaluated across multiple Illumina sequencing platforms by comparing sequencing metrics and detected variants.

Methods

DNA from cell lines or cell-free DNA (cfDNA) extracted from plasma donors was blended to create fixed proportions of known variants. Horizon Discovery created custom cell lines and verified to contain multiple key SNVs, fusions, or CNVs using digital droplet PCR. NA12878 (Coriell Institute) or cfDNA was used to dilute cell lines to contain low variant allele frequencies (AF) listed in Table 1. Indel samples consisted of blends of multiple cell lines with known insertions and deletions. Normal cfDNA from unique plasma donors was used to determine PPV.

Table 1. Sample summary

Mutation Class	Allele Frequency or Copy Number	Mixture Description	Number of Samples	AVENIO Analysis Kit
Normal	N/A	Normal cfDNA	10	Targeted Kit Expanded Kit Surveillance Kit
SNV	0.5%	cfDNA-cfDNA mixture	5	
		Custom SNV cell line	3	
Fusion	1%	Custom fusion cell line	3	
CNV	2.3 copies MET and EGFR	Custom CNV cell line	3	
	2.6 copies ERBB2	Custom CNV cell line	3	
Indel	1%	Cell lines mixtures	3	

Methods *(Continued)*

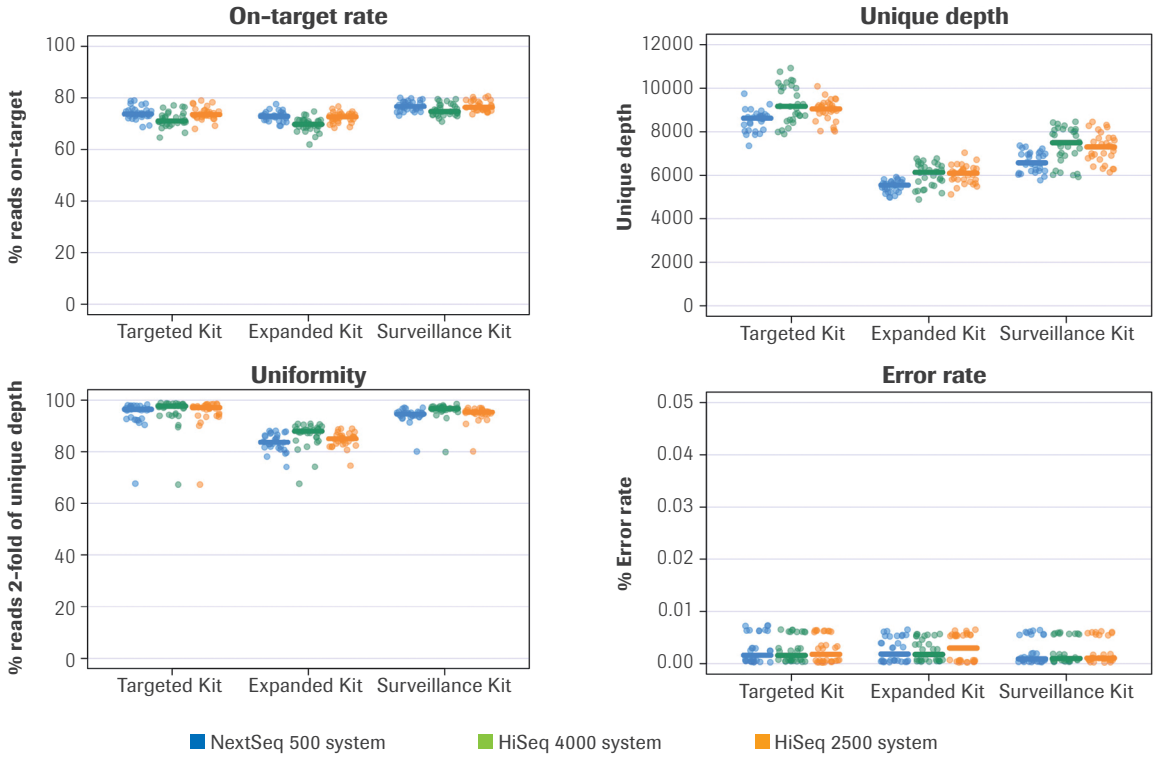
Sequencing libraries were prepared from 50 ng of input DNA per sample using the AVENIO ctDNA Analysis Kits workflow. The libraries were then sequenced on three Illumina platforms: the NextSeq 500, HiSeq 4000, and HiSeq 2500. For the Targeted Kit, 40 million paired-end reads (20 million clusters) were generated per sample, and for the Expanded and Surveillance Kits 60 million paired-end reads (30 million clusters) were generated per sample.

For the Illumina HiSeq 2500 system, the library pool was loaded on two sequencing lanes to generate equivalent sequencing depth with HiSeq 4000 and NextSeq 500 systems. All sequencing runs were loaded to attain Illumina's recommended cluster densities. In total, 90 libraries were sequenced across three platforms, generating 270 distinct sequencing data sets for variant analysis and metric comparisons. The sequencing data was analyzed using the AVENIO Oncology Analysis Software version 1.1.0.

Results

Sequencing metrics analysis: Key sequencing metrics of the sequencing libraries are plotted in Figure 1 for all three AVENIO ctDNA Analysis Kits for each Illumina sequencing platform. Median values are indicated. Metric definitions are as follows: *On-target rate:* the percentage of reads in the intended target region. *Unique depth:* the median read depth after removing duplicated reads. Unique depth is a key metric indicating the number of original genomic equivalents recovered through the library prep and sequencing process. *Uniformity:* the percentage of positions whose unique depth fall within two-folds of the median unique depth. *Error Rate:* empirically estimated as the number of non-reference over all reference base calls at <5% AF after all molecular and bioinformatic error suppression is applied. The expected values for on-target rate, unique depth, uniformity, and error rate were achieved by the AVENIO ctDNA Analysis Kits, at consistent and high-performing values. As expected, the smaller Targeted Panel (~80 kb) attained greater unique depth than the larger Expanded and Surveillance panels (~200 kb each), with all panels generating depths at very high levels. The error rates across all sequencing platforms were comparable and all well below 0.1%. Such low error rates were achieved through the analysis software's Integrated Digital Error Suppression (iDES).1 Overall, the Illumina HiSeq 4000 and HiSeq 2500 sequencing platforms generated sequencing metrics similar to the NextSeq 500/550 system.

Figure 1. Sequencing metrics



Variant analysis

SNVs: For the Targeted and Surveillance Kits,16 loci of interest SNVs at 0.5% AF were analyzed for sensitivity, and for the Expanded Kits 18 loci of interest SNVs at 0.5% AF were analyzed. All SNVs were detected across all sequencing platforms achieving sensitivities of 100%. In addition, analysis of the healthy donor cfDNA samples yielded excellent PPV.

	Single Nucleotide Variants					
	Sensitivity			PPV		
AVENIO Analysis Kit	NextSeq® 500	HiSeq® 4000	HiSeq 2500	NextSeq 500	HiSeq 4000	HiSeq 2500
Targeted Kit	100%	100%	100%	99.6%	99.6%	99.6%
Expanded Kit	100%	100%	100%	99.3%	99.6%	99.6%
Surveillance Kit	100%	100%	100%	99.6%	>99.9%	98.8%

Indels: To determine sensitivity, 5 indels at 1% AF were evaluated. All sequencing platforms achieved 100% sensitivity with PPV >99.9%.

	Indels					
	Sensitivity			PPV		
AVENIO Analysis Kit	NextSeq 500	HiSeq 4000	HiSeq 2500	NextSeq 500	HiSeq 4000	HiSeq 2500
Targeted Kit	100%	100%	100%	>99.9%	>99.9%	>99.9%
Expanded Kit	100%	100%	100%	>99.9%	>99.9%	>99.9%
Surveillance Kit	100%	100%	100%	>99.9%	>99.9%	>99.9%

Fusions: Fusion samples were analyzed for the detection of fusions at 1% AF. All panels were evaluated for the detection of EML4-ALK, RET-CCDC6, and SLC34A2-ROS1. In addition, the Expanded Kit also included detection of a TPM3-NTRK1 fusion. All fusions were detected in all samples at a sensitivity of 100% across sequencing platforms. Note that although all platforms yielded good PPV, in this sample set the Expanded Kit on the HiSeq 2500 system had slightly lower PPV based on replicates of 3 samples per test condition.

	Fusions					
	Sensitivity			PPV		
AVENIO Analysis Kit	NextSeq 500	HiSeq 4000	HiSeq 2500	NextSeq 500	HiSeq 4000	HiSeq 2500
Targeted Kit	100%	100%	100%	>99.9%	>99.9%	>99.9%
Expanded Kit	100%	100%	100%	>99.9%	>99.9%	96.8%
Surveillance Kit	100%	100%	100%	>99.9%	>99.9%	>99.9%

CNVs: CNV samples were analyzed for sensitivity with 2.3 copies of MET and EGFR and 2.6 copies of ERBB2. In this study, when evaluating all three genes, the NextSeq 500 system had slightly higher sensitivity than the HiSeq 4000 and HiSeq 2500 systems, with a replicate size of 3 samples per condition. Impressively, in this study the assay was able to detect EGFR and ERBB2 at levels lower than the stated limits of detection of 3.0 and 4.5, respectively for those genes. The AVENIO Oncology Analysis Software version 1.1.0 is aimed to have a CNV caller with an emphasis on high specificity, and is able to achieve PPV of >99.9% across all platforms.

	Copy Number Variants					
	Sensitivity			PPV		
AVENIO Analysis Kit	NextSeq 500	HiSeq 4000	HiSeq 2500	NextSeq 500	HiSeq 4000	HiSeq 2500
Targeted Kit	100%	88.9%	88.9%	>99.9%	>99.9%	>99.9%
Expanded Kit	100%	88.9%	100%	>99.9%	>99.9%	>99.9%
Surveillance Kit	100%	77.8%	100%	>99.9%	>99.9%	>99.9%

Analysis

The AVENIO ctDNA Analysis Kits and Analysis Software have been optimized for use with the Illumina NextSeq 500/550 system. The existing Analysis Software can also process data from the Illumina HiSeq 4000 and HiSeq 2500 systems, but special instructions are required.

Instructions for analyzing data from the HiSeq 4000 and HiSeq 2500 systems with the AVENIO Oncology Analysis Software 2.0.0 are available from your Roche Sequencing representative.

Conclusion

In this study, we demonstrated that libraries generated with the AVENIO ctDNA Analysis Kits achieved high sensitivity and PPV for all four mutation classes when sequenced on multiple Illumina platforms—the NextSeq 500, HiSeq 4000, and HiSeq 2500. The expected sequencing metrics of on-target rate, unique depth, uniformity, and error rate were achieved across all three platforms, extending the use of the AVENIO ctDNA Analysis Kits beyond the NextSeq 500/550 to two additional sequencing platforms. This study serves as a sample for the type of results achievable with the AVENIO ctDNA Analysis Kits, using a blend of samples consisting of cfDNA and cell lines with known mutations.

Note: for each sequencing platform, it is important that the sequencer is loaded at a library concentration that yields the instrument's recommended cluster density.

Also, the throughput for each sequencing platform should be considered, and the total number of samples loaded on the sequencer should be adjusted accordingly. Also, the specific requirements for each sequencing platform should be considered, and the total number of samples loaded on the sequencer should be adjusted accordingly. To obtain optimal mutation detection sensitivity, the recommended sequencing coverage for each sample should be approximately 40 million paired-end reads (20 million clusters) for the Targeted Panel and 60 million paired-end reads (30 million clusters) for the Expanded and Surveillance Panels. In conclusion, the AVENIO ctDNA Analysis Kits achieved similar high performance on all Illumina sequencing platforms.

1. Newman A, Lovejoy A, Klass D et al. Integrated digital error suppression for improved detection of circulating tumor DNA. *Nat Biotechnol.* 2016; 34(5):547-555. doi:10.1038/nbt.3520.

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9115 Hague Road
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