In-depth assessment reveals powerful performance and flexibility of the AVENIO ctDNA Analysis Kits

Introduction

When reporting the performance of a circulating tumor DNA (ctDNA) assay, stating the limit of detection, or the sensitivity at a given allele frequency is insufficient. Important factors to also consider in typical use cases include the cost of sequencing, multiplexing and input mass. Here we describe five factors that should be considered in direct comparisons between ctDNA sequencing assays:

Variant Allele Frequency (VAF): The amount of cell free DNA that is derived from a tumor can be very low, especially for smaller, early to mid stage tumors (at or below 0.1% VAF is common)[1]. Depending on the planned application, the necessary VAF can range from 0.05% to >1%[2]. The target VAF will vary based on the specific clinical research application of interest, panel size and sample multiplexing. Understanding the relationship between these factors will help to plan the experiments and identify the target VAF.

Sensitivity: While it can be useful to know the lowest VAF that can be detected by an assay, the likelihood of detecting a variant at a given VAF gives a better understanding of the assay performance. We suggest associating a sensitivity level for a given VAF, such as being able to call at 0.05% with 90% sensitivity, thereby giving users a level of confidence in detection at that level.

Specificity/PPV: Along with calling true variants, it is important to know how likely an assay is to call false positives. Per base specificity values tend to be very high for most sequencing technologies (> 99.99%) due to the large number of bases being queried that are correctly assigned as reference. A more stringent measure is variant level or subject level specificity —one false positive out of 1,000 known hotspot positions is a specificity of 99.9%. For ctDNA NGS applications, controlling the overall error rate through the use of molecular barcoding and advanced informatics software is essential to minimize false positives[2].

Input Mass: Plasma contains a very small amount of cell free DNA (cfDNA)—a typical amount from a 10 mL blood draw can range from 5-50 ng[1-3]. The input cfDNA for an assay will have a strong effect on the sensitivity, especially at low VAFs, so knowing the required input mass will determine an assay's utility with a typical blood draw. For example, a 10ng input sample only contains ~3,000 haploid genome equivalents, of which only 3 are tumor derived at a VAF of 0.1%. Therefore, with a 10 ng input there are on average only 3 molecules of a given mutation (10ng x 300 haploid genome equivalents per ng x 0.1% VAF = 3) and there is a 5% probability of not seeing any molecules supporting a particular mutation due to stochastic sampling at this level. In addition, no assay will recover all input molecules: typically 20-70% of the input molecules will make it through to sequencing, reflecting imperfect conversion efficiency. Conversion efficiency (aka assay efficiency) is the fraction of molecules in the input that make it through library preparation, target enrichment, and sequencing. This is a critical factor to ensure that every molecule counts.

Sequencing Reads: A primary driver of cost for any sequencing assay is the number of sequencing reads required. Knowing the number of reads necessary will enable the assessment of assay cost. If there are adequate cfDNA genome equivalents in a sample(s), then less sample multiplexing can improve sensitivity but at a higher sequencing cost.

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Methods

The Roche AVENIO ctDNA Analysis Kits are designed to allow flexibility in detecting mutations in ctDNA based on the application, the available cfDNA input, and the acceptable sequencing cost per sample. The recommended input mass is 10-50ng of cfDNA, and the recommended sequencing amount is 40 million paired-end reads for the Targeted Kit (16 samples per NextSeq High Output run) and 60 million paired-end reads for the Expanded Kit (10 samples per NextSeq High Output run). The results below show the hotspot sensitivity of the Roche AVENIO ctDNA Analysis Kits for the 81 kb Targeted Kit and 192 kb Expanded Kit for 3 different VAFs (0.5%, 0.1%, and 0.05%) achieved through mixing of cfDNA from multiple donors, using different input masses of cfDNA (1 to 100 ng) treated as completely separate samples, and using different numbers of paired-end sequencing reads from an Illumina HiSeq 4000 (5 million to 200 million reads)[4]. These plots and table enable the complete assessment of assay performance for different genotyping applications given available input and sequencing reads. All points are real data generated with the AVENIO ctDNA Analysis Kits. Specificity was calculated by counting false positive calls from a set of 252 known hotspot tumor variants for the Targeted panel and a set of 569 known hotspot tumor variants for the Expanded panel.

Results

Highly Specific: Across all samples, the variant-level specificity was 99.95% for the Targeted panel and 99.99% for the Expanded panel, with each sample having a variant level specificity greater than 99.4%.

Low Input cfDNA: With 5ng of input, the Expanded Kit yields 93% sensitivity at a VAF of 0.5% with 50 million reads, and the Targeted Kit achieved 90% sensitivity at a VAF of 0.5% with 5ng of input and 5 million reads.

Reduced Sequencing: With as few as 15 million reads, the Targeted Kit yields >99% sensitivity with 10 ng at a VAF of 0.5%. Similarly, the Expanded Kit achieves a sensitivity of 91% with 10ng of input and 20 million reads at a VAF of 0.5%.

Strong Performance: With the recommended input and sequencing for the Targeted Kit (40 million reads, 50 ng input), the hotspot sensitivity of variants at a VAF of 0.5% (medium to large tumors) is >99%, and at a VAF of 0.1% (smaller tumors) it is ~95%. With the recommended input and sequencing for the Expanded Kit (60 million reads, 50 ng input), the hotspot sensitivity of variants at a VAF of 0.5% is >99%.

Maximum Performance: For the smallest tumors or the lowest levels of residual disease, more sequencing allows higher sensitivity — with 200 million reads (3-4 samples per NextSeq High Output run) and 50ng input, the Targeted Kit gives >99% sensitivity at a VAF of 0.05% and the Expanded Kit gives near 90% sensitivity at a VAF of 0.05%.

Conclusions

Consider All the Variables: The available input DNA, the desired sensitivity at the target VAF, and the available number of sequencing reads all should be considered when planning an experiment with the AVENIO ctDNA Analysis Kits and when comparing performance to other assays.

Leverage AVENIO ctDNA Analysis Kits for Flexible Performance: With the AVENIO ctDNA Analysis Kits, customers can adjust the performance of the assay based on the panel used, the available input DNA, the target VAF, and the available sequencing reads.

Understand a Sequencing Limited vs. Molecular Biology Limited Assay: The high conversion efficiency of the AVENIO ctDNA Analysis Kits is evident in the observed sensitivity of 90% to >99% at a VAF of 0.05% with 50ng. With the AVENIO Kits one can sequence the same sample more to drive up the sensitivity, suggesting the molecular biology of the library preparation and target enrichment is not the limiting factor for sensitivity.

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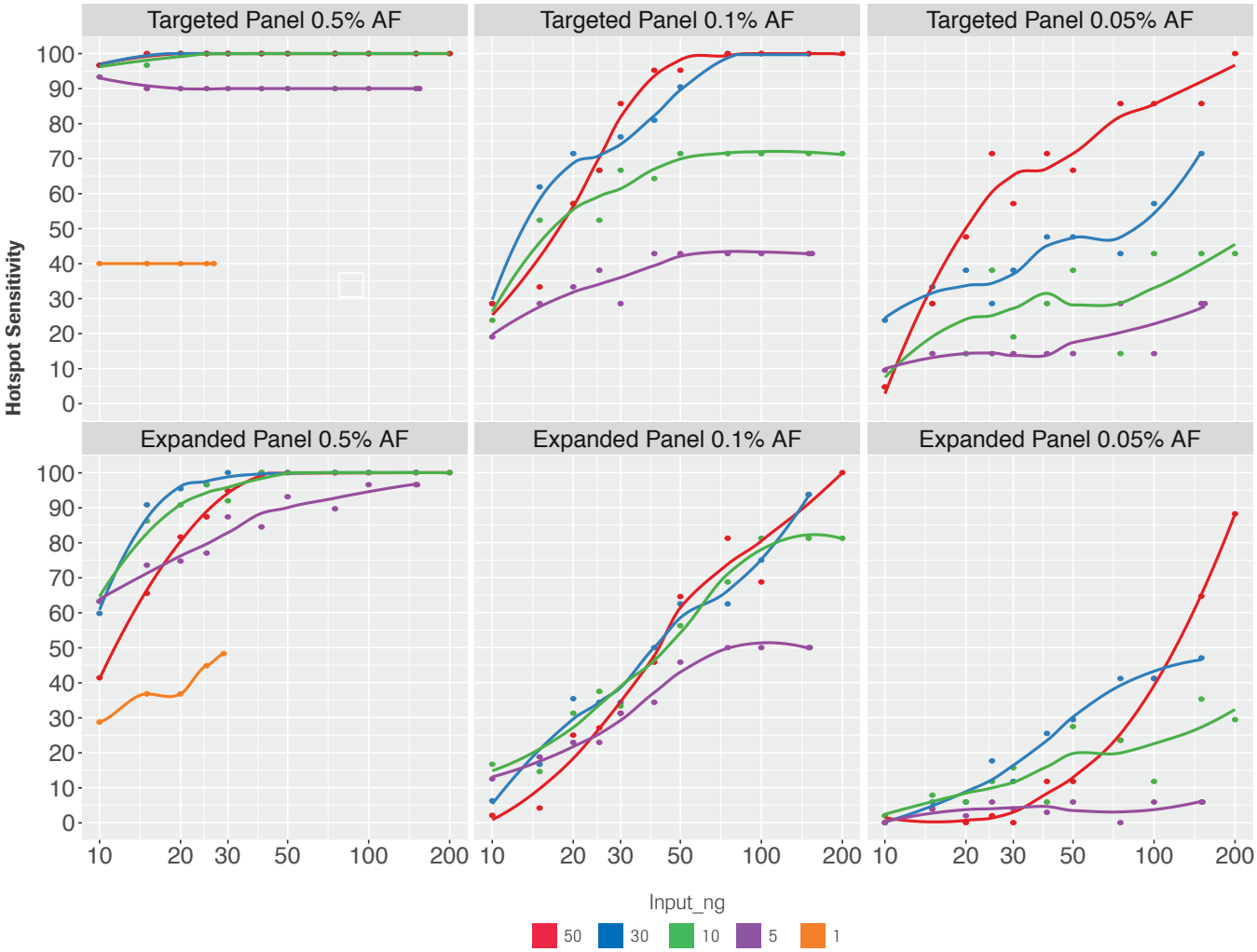
Hotspot Sensitivity vs. Sequencing Reads for Various Input Masses



These plots show the hotspot sensitivity vs. the number of sequencing reads for various cfDNA masses (line colors) used as input for library preparation. Sensitivity of the Targeted Kit and Expanded Kit for detecting mutations at 0.5%, 0.1%, and 0.05% VAFs are shown.

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Targeted and Expanded Kit Hotspot Sensitivity

Kit	Input Mass (ng)	Reads (millions)	Multiplexing on NextSeq	Sensitivity at AF%		
				0.05%	0.1%	0.5%
Targeted	1	5	>16 Mid	0%	0%	40%
	1	25	8 Mid	14%	0%	40%
	5	5	>16 Mid	10%	10%	90%
	5	25	8 Mid	14%	38%	90%
	5	50	12 High	14%	43%	90%
	5	75	8 High	29%	43%	90%
	10	5	>16 Mid	0%	5%	80%
	10	10	>16 Mid	5%	24%	97%
	10	20	11 Mid	14%	57%	100%
	10	25	8 Mid	38%	52%	100%
	10	50	12 High	38%	71%	100%
	10	100	6 High	43%	71%	100%
	30	5	>16 Mid	5%	14%	77%
	30	10	>16 Mid	24%	29%	97%
	30	15	14 Mid	33%	62%	100%
	30	25	8 Mid	29%	67%	100%
	30	50	12 High	48%	90%	100%
	30	75	8 High	43%	100%	100%
	30	150	4 High	71%	100%	100%
	50	5	>16 Mid	0%	5%	80%
	50	10	>16 Mid	5%	29%	97%
	50	25	8 Mid	71%	67%	100%
	50	50	12 High	67%	95%	100%
	50	75	8 High	86%	100%	100%
	50	200	3 High	100%	100%	100%
	100	5	>16 Mid	0%	0%	47%
	100	10	>16 Mid	5%	24%	93%
	100	15	14 Mid	29%	38%	100%
	100	20	11 Mid	24%	67%	100%
	100	25	8 Mid	52%	62%	100%
	100	50	12 High	86%	100%	100%

Kit	Input Mass (ng)	Reads (millions)	Multiplexing on NextSeq	Sensitivity at AF%		
				0.05%	0.1%	0.5%
Expanded	1	25	8 Mid	0%	0%	45%
	5	15	14 Mid	4%	19%	74%
	5	25	8 Mid	6%	23%	77%
	5	50	12 High	6%	46%	93%
	5	100	6 High	6%	50%	97%
	10	10	>16 Mid	2%	17%	63%
	10	20	11 Mid	6%	31%	91%
	10	25	8 Mid	12%	38%	97%
	10	50	12 High	27%	56%	100%
	10	75	8 High	24%	69%	100%
	10	150	4 High	35%	81%	100%
	30	10	>16 Mid	0%	6%	60%
	30	15	14 Mid	6%	17%	91%
	30	25	8 Mid	18%	34%	97%
	30	50	12 High	29%	63%	100%
	30	75	8 High	41%	63%	100%
	30	100	6 High	41%	75%	100%
	30	150	4 High	47%	94%	100%
	30	400	1 High	71%	94%	100%
	50	10	>16 Mid	0%	2%	41%
	50	25	8 Mid	2%	27%	87%
	50	50	12 High	12%	65%	100%
	50	75	8 High	24%	81%	100%
	50	150	4 High	65%	94%	100%
	50	200	3 High	88%	100%	100%
	100	10	>16 Mid	0%	0%	38%
	100	25	8 Mid	4%	21%	87%
	100	50	12 High	10%	48%	99%
	100	100	6 High	24%	75%	100%
	100	150	4 High	53%	81%	100%
	100	200	3 High	65%	100%	100%

This table shows the sensitivity of the Targeted and Expanded Kits at 0.5%, 0.1%, and 0.05% VAFs when the given mass is used as input for the assay and the resulting library is sequenced with the given number of paired-end reads. The approximate maximum number of samples that can be multiplexed on a NextSeq run to ensure the given number of paired-end sequencing reads per sample is listed. High corresponds to the High Output sequencing run and Mid corresponds to the Mid Output sequencing run on the NextSeq.

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References and Notes

1. Newman AM, Bratman SV, et. Al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nature Medicine. 2014; 20: 548-54.
2. Newman AM, Lovejoy AF, Klass DM, et. Al. Integrated digital error suppression for improved detection of circulating tumor DNA. Nature Biotechnology. 2016; 34(5): 547–555.
3. Wan JCM, et. Al. Liquid biopsies come of age: towards implementation of circulating tumour DNA. Nature Reviews Cancer. 2017; 17:223-238.
4. Performance of the AVENIO ctDNA assay was verified on the NextSeq 500 / 550 only; more than 100 million reads per sample is not supported by the on market AVENIO ctDNA Analysis Kits.

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