An efficient and ultrasensitive next-generation sequencing solution for profiling circulating tumor DNA

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Summary

- We present an ultrasensitive targeted amplicon-based NGS workflow suitable for profiling low-level ctDNA variants through molecular barcode-assisted error correction.
- Pre- and post-analytical QC measures of normal and cancer plasma cfDNA and reference materials were compared.
- Analysis of 63 early and late-stage cancers with matched tissue and plasma using the NGS workflow and ddPCR revealed inter-specimen and inter-assay concordance.

Introduction

Next-generation sequencing (NGS) of liquid biopsies offers a minimally invasive alternative to solid tissue biopsies and a more holistic profile of intra- and inter-tumoral heterogeneity for therapy selection and disease monitoring. However, NGS analysis of circulating tumor DNA (ctDNA) is highly centralized owing to cumbersome workflows, complex assays, and a lack of high-performance kitted solutions. We present a sensitive, scalable, and efficient targeted amplicon sequencing workflow and bioinformatics pipeline for ctDNA analysis with the potential for use across laboratories.

Materials and Methods

DNA was isolated from both FFPE and plasma using optimized procedures. DNA QC was performed using a novel LINE-1 qPCR assay that quantifies representative amplifiable DNA copies. NGS libraries were prepared by LBx-Seq, a prototype multi-stage PCR workflow that uniquely barcodes each PCR extension product, and sequenced on a MiSeq® System (Illumina). NGS data were analyzed with a custom bioinformatics pipeline that utilizes statistical models to correct for polymerase and sequencing errors. Variant calls were verified by Droplet Digital™ PCR (ddPCR™) technology on a QX200™ Droplet Digital™ PCR System (BioRad).

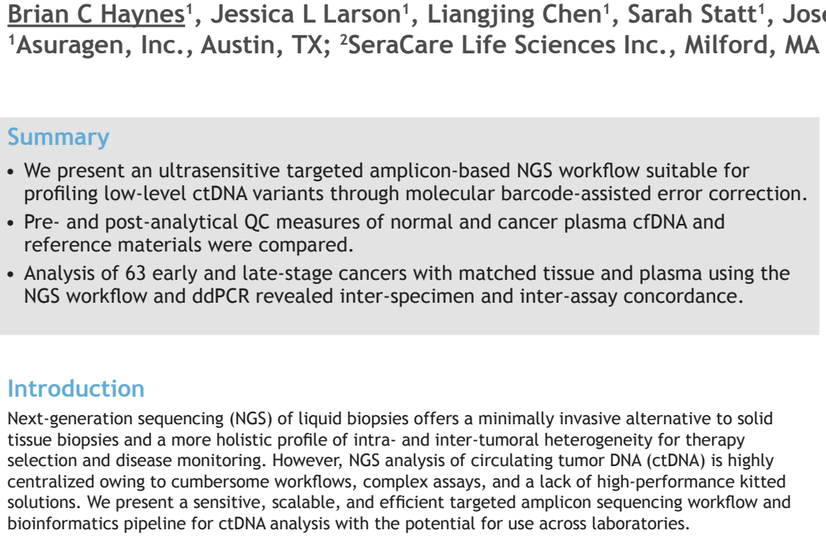

Figure 1. Summary of Study Design. Cell-free DNA standards from Horizon and SeraCare, plasma from 15 normal healthy controls and 63 cancer Subjects were evaluated using the LBx-Seq workflow. FFPE tissues from cancer subjects were profiled by the QuantideX® NGS DNA Hotspot 21 panel. Pre-analytical QC was performed with LINE-1 qPCR assays and Qubit. Plasma mutation calls were confirmed by ddPCR.

†This product is under development. Future availability and performance cannot be ensured.

‡Research Use Only. Not for use in diagnostic procedures.

‡Proof-of-concept data only. Future availability and performance cannot be ensured.

Presented at AMP2017 - ST115

Results

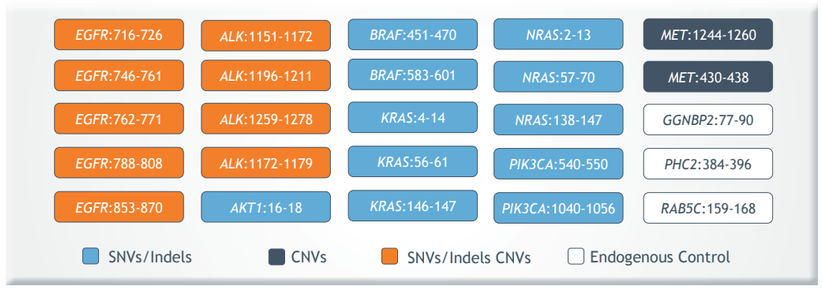

Figure 2. The Prototype LBx-Seq NGS Panel Content. Design covers over 500 DNA mutation hotspots with additional endogenous normalizer amplicons that may be used for CNV detection. Amplicons are 83 to 129 nt in length.

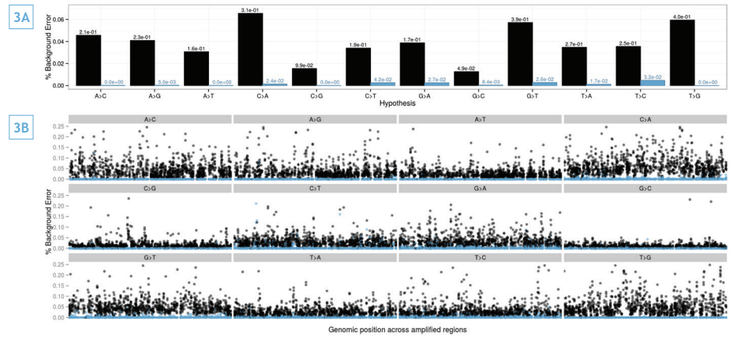

Figure 3. Quantification of Sequencing Background Errors Across 15 Normal Healthy Plasma Donors by Conventional NGS (black) and the LBx-Seq Workflow (blue). A) Barcode-assisted error correction resulted in a 40-fold average reduction in average residual error (y-axis) but varied from 8-fold (T>C) to 3,300-fold (T>G). Residual 99th percentile error by hypothesis shown above bars. **B)** Relative to conventional NGS, LBx-Seq barcode-assisted error correction reduced residual error across all hypotheses for nearly every locus.

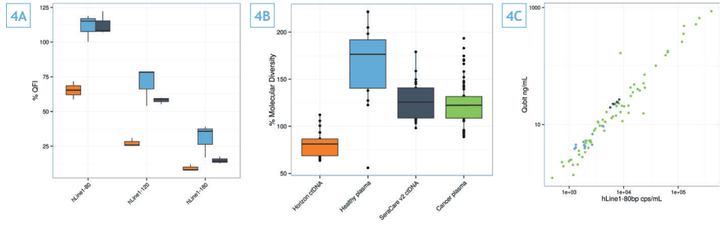

Figure 4. Comparison of Pre- and Post-Analytical QC Results for the Horizon Multiplex I cfDNA Reference Standard Set (orange), Normal Healthy Plasma (blue), Seraseq ctDNA v2 Reference Materials (dark gray), and Cancer Plasma (green). A) Pre-analytical QC analysis using the QFI™ metric (Quantitative Functional Index: genome equivalent copies relative to mass estimated copies by Qubit at 330 cp/ng) with LINE-1 qPCR 80, 120, and 180 bp assays. **B)** Post-sequencing molecular diversity: barcoded molecules relative to mass input by Qubit (330 cps/ng) **C)** Relationship between Qubit mass and LINE-1 80 bp qPCR cps per plasma mL equivalent, with Seraseq ctDNA v2 full process controls shown in dark gray.

	# Libraries	TP	FN	FP	Sn	PPV
Horizon cfDNA Standards ≥ 0.5%	10 (7)	75 (53)	5 (3)	3	93.75%	96.15%
Seraseq ctDNA v2 Mutation Mix ≥ 0.5%	6 (2)	56 (18)	4 (2)	1	93.33%	98.25%
Seraseq ctDNA v2 Reference Material ≥ 0.5%	3 (1)	29 (9)	1 (1)	2	96.67%	93.55%
Overall ≥ 0.5% VAF	19 (10)	160 (80)	10 (7)	6	94.12%	96.39%

Table 1. Analysis of SNV and INDEL Sensitivity and PPV on Horizon ctDNA and Seraseq ctDNA v2 Reference Standards. Libraries were prepared from 15 ng of input at 2% down to 0.5% variant allele frequencies. Libraries and variants at the 0.5% admixture are indicated in parentheses.

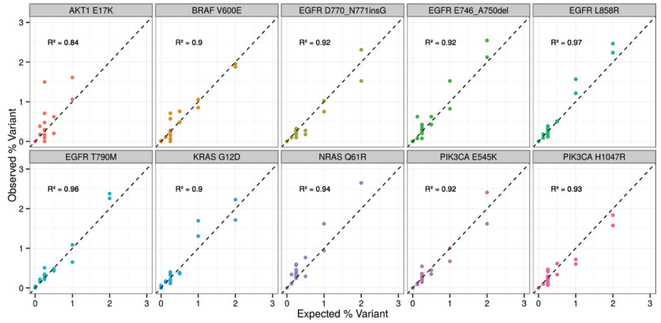

Figure 5. Analytical Concordance with Expected Variant Allele Frequencies from 0%, 0.125%, 0.25%, 0.5%, 1%, and 2% for Seraseq ctDNA v2 Reference Materials. Libraries for ≥0.5% VAF were prepared with 15 ng input, whereas <0.5% were prepared with 30 ng.

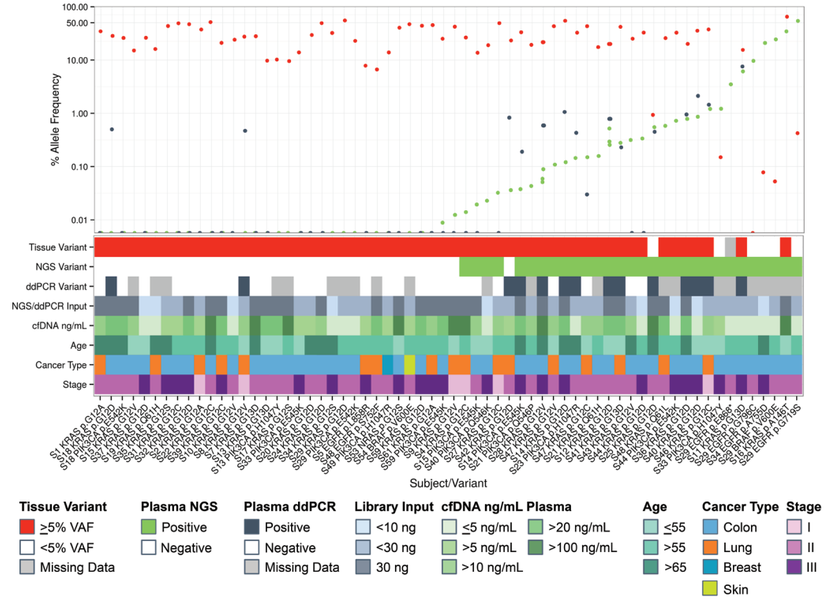

Figure 6. LBx-Seq Plasma NGS (green) Concordance with Tissue (red) and Plasma ddPCR (dark grey) for 63 Colon, Lung, Breast, and Skin Cancer Subjects. 61 tissue variants were detected in 47 subjects. Of the 61 tissue mutations, 27 were detected by plasma NGS, representing 18 subjects. Mutations were detected in an additional 4 subjects by plasma NGS that were present at trace or non-detectable levels in tissue. A total of 15/21 mutations that were positive by NGS were also positive by ddPCR (positive calls determined by three or more positive droplets in the alternate allele channel). By contrast, 21/24 mutations that were negative by NGS were also negative by ddPCR. All variants that were discordant between ddPCR and NGS were detected in the tissue.

Conclusions

- We describe a highly sensitive NGS workflow capable of detecting ctDNA variants in early- and late-stage cancers.
- Our approach overcomes the sensitivity and specificity challenges of detecting low-level variants down to 0.5% VAF from plasma DNA while maintaining a streamlined workflow free of ligation, hybridization capture, or specialized instrumentation.
- The modularity of our workflow can spur the rapid development of new panel designs in support of clinical trial research and precision diagnostics and help hasten the uptake and decentralized testing of liquid biopsies.

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