Analytical and methodological validation of the LiquidPlex™ assay

Overview

Invitae conducted an analytical and methodological validation of the LiquidPlex™ assay for solid tumors. This next-generation sequencing (NGS) assay is designed to identify variants within circulating tumor DNA (ctDNA) following extraction from the plasma fraction of blood. While the genomic targets covered by the assay are broad, the scope of this validation (version 1.0) includes 58 genomic positions across 25 genes.

Introduction

Cancer is a complex genetic disease that requires the accumulation of mutations over time.[1] These mutations may be inherited or acquired throughout an individual's lifetime. Advances in our understanding of molecular biology and advancements in genomic technologies have made it possible to detect these mutations in patient-derived tumor specimens.

Recent studies have demonstrated that some tumors shed DNA into circulation, allowing for the detection of tumor specific DNA in plasma.[2,3,4] Using NGS to sequence cell-free DNA (cfDNA) enables identification of mutations indicative of ctDNA.[5] This method has been shown to be a valuable tool in situations where the primary method of testing the tumor tissue fails to generate needed information.[5]

Materials and methods

To validate this assay, 125 libraries were generated with the LiquidPlex assay and sequenced on the Illumina® NextSeq platform using NextSeq 500/550 High Output Kit v2.5. Analysis was performed using Archer® Analysis v6.1.

The validation study focused on precision and reproducibility, sensitivity and specificity, and limit of detection. Quality control checks have been recorded in the process such as ctDNA extraction, library preparation, target capture, and sequencing. The validation study used real-world patient samples, in addition to well-characterized reference materials. The bioinformatics analysis was performed using Archer Analysis. Filter settings of [AO≥15 OR DAO≥3] AND AF Outlier P-value ≤0.001 are used to filter out sequencing and PCR artifacts. Targets enriched in this test are listed in **Table 1**.

Table 1: Gene target list

AKT1	**ESR1**	**NRAS**
BRAF	**FGFR3**	**PDGFRA**
CTNNB1	**IDH1**	**PIK3CA**
EGFR	**KIT**	**RET**
ERBB2	**KRAS**	**TP53**

Acceptance criteria

Final sensitivity and specificity were defined at each input mass and allele fraction level based on the results of this study. Acceptance criteria as defined by the validation plan:

- Specificity should be greater than 98% while sensitivity must exceed 90%. The assay may be validated to an LOD with sensitivity and specificity below these thresholds, however the limitation of the assay must be clearly defined in the final report.
- At least 90% of the samples must generate libraries that pass library preparation QC.
- Reproducibility must exceed 90% for each variant type at the lower limit of detection.

In addition to the quality metrics / acceptance criteria defined in the validation plan, the validation experiments drove additional post-sequencing quality control. In order to pass library preparation QC, each library must quantify above 4 nanomolar (nM) by qPCR for inclusion in a sequencing batch. Post sequencing, QC metrics for each sequencing batch require at least 75% of bases to have a quality score above Q30. Individual QC metrics are a minimum of 6M reads and a minimum of 50,000 unique fragments per sample.

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Results

Reproducible and accurate variant calling was achieved for somatic variants detected in this validation. The accuracy of the assay was investigated using 50 replicates of controls containing 24 cancer-causing variants at allele fractions as low as 0.13% using a range of read depths from 4-10M reads **(Tables 2 and 3)**. Accuracy of real-world variant detection was 100% down to the 0.25% AF LLOD at a read depth of 6M. All variants detected in real-world samples were orthogonally confirmed using digital droplet PCR.

Table 2: Reference sample results: LLOD was determined to be 0.25% AF when using a minimum of 6M reads with 50ng of input*

	0.25% AF	>0.5% AF
Sensitivity	96.5%	100%
Precision	91.3%	100%
Reproducibility	91.3%	100%

**For all variants except PIK3CA p.N1068fs*4*

Table 3: Reference sample results: LLOD was determined to be 2% AF when using 4M reads with 20ng of input

	2% AF
Sensitivity	99.1%
Precision	95.7%
Reproducibility	100%

Conclusions

We tested and validated the LiquidPlex assay. This test sequences select coding sequences containing variants that are common drivers of solid tumor cancers as well as common resistance alterations that are relevant to treatment selection. The assay as validated has a high degree of analytical performance for the detection of SNVs, small insertions and deletions. These results serve to verify that the assay is robust and appropriate for use in research settings.

References

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