AVENIO ctDNA Analysis Kits Performance Across Illumina Sequencing Platforms

Introduction

The AVENIO ctDNA Analysis Kits are verified to produce enriched circulating tumor DNA libraries, ready to be sequenced on the 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 are available to sequence selected genes of interest (AVENIO Targeted Kit, Expanded Kit, and Surveillance Kit), all demonstrating high sensitivity and positive predictive value (PPV).

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. Ninety sequencing libraries, containing variants with low allele frequencies, were sequenced using the three sequencing platforms, and analyzed with the AVENIO Oncology Analysis Software version 1.1.0. The resulting sequencing metrics and detected variants were used to evaluate assay performance across the Illumina sequencing platforms for each of the three AVENIO ctDNA Analysis Kits.

Methods

DNA from cell lines or cell free DNA (cfDNA) extracted from plasma donors were blended to create fixed proportions of known variants. Horizon Discovery created customer cell lines and verified them by digital droplet PCR to contain multiple key SNVs, fusions, or CNVs. NA12878 (Coriell Institute) or cfDNA were 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 were 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)*

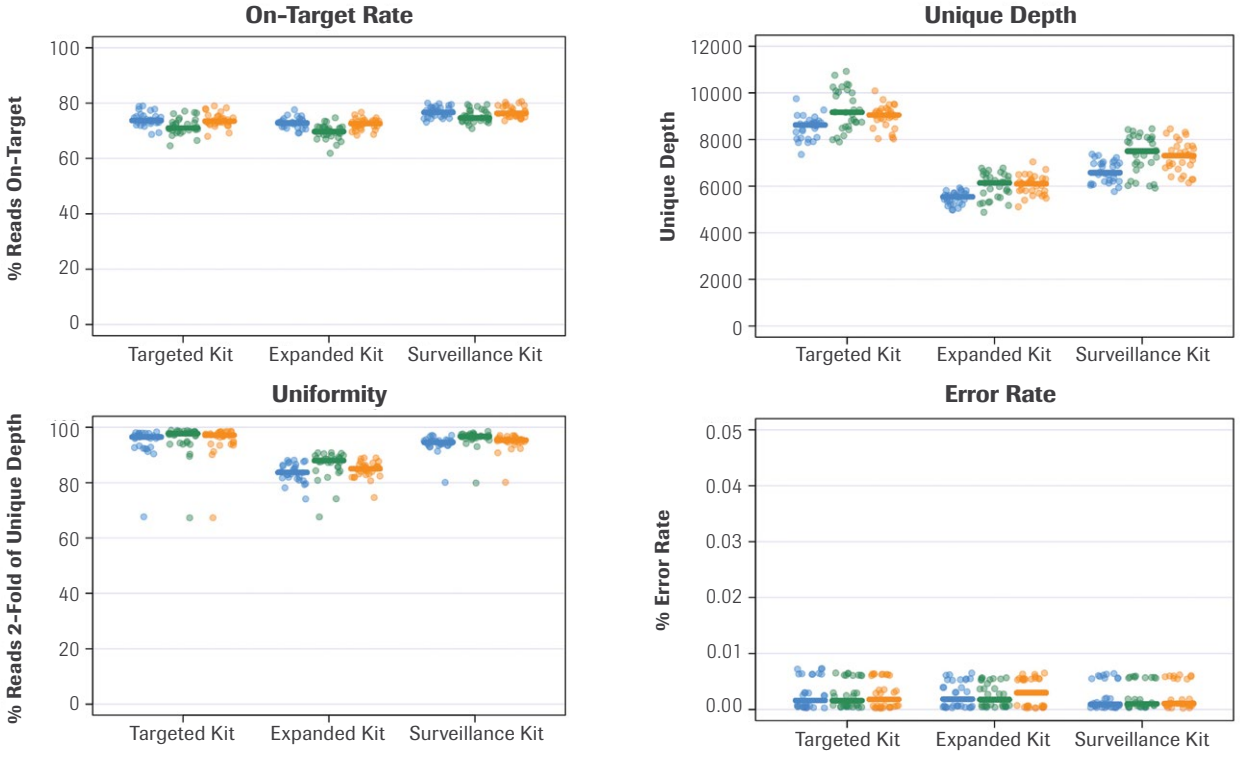
Sequencing libraries were prepared using the AVENIO ctDNA Analysis Kits workflow utilizing 50ng of input DNA. The same sequencing libraries were sequenced across all three platforms. 40 million paired-end reads (20 million clusters) for the Targeted Kit and 60 million paired-end reads (30 million clusters) for the Expanded and Surveillance Kits 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 each of the 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 are achieved by the AVENIO ctDNA Analysis kits, at consistent and high performing values. As expected, the smaller Targeted Panel (~80kb) attains greater unique depth than the larger Expanded and Surveillance panels (~200kb 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 rate is achieved through the analysis software's Integrated Digital Error Suppression (iDES).[1] Overall, the Illumina HiSeq 4000 and HiSeq 2500 sequencing platforms are able to generate sequencing metrics similar to the NextSeq 500/550 system.

Figure 1. Sequencing Metrics



Variant Analysis

SNVs: 16 loci of interest SNVs at 0.5% AF for the Targeted and Surveillance Kits, and 18 loci of interest SNVs at 0.5% AF for the Expanded Kits were analyzed for sensitivity. All SNVs were detected across all sequencing platforms achieving sensitivities of 100%. Analyzing 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: 5 indels at 1% AF were evaluated for sensitivity. 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 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 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 1.1.0 and 2.0.0 are available from your Roche representative.

Conclusion

In this study, the AVENIO ctDNA Analysis Kits created libraries that, when sequenced on multiple Illumina platforms, achieved high sensitivity and PPV for all four mutation classes. The AVENIO ctDNA Analysis Kits were evaluated across three Illumina sequencing 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 in an equivalent manner, extending the use of the AVENIO ctDNA Analysis Kits to additional sequencing platforms. This study serves as an example 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, it is important that the user loads the sequencer at a concentration that yields the instrument's recommended cluster density. Also, for each sequencing platform, the total number of samples loaded on the sequencer should be considered. To obtain optimal mutation detection sensitivity, aim for each sample to receive sequencing coverage of 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 three platforms.

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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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