Detecting Ultra-Low Frequency ESR1 Mutations in Liquid Biopsy Samples Using the oncoReveal® Essential LBx Panel

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INTRODUCTION

Introduction. Cell-free DNA (cfDNA) sequencing enables sensitive detection of low-frequency somatic mutations in liquid biopsy samples, supporting applications such as treatment response monitoring and minimal residual disease detection. Mutations in ESR1, particularly in the ligand-binding domain, are associated with endocrine therapy resistance in hormone receptor-positive breast cancer and play a growing role in guiding treatment selection. We developed the oncoReveal® Essential LBx Panel as a single-tube, multiplexed NGS assay targeting somatic variants across 34 cancer-related genes, including ESR1. The panel is optimized for variant detection from cfDNA, combining a one-step target amplification protocol that preserves the original template, an ultra-HiFi enzyme that minimizes PCR errors, and a machine learning error suppression algorithm. Together, they enable detection of variants with allele frequencies as low as 0.05%, using a simplified workflow without utilizing molecular barcodes.

Methods. Amplicons were designed using Pillar VersaTile™ software to reflect the size profile of cfDNA, averaging 87bp [55-131bp]. Pillar SLIMamp® chemistry allows for streamlined, single-tube target amplification followed by dual-index adapter PCR. A dilution series of Seraseq® ctDNA ESR1 Mutation Mix AF 1% with Seraseq WT sample yielded samples with VAFs of 0.5%-0.05%. Libraries were generated with DNA inputs of 5-30ng and sequenced on the Illumina NextSeq 550 platform as 2x121bp. Secondary analysis was performed with PiVAT®, which applies paired-end read assembly, trinucleotide-context noise modeling, and variant-specific filtering to maximize specificity.

Results. High-quality libraries were consistently generated across the full input and dilution range, with yields ranging from 13-128nM. Sequencing metrics showed mean Q30 >90%, mapping >99%, and on-target rates >90%. Samples had a uniform amplicon coverage with >97% of bases at ≥0.2x mean. All expected ESR1 mutations were detected across replicates with VAF >0.5%. For ≥10ng inputs, the PPA at 0.1% VAF was 98.1% [CI: 93.5-99.8%]; for 30ng inputs, the PPA at 0.05% VAF was 82.4% [CI: 73.9-89.1%]. No false positives were observed in wild-type or healthy donor cfDNA samples, resulting in 100% NPA at all tested sites. Observed VAFs correlated strongly with expected values, increasing monotonically with DNA input.

Conclusion. The oncoReveal® Essential LBx Panel is a kitted, accurate, and streamlined NGS-based solution for detecting low-frequency ESR1 mutations from cfDNA. With high analytical sensitivity and specificity down to 0.05% VAF, minimal DNA input, no molecular barcode, and a same-day workflow, the assay is well-suited for liquid biopsy applications in precision oncology.

MATERIALS AND METHODS

- Samples: Reference standards (Seraseq® ESR1 Mutation Mix and WT) and cfDNA from healthy donors were used to generate serial dilutions (1%–0.05% VAF) for performance testing.
- cfDNA extraction: Plasma cfDNA was isolated using the QIAamp Circulating Nucleic Acid Kit, with inputs ranging from 5–30 ng.
- Library preparation: Libraries were prepared with SLIMamp® single-tube multiplex PCR and dual-index adapter addition, enabling amplification of short amplicons (55–131 bp; avg 87 bp) optimized for ctDNA.
- Sequencing: Pooled libraries were sequenced on the Illumina NextSeq 550 using a 2 × 121 bp paired-end configuration.
- Data analysis: Sequencing reads were processed using PiVAT®, which performs alignment, paired-end consensus assembly, trinucleotide-context noise modeling, and variant calling with VEP annotation.

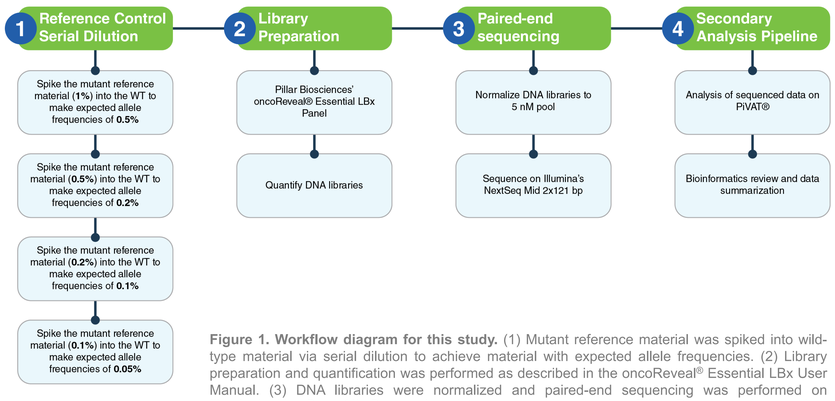

Figure 1. Workflow diagram for this study. (1) Mutant reference material was spiked into wild-type material via serial dilution to achieve material with expected allele frequencies. (2) Library preparation and quantification was performed as described in the oncoReveal® Essential LBx User Manual. (3) DNA libraries were normalized and paired-end sequencing was performed on Illumina's NextSeq. (4) Sequencing data were analyzed using Pillar Biosciences' secondary analysis software PiVAT®.

RESULTS

Dilution series preparation

Sample	Spiked-in Allele Frequency	Input (ng)	# of Reps
Seraseq® ctDNA ESR1 Mutation Mix AF 1% (Cat# 0710-3565) spiked into Seraseq® ctDNA ESR1 Mix WT (Cat# 0710-3564)	0.01	30	2
	0.005	30	3
		20	3
		10	3
		5	3
	0.002	30	3
		20	3
		10	3
		5	3
	0.001	30	3
		20	3
		10	3
		5	3
	0.0005	30	3
		20	3
		10	3
		5	3
cfDNA Polishing Control	N/A	30	1
Healthy Donor cfDNA	N/A	20	23†

Table 1. Positive and negative samples used in this study, including expected allele frequencies, DNA input amounts, and number of replicates.
†23 replicates were from a set of 13 healthy donors.

Sequencing library performance

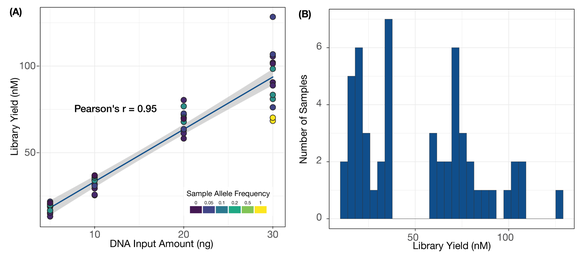

Figure 2. High yield library from oncoReveal® Essential LBx Panel. (A) Correlation between DNA input (ng) vs library yield (nM) for each of the dilutions used in this study. Library yield increases linearly for each of the sample as DNA input amount increases. Allele frequency of each sample is color-coded as shown in the legend. (B) Histogram showing the distribution of library yield across samples.

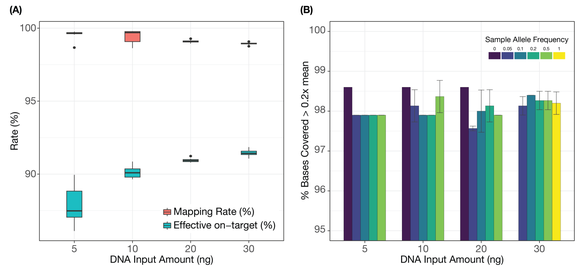

Figure 3. Panel amplicon performance (A) Mapping rate and Effective on-target rate (eOTR) for each DNA input amount (ng). Mapping rates stay relatively constant across DNA input amounts. eOTR increases with increasing DNA input amounts. (B) Uniformity of amplicons covered at >0.2x relative to mean coverage of the sample. Uniformity becomes more consistent across sample allele frequencies as DNA input amount increases.

RESULTS

Sensitivity and specificity at different inputs

Positive Samples					
Samples Allele Fraction	Reps	PPA (observed n/ expected N) [95%CI]			
		5 ng	10 ng	20 ng	30 ng
1% (Stock)	2	- -	- -	- -	100% (42/42) [91.6%, 100%]
0.5%	3	**100% (63/63) [94.3%, 100%]**	**100% (63/63) [94.3%, 100%]**	100% (63/63) [94.3%, 100%]	100% (63/63) [94.3%, 100%]
0.2%	3	**93.7% (59/63) [84.8%, 97.5%]**	**100% (63/63) [94.3%, 100%]**	100% (63/63) [94.3%, 100%]	100% (63/63) [94.3%, 100%]
0.1%	3	68.3% (43/63) [56%, 78.4%]	**95.2% (60/63) [86.9%, 98.4%]**	**98.4% (62/63) [91.5%, 99.7%]**	98.4% (62/63) [91.5%, 99.7%]
0.05%	3	55.6% (35/63) [43.3%, 67.2%]	66.7% (42/63) [54.4%, 77.1%]	**82.5% (52/63) [71.4%, 90%]**	**81% (51/63) [69.6%, 88.8%]**
Negative Samples					
Sample Type	Samples	Negative Agreed	Negative Expected	NPA (n/N) [95%CI]	
Healthy donor cfDNA	24	504	504	100.0% (504/504) [99.2%, 100.0%]	

Table 2. Panel performance for positive and negative samples. Positive percent agreement (PPA) and negative percent agreement (NPA) for the oncoReveal® Essential LBx Panel for the Seraseq® ctDNA ESR1 Mutation Mix AF 1% serial dilutions and negative samples. The bold cells highlight the lowest two inputs at which best performance is observed for each dilution.

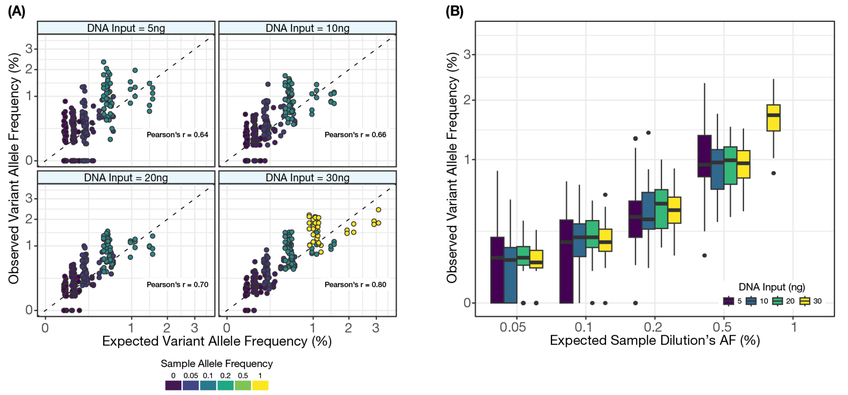

Figure 4. Correlation of observed variant allele frequency (VAF) compared to the expected dilution VAF across multiple DNA input amounts. (A) Each point represents a single variant in each sample/replicate, color coded by the allele frequency of the sample as shown in the figure's legend. Both axes are in square root transformation to visually differentiate low VAF calls. As expected, Pearson's correlation increases monotonically as the DNA input of the sample increases. (B) Boxplot summarizing the observed VAF across the DNA inputs. As with the variant correlation, the boxplot shows increase in the median VAF with the sample dilutions.

CONCLUSIONS

- The oncoReveal® Essential LBx Panel enables sensitive detection of key ESR1 ligand-binding domain mutations and other actionable variants across solid tumors.
- The streamlined, single-tube workflow supports same-day library preparation and sequencing.
- The assay generated high-yield, high-quality libraries with excellent mapping, on-target rates, and uniform coverage.
- Demonstrated >95% PPA at 0.1% VAF and >85% PPA at 0.05% VAF, with 100% NPA across tested sites.
- Independent external validation confirmed performance and reproducibility down to 10 ng cfDNA input.
- Overall, the assay provides a robust, reproducible, and sensitive solution for detecting low-frequency SNVs and indels in liquid biopsy applications.