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WHITE PAPER

Pillar® oncoReveal® Essential LBx Panel

Investigating the panel performance for hotspot ESR1 variants

Introduction

Ultra-deep targeted sequencing of cell-free DNA (cfDNA) has emerged as a powerful tool for non-invasive detection of low frequency somatic variants, specifically in applications such as liquid biopsy. This approach allows for the detection of genetic mutations, tumor heterogeneity, and minimal residual disease without the need for invasive procedures, offering patients and clinicians a more accessible and dynamic method for monitoring disease progression, treatment response, and the emergence of therapeutic resistance (Wan et al., 2017).

Liquid biopsy (LBx) panels, such as Pillar's **oncoReveal® Essential LBx**, are designed to target specific genetic hotspots within cancer genomes and have become particularly valuable in precision medicine, as they can identify clinically actionable mutations that may guide targeted therapeutic interventions. For example, mutations in the ESR1 gene, particularly those in the ligand-binding domain such as D538G, E380Q, and D537S/N/C, have been identified as key contributors to resistance to endocrine therapies, including Selective Estrogen Receptor Modulators (SERMs), Selective Estrogen Receptor Degraders (SERDs), and aromatase inhibitors (Crucitta, et al., 2022; Bidard, et al, 2022; Bardia et al 2021; oncokb.org/gene/ESR1). These mutations, often occurring after long-term treatment, lead to constitutive activation of the ERα pathway allowing the cancer cells to evade the therapeutic effects of endocrine therapies (Toy et al., 2017). The detection of ESR1 mutations has become an important clinical tool in guiding treatment decisions for breast cancer patients, as these mutations have been linked to poor prognosis and a higher likelihood of disease recurrence despite initial responsiveness to therapy (Reinert et al., 2017).

Pillar's **oncoReveal® Essential LBx** is a streamlined, highly specific and sensitive Next-Generation Sequencing (NGS) assay for liquid biopsy. Key factors that enable the assay's performance are (1) a one-step target amplification protocol preserves the original template, (2) an ultra-high Fidelity enzyme system, which minimizes PCR errors, and (3) a proprietary error correction algorithm with dynamically trained model leveraging normal samples to correct sequencing, mapping, and PCR errors. Here, we demonstrate the performance of the oncoReveal® Essential LBx panel for ESR1 variant detection at low variant allele frequencies (≥0.05%) using a standard reference control sample. Additionally, we validate the performance of the panel via an independent third-party evaluation by a European diagnostic laboratory with no assistance from Pillar Biosciences, Inc.

oncoReveal® Essential LBx Panel (34 Genes)				
AKT1	EGFR	GNA11	NRAS	RET
ALK	ERBB2	GNAQ	PDGFRA	RNF43
AR	ERBB3	GNAS	PIK3CA	SF3B1
ATM	ESR1	HRAS	PPP2R1A	SMAD4
BRAF	FGFR1	KIT	PTCH1	TERT
CDK4	FGFR2	KRAS	PTEN	TP53 *(Full CDS)*
CTNNB1	FGFR3	MET	RAC1	

Table 1. Genes included in the oncoReveal® Essential LBx Panel. TP53 has full CDS coverage; the remaining genes have hotspot coverage. ESR1 (NM_00125.4) has full coverage of exons 5-7 and partial coverage of exons 4 and 8; oncogenic/likely oncogenic mutations covered are between codons 310-547.

Materials and Methods

Panel design

Pillar Biosciences' oncoReveal® Essential LBx is a liquid biopsy panel that interrogates hundreds of somatic variants across 34 genes of interest (**Table 1**) from multiple solid tumor cancer types, including hotspot mutations in ESR1. Hotspot targets were selected based on NCCN guidelines, literature review, and variants indicated in clinical trials where possible. The final panel targets were supplemented with pathologists' review. Amplicons for the panel were designed using Pillar's AI-empowered VersaTile™ Primer Design tool, for efficient single-tube target enrichment. Amplicons were designed to be as small as possible to facilitate detection of tumor ctDNA, within the predicted ctDNA length of ~168 bp based on the size of DNA fragment protected by the nucleosome. Amplicon length ranged from 55-131 bp with an average of 87 bp. The panel can detect four types of variants from cell-free DNA (cfDNA) extracted from plasma: single nucleotide

variants (SNVs), small insertion/deletion (indel) variants, copy number amplification (CNA), and microsatellite instability (MSI).

Gene	Nucleotide change	Amino Acid change	Transcript	GRCh37 location	Alteration Type
ESR1	c.1138G>C	E380Q	NM_000125.4	6:152332832	SNV
	c.1387T>C	S463P	NM_000125.4	6:152415537	SNV
	c.1603C>A	P535T	NM_000125.4	6: 152419916	SNV
	c.1607_1608delinsAT	L536H	NM_000125.4	6:152419920-152419921	INDEL
	c.1607T>A	L536H	NM_000125.4	6:152419920	SNV
	c.1607T>C	L536P	NM_000125.4	6:152419920	SNV
	c.1607T>G	L536R	NM_000125.4	6:152419920	SNV
	c.1607_1608delinsAG	L536Q	NM_000125.4	6:152419920-152419921	INDEL
	c.1610_1611delinsCA	Y537S	NM_000125.4	6:152419923-152419924	INDEL
	c.1609_1610delinsAG	Y537S	NM_000125.4	6:152419922-152419923	INDEL
	c.1610A>C	Y537S	NM_000125.4	6:152419923	SNV
	c.1609T>A	Y537N	NM_000125.4	6:152419922	SNV
	c.1608_1609delinsTA	Y537N	NM_000125.4	6:152419921-152419922	INDEL
	c.1610A>G	Y537C	NM_000125.4	6:152419923	SNV
	c.1609T>G	Y537D	NM_000125.4	6:152419922	SNV
	c.1613A>G	D538G	NM_000125.4	6:152419926	SNV
	c.1610_1615dupATGACC	D538_L539insHD	NM_000125.4	6:152419923-152419928	INDEL
	c.1625A>G	E542G	NM_000125.4	6:152419938	SNV
PIK3CA	c.1624G>A	E542K	NM_006218.4	3:178936082	SNV
	c.1633G>A	E545K	NM_006218.4	3:178936091	SNV
	c.3140A>G	H1047R	NM_006218.4	3:178952085	SNV
	c.3203dupA†	p.N1068Kfs*5†	NM_006218.4†	3:178952148†	INDEL†

Table 2. Variants in the Seraseq® ctDNA ESR1 Mutation Mix AF 1% (Cat# 0710-3565). The testing material contains 22 variants: 18 ESR1 variants and 4 PIK3CA variants.
† Variant not covered by the oncoReveal® Essential LBx Panel.

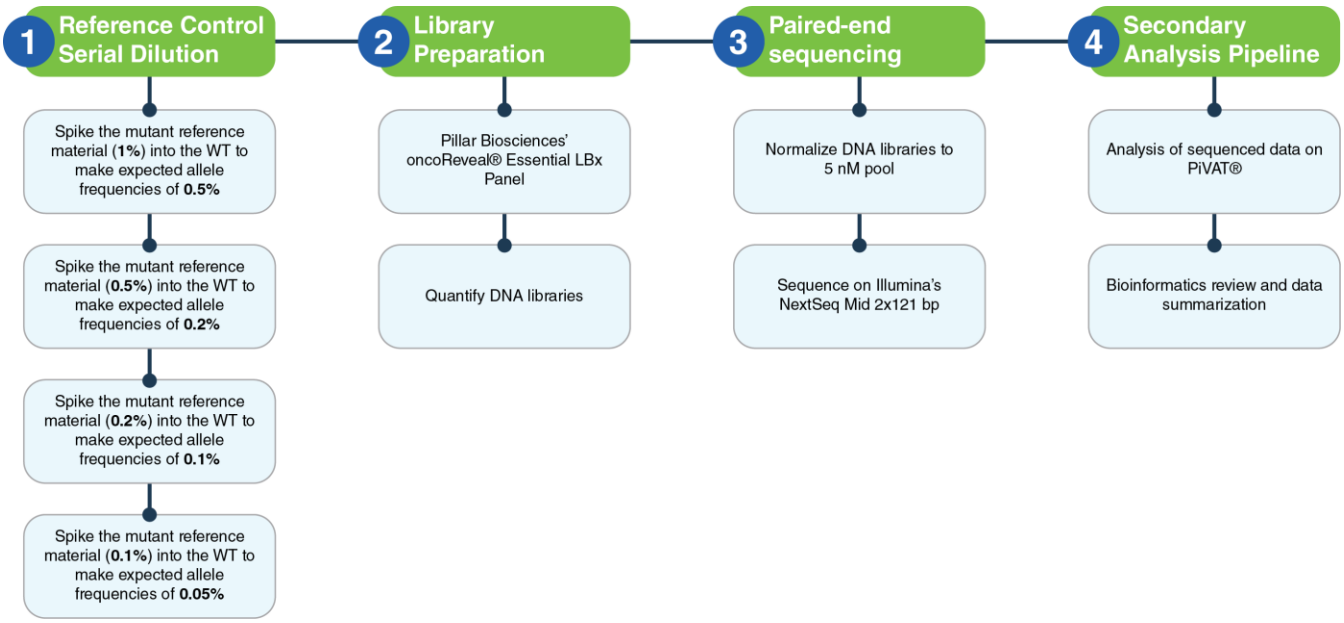

Figure 1. Workflow diagram for this study. (1) Mutant reference material was spiked into wild-type material via serial dilution to achieve material with expected allele frequencies. (2) Library preparation and quantification was performed as described in the oncoReveal® Essential LBx User Manual (*Doc. No. UM-0079*). (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®.

COSV	Tube	Exon	HGVS(p)	HGVS(c)
COSV52795259	1	8	L536H	1607T>A
COSV52782924	1	8	Y537C	1610A>G
COSV52782930	2	8	L536P	1607T>C
COSV52783938	2	8	Y537S	1610A>C
COSV52787207	3	8	L536R	1607T>G
COSV52781024	3	8	D538G	1613A>G
COSV52782264	4	5	E380Q	1138G>C
COSV52784970	4	7	S463P	1387T>C
COSV52784978	4	8	Y537N	1609T>A
Wildtype	5	-	-	-

Table 3. Mutations present in each tube of the SensID ESR1 Reference Set 1% AF cfDNA.

Sample	Spiked-in Allele Frequency	Input (ng)	# of Replicates
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 4. 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.

Samples

Internal performance assessment

Standard reference samples and cfDNA from healthy donors were used in this study. The following molecular standards were used to assess DNA performance:

- Seraseq® ctDNA ESR1 Mutation Mix AF 1% (Cat# 0710-3565) consists of 22 clinically relevant mutations in ESR1 (n=18) and PIK3CA (n=4), each at approximately 1% variant allele frequency. The fragment size ranges from 155-200 bp. The mutations, as listed by the vendor, are listed in **Table 2**.
- Seraseq® ctDNA ESR1 Mix WT (Cat# 0710-3564) is the corresponding wildtype sample that was used for dilution as well as the negative sample for specificity calculation.
- Anchor Molecular's AM Normal cfDNA (Cat# 60133001) sample was used for polishing control (described below).
- 23 cfDNA samples from 13 healthy donors were collected in-house. 10 mL blood samples were collected in Streck Cell-Free DNA BCT tube; 4 mL plasma was isolated from each tube. QIAamp Circulating Nucleic Acid kit (Cat#55114) was used for extraction. The yields of cfDNA from 4 mL plasma range from 15 to 54 ng. The cfDNA input used for the Essential assay varies from 5 ng to 30 ng.

Independent external validation

The third-party evaluation was performed using the SensID ESR1 Reference Set 1% AF cfDNA. This reference set includes ESR1 mutations at 1% allele frequency (AF), across four vials (tubes), each harboring different clinically relevant variants (**Table 3**).

Experiment workflow

Internal performance assessment

The experiment workflow is delineated in **Figure 1**. Briefly, a dilution series of the Seraseq® ctDNA ESR1 Mutation Mix AF 1% standard was generated samples with AF 0.5%, 0.2%, 0.1%, and 0.05% (**Table 4)**. Libraries of the original stock and the four dilutions were prepared as per the kit protocol (see "Panel chemistry") with DNA inputs of 5 ng, 10 ng, 20 ng, and 30 ng. DNA libraries were normalized and loaded on Illumina's NextSeq mid-output kit with 2x121 bp configuration according to the user manual (*Doc. No. UM-0079*). Sequencing reads were processed on PiVAT 24.2.3 (see "Secondary Analysis Pipeline PiVAT") and the results were analyzed for performance estimation (see "Performance estimators").

Independent external validation

An experimental workflow similar to that detailed in Figure 1 was followed. The four tubes of the SensID ESR1 Reference Set 1% AF cfDNA were pooled and serially diluted to achieve final allele frequencies of 0.5%, 0.2%, 0.1%, and 0.05% (**Table 5**), providing a rigorous, titrated series to test the lower limit of detection (LoD) of the panel under controlled conditions. Each dilution of the SensID mix was tested using two input amounts: 10 ng and 20 ng of cell-free DNA (cfDNA). Variant calling was performed across the mixed ESR1 mutant background using standard assay protocols. The resulting data were analyzed for total variant detection, allele frequency correlation, and missed call rates stratified by both input mass and allele fraction. DNA libraries were normalized and loaded on Illumina's NextSeq 500 mid-output kit according to the user manual (*Doc. No. UM-0079*). Sequencing reads were processed on PiVAT 25.1.0 (see "Secondary Analysis Pipeline

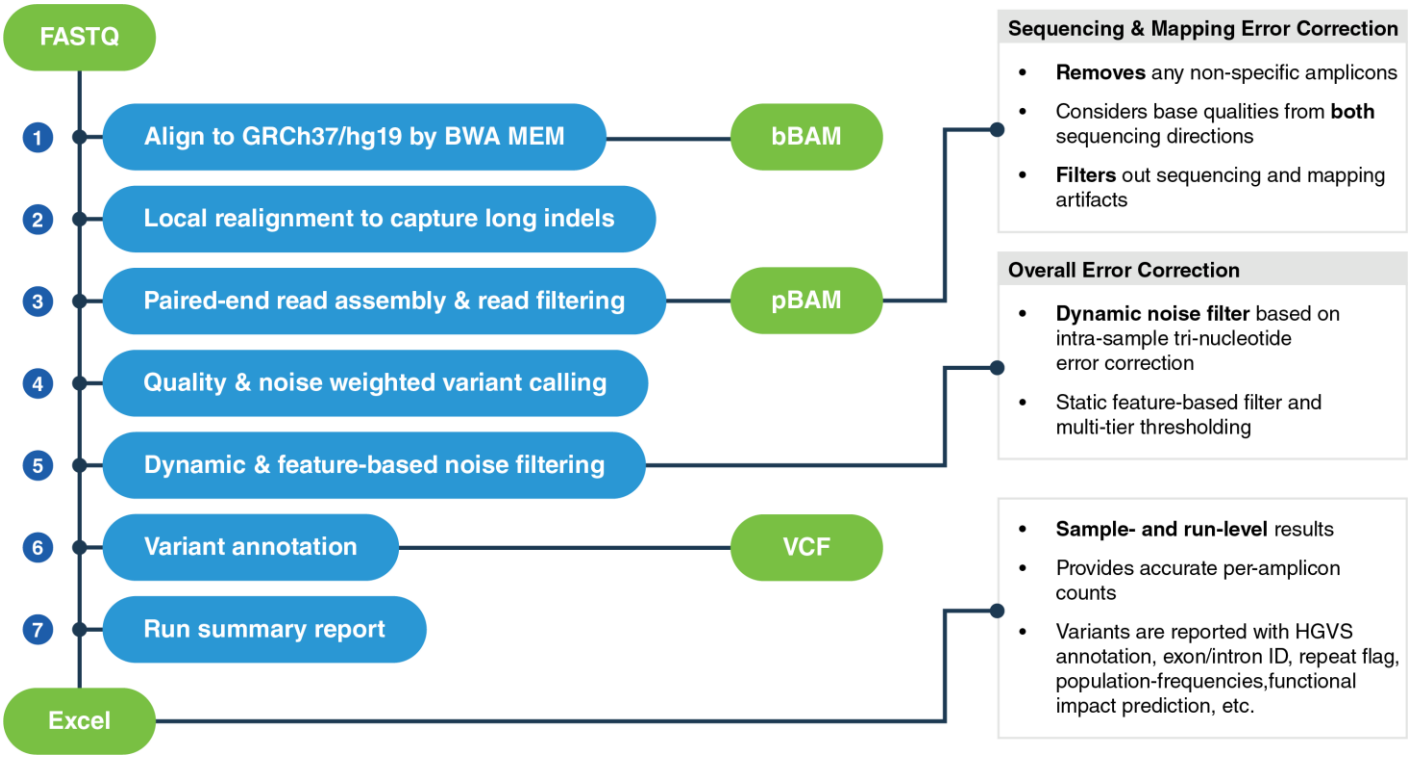

Figure 2. PiVAT® data analysis workflow. PiVAT® software uses both standard BAM and additional enhanced pBAM alignment file outputs to calculate and report sequencing quality metrics.

PiVAT") and the results were analyzed for performance estimation (see "Performance estimators"

Panel chemistry

The oncoReveal® Essential LBx Panel (*Part No. HLA-HS-1006-24*) utilizes our SLIMamp® (stem-loop inhibition-mediated amplification) technology allowing researchers to amplify regions of interest in a simple, single-tube multiplex PCR reaction. Pairs of DNA oligos designed for each region of interest, or hotspot, are used in the first round of gene-specific PCR (GS-PCR), and the products are subsequently purified via size selection. After purification, a second round of PCR adds unique dual barcode index adaptors for sample tracking and sequencing (*Part Nos. IDX-PI-1013-96 or IDX-PI-1014-96*). The oncoReveal® Essential LBx Panel contains sufficient reagents to prepare 24 libraries. Use of this panel requires a unique dual barcode indexing kit and the resulting libraries are designed for sequencing on the Illumina platform using a paired-end read length of at least 2 × 121 bp. The workflow of this panel can be performed and loaded onto the sequencing instrument within one day, and the protocol contains numerous stopping points for users who have time limitations.

Secondary Analysis Pipeline PiVAT®

Pillar Variant Analysis Toolkit (PiVAT®) is a clinical grade, research use only (RUO), secondary analysis bioinformatics software. Designed to pair with Pillar's PCR chemistry, PiVAT® provides a robust LBx pipeline that identifies low frequency variants with high specificity in LBx samples' next-generation sequencing data. PiVAT® is designed for clinical applications with minimal human review. PiVAT's LBx bioinformatics pipeline consists of the following steps (**Figure 2**):

1. Read-to-genome alignment: Reads in FASTQ files are aligned to the human genome (GRCh37/hg19) using BWA-MEM.
2. Local realignment: Local realignment is performed using Smith-Waterman and a proprietary algorithm to improve the precision for detecting insertions and deletions at the edge of the reads.
3. Unique positional paired-end read assembly and read filtering: To maximize the base accuracy and minimize the sequencing noise, paired-end reads are assembled into consensus reads, weighted with the base quality scores from both mates. The assembled reads correspond to the gene-specific amplicon positions on the genome. A set of filters are then applied to remove non-uniquely mapped reads (e.g., pseudogenes) and reads that do not match the amplicon positions (e.g., primer-dimers or non-specific amplifications).

4. Variant calling: Variants are identified for each position, accounting for variant quality and contextual noise. Low quality and low confidence variants are filtered.
5. Post-call filtering: Based on pre-defined heuristics, a set of decision rules are employed to filter frequently observed, position independent mutational changes. E.g., indel variant types require higher variant allele frequency threshold than single nucleotide variants. PiVAT® utilizes at least one normal sample, sequenced in the same manufacturing lot as the rest of the samples, to estimate the background error and use it to apply dynamic noise correction. PiVAT® implements a rigorous statistical model based on the trinucleotide context of each mutation to dynamically set filtering thresholds for all variants. Only variants passing all the above filters are retained and treated as true calls.
6. Variant annotation: Variant annotation is performed using Ensembl Variant Effect Predictor (VEP). Each variant is annotated with HGVSc, HGVSp, exon/intron ID, homopolymer and simple sequence repeat, population-level frequencies, functional impacts, etc.
7. Run summary report: The quality metrics are assessed for each run and each sample. The criteria for passing the quality metrics are pre-assigned within the panel in PiVAT®. PDF and Excel reports are created for each sample. A combined Excel report is created for the run.

Tube	Input (ng)	%AF	# Reps
TUBE 1	10	1.00	1
TUBE 2	10	1.00	1
TUBE 1+2	10	0.50	1
TUBE 3	10	1.00	1
TUBE 4	10	1.00	1
TUBE 3+4	10	0.50	1
TUBE 1+2+3+4	10	0.20	2
TUBE 1+2+3+4	10	0.10	3
TUBE 1+2+3+4	10	0.05	3
TUBE 1+2+3+4	20	0.20	2
TUBE 1+2+3+4	20	0.10	3
TUBE 1+2+3+4	20	0.05	3
TUBE 5	10	-	1

Table 5. Schematic of the dilution and input strategy used in the SensID-based validation. ESR1 Reference Set 1% AF cfDNA from SensID was pooled and diluted to target allele fractions of 0.5%, 0.2%, 0.1%, and 0.05%, and tested at two cfDNA input levels (10 ng and 20 ng) using the oncoReveal® Essential LBx assay. Each combination was assessed for variant calling efficiency relative to known inputs.

Performance estimators

Panel performance was calculated using the following metrics:

$$\text{Positive Percent Agreement (PPA)} = \frac{\text{True positives} \times 100}{\text{True positives + False negatives}}$$

$$\text{Negative Percent Agreement (NPA)} = \frac{\text{True negatives} \times 100}{\text{True negatives + False positives}}$$

Confidence intervals were calculated using Wilson's method.

Results and Discussions

Library yields and sequencing metrics

High library yield, ranging between 13.2-128.4 nM across all the samples, provided sufficient concentration for loading. The library yield ranged from 68-128 nM for 20-30 ng samples. A strong correlation was observed between sample DNA input (ng) and library yield, supporting the robustness of the panel (**Figure 3**). The libraries were loaded on Illumina's Nextseq mid-output kits, producing 9.7-22.9M clusters per sample (mean = 14.2M).

Overall % Q30 and % Q20 ranges were 84.9 – 92.9 and 90.6 – 95.6, respectively.

Mapping rate is defined as the percentage of sequenced reads that map to the human genome (hg19). Effective on-target rate is defined as the percentage of total sequenced reads that map to target amplicon regions. The mapping and on-target rates were 99.3% ± 0.1% and 90.1% ± 0.2% respectively (**Figure 4A**). Coverage uniformity is measured by the percentage of bases in target regions covered at >0.2x mean coverage for the given sample. Greater than 97% of bases were covered at >0.2x mean for all the samples (**Figure 4B**).

Performance comparison

All expected variants were consistently called across replicates and DNA sample inputs in 1% stock standard sample as well as in the 0.5% dilutions (**Table 6**). As the sample concentration reduces, a higher Positive Percent Agreement (PPA) is observed with larger DNA inputs. The limit of detection (LoD) for ≥10 ng of the panel is estimated to be in the 0.05%-0.1% range. The PPA (observed n/ expected N) [95% confidence interval; CI] at 0.1% for ≥10 ng samples is 98.1% (106/108) [93.5%, 99.8%]. Whereas the PPA at 0.05% for 30 ng samples is 82.4% (89/108) [73.9%, 89.1%].

For the positions tested in this study, no false positive calls were observed in the healthy donor cfDNA samples, leading to a 100% NPA for these sites (**Table 6**). A detailed, per-site observed VAF (%) data for 30 ng input is provided in **Table S1**. The correlation between observed variant allele frequency (VAF) and expected VAF for each variant call also increases monotonically with DNA sample input (**Figure 5A**).

Finally, the overall sample VAF was also observed to increase monotonically with expected sample dilution (**Figure 5B**).

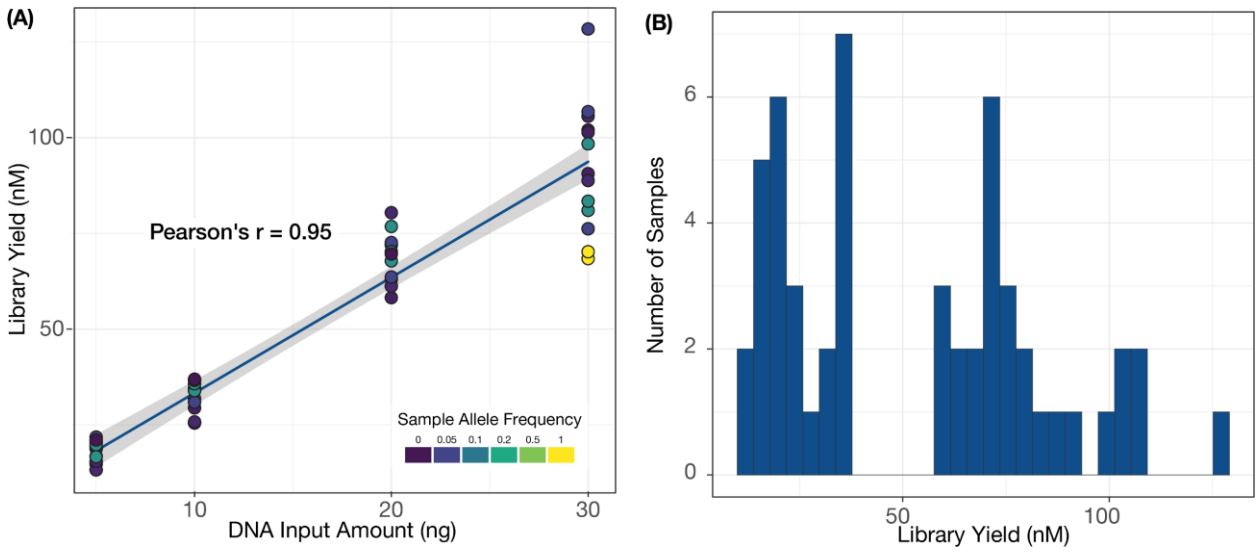

Figure 3. High yield library from oncoReveal® Essential LBx Panel. (A) Correlation between DNA input (ng) vs library yield (nM) for each of the dilutions used in this study. Library yield increases linearly for each of the sample as DNA input amount increases. Allele frequency of each sample is color-coded as shown in the legend. (B) Histogram showing the distribution of library yield across samples.

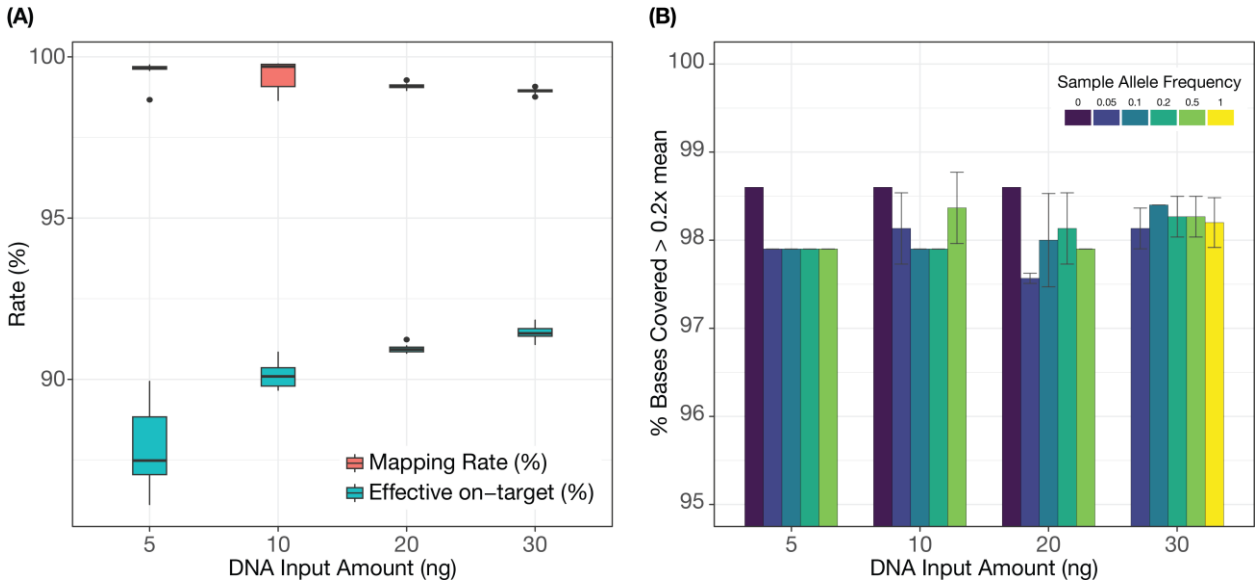

Figure 4. Panel amplicon performance (A) Mapping rate and Effective on-target rate (eOTR) for each DNA input amount (ng). Mapping rates stay relatively constant across DNA input amounts. eOTR increases with increasing DNA input amounts. (B) Uniformity of amplicons covered at >0.2x relative to mean coverage of the sample. Uniformity becomes more consistent across sample allele frequencies as DNA input amount increases.

Positive Samples					
Samples Allele Fraction	Replicates	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 6. 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.

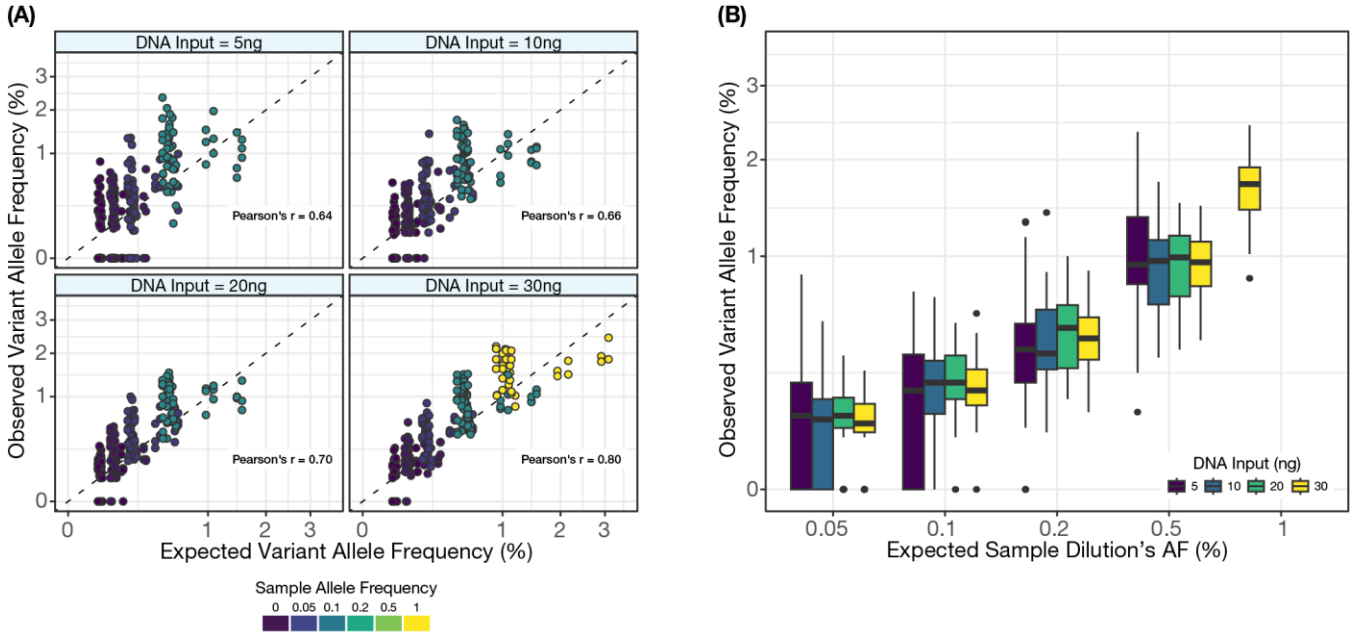

Figure 5. Correlation of observed variant allele frequency (VAF) compared to the expected dilution VAF across multiple DNA input amounts. (A) Each point represents a single variant in each sample/replicate, color coded by the allele frequency of the sample as shown in the figure's legend. Both axes are 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.

Independent external validation

The Pillar® oncoReveal® Essential LBx Panel demonstrated high concordance with the expected mutations in the SensID ESR1 reference standard, particularly at allele frequencies of ≥0.1% with DNA input levels of ≥10 ng (**Table 7**). All missed variant calls occurred exclusively in samples with very low AF (≤0.05%) and/or minimal input mass (10 ng), consistent with performance expectations and below the previously estimated LoD (**Table S2**).

Notably, the assay's estimated LoD was empirically confirmed at ~0.1% with 10 ng input, and even lower (approaching 0.05%) when 20 ng of DNA was used. Performance improved in a dose-dependent fashion with increasing input DNA and target AF – a pattern that mirrors internal titration data and reinforces the robustness of the assay across clinically relevant ranges.

These findings validate the internal performance claims of the assay and support its deployment in cfDNA contexts where mutation burden may be low.

Conclusions

In this study, we evaluated the performance of Pillar Biosciences' oncoReveal® Essential LBx Panel for detecting key mutations in the ESR1 ligand binding domain. The panel covers a broad range of clinically actionable mutations in multiple solid tumors and has a streamlined workflow, with same day loading of libraries. When tested on a dilution series of Seraseq® ctDNA ESR1 Mutation Mix AF 1%, the assay produces high yield libraries across the dilutions prepared in this study and shows excellent mapping rates, on-target rates, and amplicon uniformity. The panel shows >95% PPA in 0.1% samples and >85% PPA in 0.05% samples, while maintaining 100% NPA on the tested sites. When tested by an independent external laboratory, in a previously untested reference material, the results are replicable down to 10 ng input, further supporting the robustness of the panel. In conclusion, our study demonstrates that oncoReveal® Essential LBx Panel is a robust liquid biopsy assay for the investigation of SNVs and indels, particularly in ESR1.

Tubes	Input (ng)	% AF	# Reps	Expected	Detected	Missed	Additional	PPA	CI
TUBE 1	10	1.00	1	2	2	-	-	100%	[34.2-100%]
TUBE 2	10	1.00	1	2	2	-	1	100%	[34.2-100%]
TUBE 3	10	1.00	1	2	2	-	1	100%	[34.2-100%]
TUBE 4	10	1.00	1	3	3	-	3	100%	[43.8-100%]
TUBE 1+2	10	0.50	1	4	4	-	-	100%	[51-100%]
TUBE 3+4	10	0.50	1	5	5	-	1	100%	[56.6-100%]
TUBE 1+2+3+4	10	0.20	2	18	18	-	2	100%	[82.4-100%]
TUBE 1+2+3+4	10	0.10	3	27	25	2	1	92.6%	[76.6-97.9%]
TUBE 1+2+3+4	10	0.05	3	27	21	6	3	77.8%	[59.2-89.4%]
TUBE 1+2+3+4	20	0.20	2	18	18	-	3	100%	[82.4-100%]
TUBE 1+2+3+4	20	0.10	3	27	27	-	3	100%	[87.5-100%]
TUBE 1+2+3+4	20	0.05	3	27	25	2	2	92.6%	[76.6-97.9%]
Total				**162**	**152**	**10**	**20**	**93.8%**	**[88.9-96.6%]**

Table 7. Variant detection sensitivity by independent external validation. Observed Percent Positive Agreement (PPA) is listed for each mix tube, along with the tube's expected % allele fraction, DNA input amount, and number of replicates. Also provided is the breakdown of number of calls expected in each tube, detected, missed, and called additionally in the following four columns. Concordance improves with increased DNA input. Even at 10 ng, a high PPA of 92.6% was achieved at 0.1%.

References

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Table S1. Observed variant allele frequency (%) for each dilution's replicate at 30 ng sample input. Only one call is missed in 0.10% replicates, whereas 12 variants are missing for 0.05% (shown in **bold red** font).

Gene	Genomic change	Amino acid change	1.00%		0.50%			0.20%			0.10%			0.05%			Negative Sample
			Rep 1	Rep 2	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	
ESR1	c.1138G>C	E380Q	1.69	1.68	0.72	0.86	0.76	0.24	0.27	0.36	0.11	0.15	0.16	0.06	0.08	0.14	0
	c.1387T>C	S463P	1.48	1.24	0.49	0.63	0.8	0.25	0.15	0.21	0.12	0.14	0.19	0.07	**0**	0.12	0
	c.1603C>A	P535T	1.84	2.08	1.31	1.22	1.2	0.55	0.54	0.88	0.3	0.16	0.57	0.17	0.15	0.09	0
	c.1607_1608delinsAT	L536H	1.87	1.95	1.47	1.08	0.78	0.59	0.44	0.3	0.12	0.38	0.27	0.13	0.07	0.07	0
	c.1607T>A	L536H	1.92	1.78	0.91	0.9	1	0.65	0.44	0.58	0.13	0.16	0.17	0.15	0.14	0.26	0
	c.1607T>C	L536P	1.34	1.51	1.05	1.01	0.82	0.35	0.38	0.34	0.14	0.25	0.18	0.08	0.07	0.12	0
	c.1607T>G	L536R	1.83	1.82	0.97	1.3	1.01	0.42	0.47	0.39	0.37	0.2	0.21	0.13	0.1	0.06	0
	c.1607_1608delinsAG	L536Q	2.19	1.6	1.46	1.32	1.31	0.63	0.55	0.58	0.2	0.26	0.36	**0**	0.11	**0**	0
	c.1610_1611delinsCA	Y537S	1.74	1.68	1.1	1.01	0.96	0.45	0.52	0.59	0.21	0.28	0.15	0.08	0.14	0.24	0
	c.1609_1610delinsAG	Y537S	2.11	1.97	1.06	1.13	1.24	0.28	0.48	0.63	0.24	0.28	0.26	0.12	0.16	0.11	0
	c.1610A>C	Y537S	1.55	1.43	0.78	0.82	0.93	0.46	0.42	0.43	0.3	0.19	0.12	**0**	0.08	0.04	0
	c.1609T>A	Y537N	2.44	1.84	1.13	1.14	1.05	0.5	0.69	0.46	0.32	0.2	0.32	0.13	**0**	**0**	0
	c.1608_1609delinsTA	Y537N	1.84	2.07	0.78	1.48	1.23	0.67	0.52	0.39	0.33	0.18	0.13	**0**	0.16	0.17	0
	c.1610A>G	Y537C	1.8	1.47	1.46	1.04	0.76	0.6	0.65	0.43	0.45	0.25	0.07	0.1	0.17	0.16	0
	c.1609T>G	Y537D	2.1	1.53	0.9	1.39	1.17	0.37	0.38	0.72	0.29	0.12	0.1	0.11	0.26	**0**	0
	c.1613A>G	D538G	1.99	1.6	0.93	1.13	0.83	0.4	0.51	0.39	0.27	0.25	0.36	0.14	0.16	0.08	0
	c.1610_1615dup	D538_L539insHD	1.85	2.11	0.95	1.12	0.93	0.38	0.36	0.62	0.14	0.2	0.15	0.06	0.03	0.12	0
	c.1625A>G	E542G	1.01	0.98	0.61	0.63	0.52	0.4	0.27	0.25	0.08	0.11	0.15	0.07	0.07	0.06	0
PIK3CA	c.1624G>A	E542K	0.82	1.03	0.53	0.46	0.41	0.16	0.11	0.26	0.13	0.15	0.06	**0**	**0**	0.11	0
	c.1633G>A	E545K	1.13	1.38	0.46	0.45	0.55	0.19	0.16	0.19	0.13	**0**	0.1	0.07	0.07	0.09	0
	c.3140A>G	H1047R	1.03	1.02	0.44	0.6	0.41	0.16	0.38	0.16	0.21	0.08	0.12	**0**	0.05	**0**	0

Table S2. Summary of variant call success across all dilution and input combinations in the independent external validation. Each cell represents the detection status of a specific ESR1 mutation at a given condition. Missed calls are predominantly confined to the 0.05% AF group, confirming the LoD boundary.

Tube	Input (ng)	%TC	Replicates	TUBE 1 Variants		TUBE 2 Variants		TUBE 3 Variants		TUBE 4 Variants		
				c.1607T>A	c.1610A>G	c.1607T>C	c.1610A>C	c.1607T>G	c.1613A>G	c.1138G>C	c.1387T>C	c.1609T>A
				L536H	Y537C	L536P	Y537S	L536R	D538G	E380Q	S463P	Y537N
TUBE 1	10	0.01	1	1	1	-	-	-	-	-	-	-
TUBE 2	10	0.01	1	-	-	1	1	-	-	-	-	-
TUBE 1+2	10	0.005	1	1	1	1	1	-	-	-	-	-
TUBE 3	10	0.01	1	-	-	-	-	1	1	-	-	-
TUBE 4	10	0.01	1	-	-	-	-	-	-	1	1	1
TUBE 3+4	10	0.005	1	-	-	-	-	1	1	1	1	1
TUBE 1+2+3+4	10	0.002	2	2	2	2	2	2	2	2	2	2
TUBE 1+2+3+4	10	0.001	3	3	3	3	3	3	3	3	2	2
TUBE 1+2+3+4	10	0.0005	3	3	3	3	2	3	2	2	0	3
TUBE 1+2+3+4	20	0.002	2	2	2	2	2	2	2	2	2	2
TUBE 1+2+3+4	20	0.001	3	3	3	3	3	3	3	3	3	3
TUBE 1+2+3+4	20	0.0005	3	3	2	2	3	3	3	3	3	3