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Robust detection of Copy Number Amplifications using Pillar's oncoReveal™ Core LBx panel with low input from liquid biopsy samples

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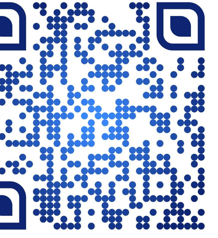

ABSTRACT

Introduction. Liquid biopsy (LBx) testing from cell free DNA (cfDNA) is the future of cancer diagnosis, therapy selection, and disease monitoring. In this setting, reliably calling mutations with limited input material and standard low-mid throughput NGS platforms remains a significant challenge. Among all mutations, Copy Number Amplifications (CNA) are the most difficult to detect, typically requiring large amounts of circulating tumor DNA (ctDNA) and high tumor content. This is especially challenging in amplicon-based panels where only a subset of the genome is covered. Here we report oncoCNA, a new, highly sensitive, specific, and interpretable CNA variant caller in Pillar's PiVAT® (**Pi**llar Biosciences **V**ariant **A**nalysis **T**oolkit). oncoCNA utilizes Bayesian non-parametric methods for accurate detection of CNAs in samples with low input amount cfDNA. Using this approach, we demonstrate accurate quantification of copy number (CN) in multiple genes with only 10ng of cfDNA at copy number ratios as low as 1.1.

Methods. Seracare® ctDNA Complete™ Mutation Mix (CMM) reference material with AF0.5%, AF1%, AF2.5%, and AF5% were selected for this experiment. AF0.5% sample was diluted to create AF0.25% using Seraseq® ctDNA Complete™ Mutation Mix WT as background. Expected CNAs for three genes: ERBB2, MET, and MYC, were obtained from vendor's ddPCR reported results. To measure the effect of choice of CN normal sample, Anchor Molecular normal reference of gDNA (AM), Seracare® Wild Type (SCCWT), and/or humaAM, HCN, as well as CN normal Seracare® ctDNA Mutation Mix v2. Sequencing libraries were prepared with three DNA inputs: 10ng, 20ng, and 30ng, on Pillar's 104-gene oncoReveal™ Core LBx research-use-only (RUO) panel and sequenced on Illumina's NextSeq™ machine. Analysis was performed with oncoCNA, as part of Pillar's PiVAT® pipeline.

Results. PiVAT's oncoCNA achieved 100% sensitivity and 100n clinical normal cfDNA (HCN) were used as negative controls. Specificity was assessed using % specificity in AF1% and higher samples (copy number ratio ≥1.35-1.44), and 75% sensitivity and 99% specificity at AF0.5% sample (copy number ratio 1.1-1.5). No CNs were detected at AF0.25%. Performance was comparable between 10ng and 30ng input. Regardless of the choice of normal, oncoCNA reported CNs in the 1.3-1.4 copy ratio. However, when an ensemble of normals was used, we were able to push our detection limit to 1.1. Batch-to-batch effect had no impact on CN calling, as measured by using out-of-batch normals.

Conclusion. CNA calling is challenging for focused panels with low DNA input and low CNs. oncoCNA addresses this challenge by accurately and consistently calling CNs with as little as 10ng cfDNA and 1.2 copy number ratio, enabling rapid and cost-effective on-site LBx NGS testing at any pathology lab.

EXPERIMENT DESIGN

- **Panel gene coverage for CNA:** EGFR, ERBB2, FGFR1, FGFR2, FGFR3, KIT, MAP2K1, MET, PDGFRA, PIK3CA
- Amplicons are designed to be as small as possible to facilitate detection of tumor ctDNA, which is predicted to average around 145bp in length based on the size of DNA fragment protected by the nucleosome.
- Amplicons range from 54 to 129 bp in length, avg 79.2, med 71.

- **Positive sample:**
  - SeraCare's Seraseq® ctDNA Complete™ Mutation Mix v1 AFs 0.125 to 5%
  - Number of CN calls per sample = 2 out of 11 designed
  - Approximate CN ratio range from 1.05 to 3

- **Targeted LoD:** 1.4 CN ratio (2.8 CNA) at 30ng and 1.8 CN ratio (3.6 CNA) at 10ng

- **CN Negative samples:** SeraCare's Seraseq® ctDNA Mutation Mix v2

- **Negative control samples (run in- and out-of-batch):**
  - Synthetic normal: Anchor Molecular Reference
  - Synthetic normal: SeraCare's Seraseq™ ctDNA Complete™ Reference Material WT
  - Clinical normal: Human cfDNA normal sample

EXPERIMENT DESIGN

Table 1. Breakdown of various allele frequency samples for Seraseq® ctDNA Complete™ Mutation Mix v1 tested in this study. Sample breakdown is shown for the two DNA inputs tested as part of this study. Manufacturer's listed sample allele frequency is provided and each gene's ddPCR screened copy number ratio is listed. Sample with allele frequency within the targeted limit of detection (LoD) range is shaded in light blue. Additionally, copy number neutral samples were also tested.

	Seraseq® ctDNA Complete™ Mutation Mix v1 AFs					CNA Positives	CNA Negatives
CN ratio	~1.05-1.08	~1.1-1.25	~1.3-1.4	~1.8-2.3	~3.05-3.8		
DNA input	0.25	0.5	1	2.5	5		
10ng	1	4	4	3	1	13	5
30ng	2	1	1	1	1	6	6
Total	3	5	5	5	2	21	11

RESULTS

Schematic of PiVAT's Algorithm

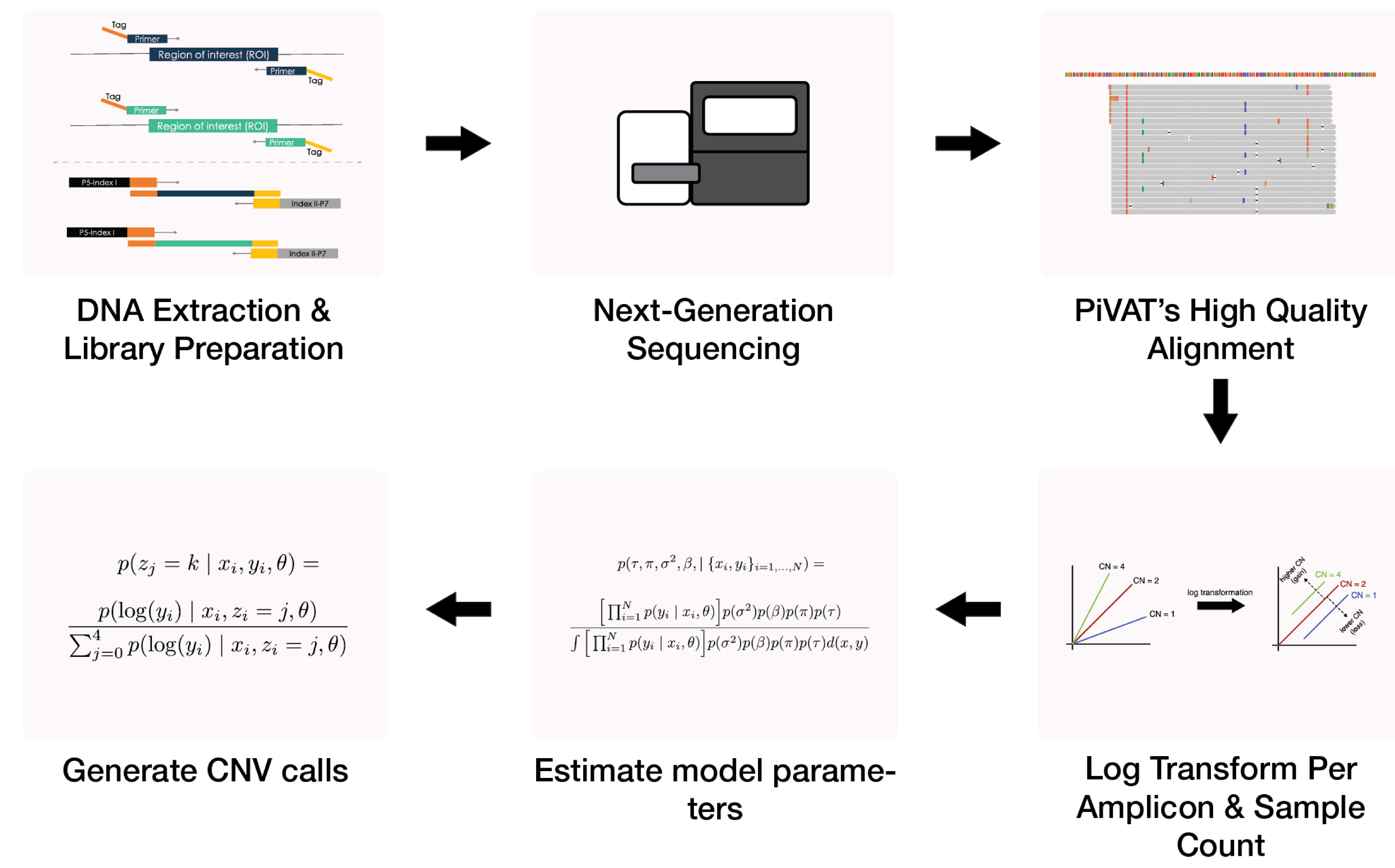

Figure 3. Schematic representation of PiVAT's copy number amplification calling algorithm. The data ingested by PiVAT LBx Copy Number Amplification (CNA) algorithm originates from a targeted sequencing, cfDNA panel. The data undergoes several quality control, alignment, and refinement steps to ensure high quality data is passed to downstream processes. The input to CNA calling algorithm are per amplicon and per sample coverages. As with most targeted sequencing data, a natural variation in the sequencing coverage exists, that is not necessarily reflect the true copy number of the genomic fragment sequenced. Consequently, a CN-free negative control sample is required to normalize the counts. The normalized counts are processed through an unsupervised clustering model to detect CNAs and assign each gene to a specific copy number value.

Sequencing Summary

Table 2. Summary of key sequencing metrics. Each sample was sequenced to an average depth of 43M sequencing reads.

Panel Statistics	Average ± S.E.
Overall Q30	92.6% ± 0.09%
Overall Q20	95.0% ± 0.06%
Mapping rate	99.6% ± 0.01%
On-target rate	96.4% ± 0.04%
Coverage uniformity	95.6% ± 0.29%

RESULTS

Performance of PiVAT with Different Normal Backgrounds

Input (ng)		Seraseq® ctDNA Complete™ Mutation Mix v1 AFs:				
	CN ratio (approx)	1.1-1.14	1.2-1.28	1.3-1.4	1.8-2.3	3.05-3.8
	Selected Normal	0.25	0.5	1	2.5	5
Positive Percent Agreement (PPA)						
10	AM	0.00%	25.00%	62.50%	100.00%	100.00%
10	SCC WT	50.00%	100.00%	100.00%	100.00%	100.00%
10	Clinical normal	0.00%	87.50%	62.50%	100.00%	100.00%
30	AM	0.00%	0.00%	50.00%	100.00%	100.00%
30	SCC WT	0.00%	100.00%	100.00%	100.00%	100.00%
30	Clinical normal	0.00%	50.00%	100.00%	100.00%	100.00%
Negative Percent Agreement (NPA)						
10	AM	89.50%	91.90%	93.50%	92.60%	89.50%
10	SCC WT	93.10%	91.50%	91.50%	93.20%	93.10%
10	Clinical normal	100.00%	100.00%	100.00%	100.00%	100.00%
30	AM	100.00%	100.00%	100.00%	100.00%	100.00%
30	SCC WT	93.90%	93.10%	93.10%	93.10%	93.10%
30	Clinical normal	100.00%	100.00%	100.00%	100.00%	100.00%

Table 3. Performance metrics summary from PiVAT CNA algorithm when run on positive and negative reference samples. The table summarizes the positive percent agreement (PPA) and negative percent agreement (NPA) for the positive and normal standard reference samples tested in this study. The performance is broken down by DNA input tested and the negative control sample chosen. Three different negative control sample types were tested: synthetic normal samples from Anchor Molecular (AM), synthetic wild-type sample from SeraCare (SCC WT), and clinical normal sample from blood draws. Negative controls were run in a combination of in- and out-of-batch manner.

Impact of Choice of Negative Control Sample

In our testing, we observed that synthetic normal samples consistently had relatively lower performance compared with clinical normal samples. With clinical normal samples selected as negative control, we were able to lower our LoD.

Table 4. False positives generated per sample across the normal samples. All the normal samples were processed using different negative control samples. False positive calls detected in each of normal sample was recorded. When Seracare WT sample is used as a negative control, we identify a much higher number of false positive calls with the same samples, than compared to other negative control samples.

Selected Normal	Samples Tested	FPs	FP / sample
AM	4	4	1
SCC WT	5	9	1.8
Clinical normal	2	0	0
Total	**11**	**13**	**1.2**

CONCLUSIONS

- Pillar's robust SLIMamp® multiplex amplicon-based chemistry produces consistent within sample-type amplicon coverage, enabling out-of-batch normal samples
- Regardless of the choice of the negative control sample, PiVAT™ can accurately detect CNs, with high sensitivity and specificity across the positive and negative reference samples tested in this study
- The best calling performance was observed when clinical normal samples were used as negative control, enabling lower LoD CNA calling
- Larger studies would be needed to establish the clinical utility of this approach