Illumina Cell-Free DNA Prep with Enrichment: A custom enrichment assay that provides high sensitivity for somatic variants detection

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INTRODUCTION

Cell-free DNA (cfDNA) derived from plasma combined with next-generation sequencing (NGS) is an emerging tool for non-invasive evaluation of biomarkers in cancer. Genomic variants that arise in tissues are typically present at very low abundance in cfDNA and require detection methods with high analytical sensitivity and specificity. Illumina cfDNA Prep with Enrichment is a novel and flexible custom enrichment library preparation assay with integrated analysis pipeline that leverages Illumina NGS platforms for detection of low frequency single nucleotide variants (SNV), insertions and deletions (Indels), copy-number variations (CNV) and gene fusions. The integrated workflow is compatible with user-defined range of custom enrichment panels with high analytical sensitivity and specificity from as little as 10 ng cfDNA input.

MATERIALS & METHODS

For this study, libraries were generated using 20 ng input, unless noted. Input was derived from patient cfDNA, commercially available cfDNA reference standards, cfDNA-like contrived sample (variants from cfDNA reference standard diluted in cfDNA), and nucleosome preparations (npDNA) from cell lines. Following library preparation, libraries were enriched with panels of different sizes and formats and sequenced on the NovaSeq™ 6000, NextSeq™ 2000 and NextSeq™ 550 instruments and analyzed with the DRAGEN™ for ILMN cfDNA Prep with Enrichment App on BaseSpace™ Sequence Hub. Assay analytical sensitivity was evaluated with different panels for different variant types diluting targeted variants to at least 3 desired VAF levels or fold change (FC). 20 ng libraries were prepared by two operators using two lots of reagents and sequenced on two instruments for a total of 24 observations per variant per level. Specificity for small DNA variants was established from 120 libraries of 20ng input prepared from cfDNA from healthy donors using two different custom panels (80bp-ssDNA 55 Kb and 2000 Kb). The assay precision was evaluated by testing a panel of samples with targeted variant types across multiple operators, reagent lots, and sequencing instruments.

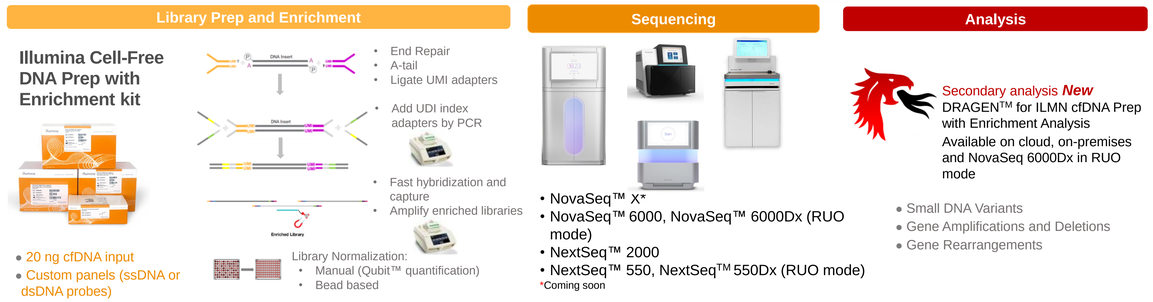

Figure 1. Illumina cell-free DNA Prep with Enrichment workflow

RESULTS

Variant Category	Variant Sensitivity	Variant Specificity
SNV ≥ 0.2% VAF	≥ 90%	≤99.98%
Indels ≥ 0.5% VAF	≥ 90%	
Gene amplifications ≥1.3-fold change	≥ 95%	ND[1]
Gene deletions ≤ 0.6-fold change	≥ 95%	
Gene rearrangements at 0.5% VAF	≥ 95%	

Table 1. Analytical sensitivity and specificity for different variant types
[1]Non-determined

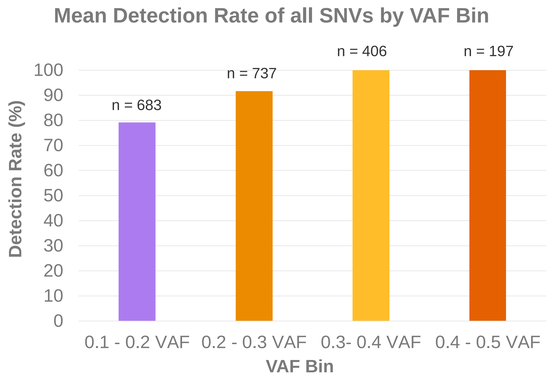

Figure 2. Observed hit rate for SNVs tested across cell line and contrived sample titrations

Analytical Sensitivity and Specificity

CNV and Gene Rearrangement Detection

Sample type	Variant type	Gene/s	VAF or FC achieved with indicated panel (size, Kb)	
			Panel A (2000)	Panel B (300)
cfDNA-like	Gene fusion	CD74:ROS1	0.36%	0.45%
		NCOA4:RET	0.2%	0.27%
		EML4-ALK	0.24%	0.34%
	Gene Amplification	ERBB2	1.16	1.35
		MET	1.18	1.36
		MYC	1.18	1.2
npDNA	Gene Deletion	BRCA1	0.79	0.75
		BRCA2	0.83	0.80

Table 2. VAF and FC with ≥95% Detection Rate for gene fusions and CNVs using panels of different size

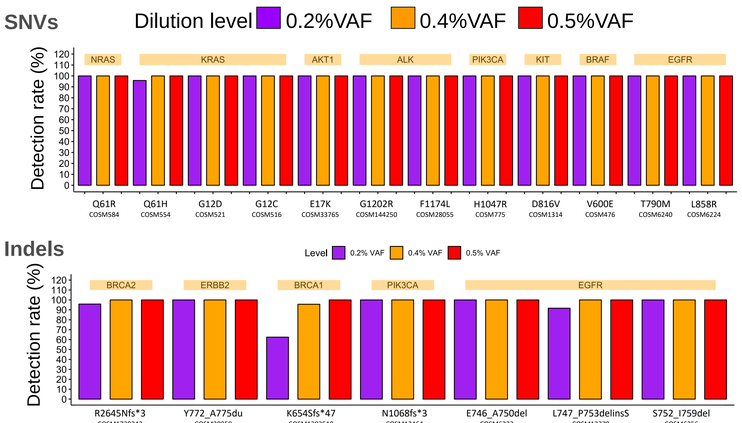

Figure 3. Hit rate for SNVs and Indels tested across cfDNA-like sample titrations

Exceptional analytical sensitivity with custom panels

(Illumina and third-party vendors)

Panel	Size (Kb)	Probe format	Variant types targeted
Small	55	80 bp - ssDNA	SNVs, Indels
Medium-A	250	120bp - dsDNA	SNV, Indels, Fusions
Medium-B	300	80 bp - ssDNA	SNV, Indels, Fusions, CNVs
Large	2000	80bp - ssDNA	SNV, Indels, Fusions, CNVs

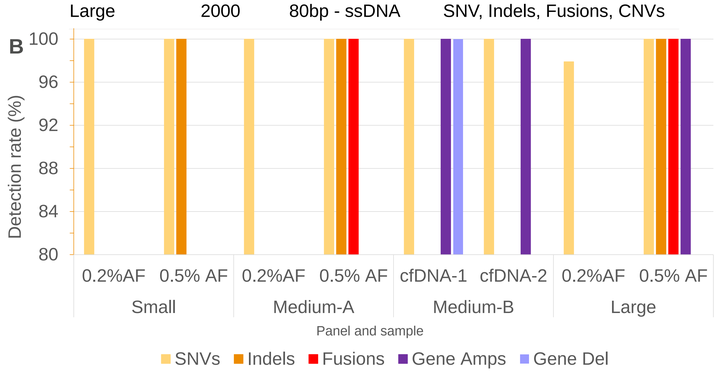

Figure 4. Variant detection across libraries prepared with cfDNA, cfDNA-like with SNVs at 0.2% VAF, or cfDNA reference standard with variants at 0.5% VAF. Libraries were enriched with panels described in **A**

Compatibility with different sequencing platforms

Excellent performance of 20ng cfDNA reference standard libraries enriched with a 120bp-dsDNA 250 Kb panel and sequenced* on mid- and high-throughput sequencing systems.

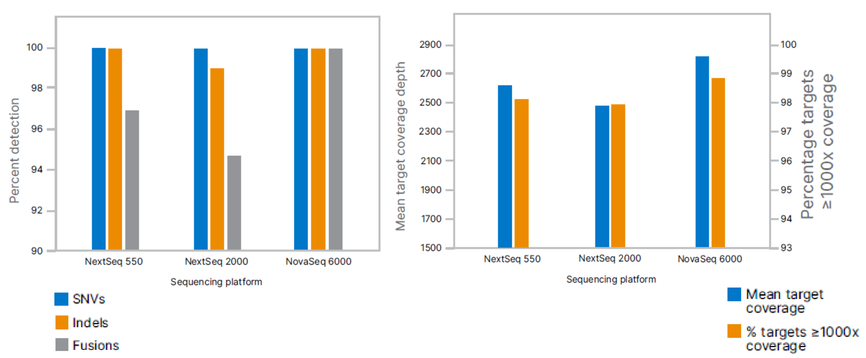

Figure 5. Comparable library performance metrics and variant detection between sequencing platforms

*Libraries were sequenced at an average read depth of 46M paired-end reads and ~30,000x on-target coverage

Input titration and impact of enrichment format

The impact of the amount of cfDNA input was evaluated preparing libraires with10ng, 20ng and 30ng input. Libraries were individually enriched (1-plex) with an 80bp-ssDNA 180 Kb panel (unless noted), and the same four libraries were re-enriched using the same panel as 4-plex enrichment. The assay delivers ≥ 90% sensitivity with as low as 10ng input for 0.2% VAF SNVs, and ≥95% for 0.5% VAF indels. High concordance for variant detection between 1-plex and 4-plex enrichment was shown using custom panels with ssDNA probes or dsDNA probes (20 ng only; 1-plex-B, 4-plex-B).

[1]20ng libraries were re-enriched with the dsDNA version of the panel (1-plex-B and 4-plex-B).
[2]cfDNA-like contrived sample with SNVs at 0.2% VAF.
[3]Seraseq® ctDNA Complete Mutation Mix AF-0.5% (Seracare, LGC Clinical Diagnostics).

Input (ng)	Enrichment format[1]	Detection rate	
		SNVs[2]	Indels[3]
10	1-plex	96%	100%
	4-plex	96%	100%
20	1-plex	100%	100%
	4-plex	97.9%	96.4%
	1-plex-B	98.7%	98.2%
	4-plex-B	98.9%	96.4%
30	1-plex	100%	100%
	4-plex	100%	100%

Precision

To evaluate variant call precision, 18 library preparation events across multiple operators, reagent lots and sequencing instruments were performed. All libraries were enriched with an 80bp ssDNA 2000 Kb panel (panel A), and a subset of libraries were re-enriched with an 80bp ssDNA 300 Kb panel (panel B). Variant call concordance was evaluated by percent positive calls (PPC) and percent negative calls (PNC). PPC calculations used a pre-defined list of targeted variants per sample, whereas for PNC it was defined as (1-FP/Negative) where FP is defined as total identified variants.

Variant Type	Panel	PPC	95% two-sided CI	PNC	95% two-sided CI
SNVs and Indels	A	**99.78%** (3592/3600)	(99.56%, 99.89%)	**99.999%** (633587050/633596400)	(99.998%, 99.999%)
	B	**99.48%** (570/573)	(98.47%, 99.82%)	**99.997%** (66906059/66908019)	(99.997%, 99.997%)
Gene Amplifications	A	**98.18%** (486/495)	(96.58%, 99.04%)	**99.72%** (353238/354230)	(99.70%, 99.74%)
	B	**97.35%** (184/189)	(93.96%, 98.86%)	**99.75%** (57552/57699)	(99.70%, 99.78%)
Gene Deletions	A	**100%** (124/124)	(97.00%, 100%)	**99.77%** (353803/354601)	(99.76%, 99.79%)
	B	**100%** (102/102)	(96.37%, 100%)	**99.75%** (57641/57786)	(99.71%, 99.79%)
Gene Fusions[1]	A	**96.91%** (157/162)	(92.98%, 98.67%)	**97.36%** (3470/3564)	(96.78%, 97.84%)

[1]Gene fusions precision not evaluated with panel B .

CONCLUSION

Together these results demonstrate that Illumina cfDNA Prep with Enrichment coupled with DRAGEN™ for IILMN cfDNA Prep with Enrichment Analysis achieve >90% analytical sensitivity for SNVs at 0.2% VAF, and >95% to 0.5% SNVs, Indels and gene rearrangements. High analytical sensitivity for low abundance gene amplifications and gene deletions was also demonstrated. The assay is a versatile custom enrichment solution optimized for low input cfDNA and shows high concordance for variant detection between 1-plex and 4-plex enrichment formats. Illumina cfDNA Prep with Enrichment supports a range of panel sizes and is compatible with probe formats from Illumina or third-party providers.

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