Abstract #432

Novel method for NGS analysis of actionable mutations in circulating tumor DNA specimens: Improved quality control and 20-fold lower sequencing required

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on Behalf of the Onco-panel Working Group of the Sequencing Quality Control Phase 2 (SEQC2) Consortium
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Background

Identification of actionable mutations in circulating tumor DNA (ctDNA) enables gene-targeted therapy of solid tumors based on a simple blood test. NGS methods that attach a unique molecular identifier (UMI) to each DNA molecule control for sequencing technical error and thereby reduce variant allele fraction (VAF) limit of detection (LOD) to <0.01% at a cost of 10-20-fold higher sequencing requirement. Importantly, the VAF lower limit of detection (LOD) for analysis of ctDNA specimens does not typically extend below 0.5% due to limits of ctDNA specimen quantity. We previously reported that synthetic internal standard (IS) spike-ins control for site specific technical error to as low as 0.05% while requiring 20-fold less sequencing space.

Purpose/Hypothesis

Given the high cost and limited value of UMI NGS when ctDNA specimen size restricted VAF LOD to 0.5%, we tested the hypothesis that synthetic internal standard (IS) spike-in molecules provide reliable alternative quality control for site specific sequencing error while eliminating UMI-imposed sequencing burden in analysis of ctDNA reference samples.

Method Overview

ctDNA reference material: SEQC2 WG2 reference DNA with multiple actionable mutations at known VAFs was diluted into Sample B normal DNA 2-fold (Sample C), 5-fold (Sample D), and 25-fold (Sample E), then enzymatically degraded to a modal fragment size of 150 bp.

Synthetic Internal standard (IS) spike-ins: IS and PCR primers for 18 actionable mutations were synthesized at Accugenomics, Inc. (ACG). IS were synthesized with dinucleotide variants every 50 bp to distinguish from native sequence, cloned into pUC vectors, confirmed to be wild-type sequence except at dinucleotides, linearized, quantified, and combined at a known genome copy mixture. They were then enzymatically degraded to a modal fragment size of 150 bp.

Test Samples: Enzymatically fragmented SEQC2 Samples C, D, and E were combined with fractionated ACG IS, creating samples CfAG, DfAG, and EfAG.

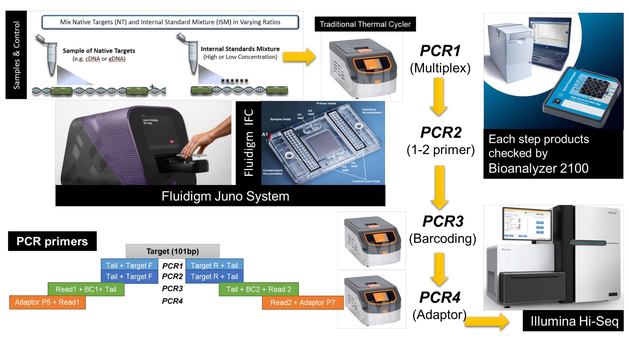
Experimental Design: Assess inter-replicate reproducibility of VAF measurement in 25 ng (7,250 genome copies) of Samples CfAG, DfAG, and EfAG.

Variant Call Relative to IS-Spike In

- IS mixture added to ctDNA reference material at 1:1 genome copy prior to library prep.
- Separation of target from IS reads into different bins using custom splitter.
- Pipeline analysis on Qiagen CLC Genomics Workbench.
- Variant calls
  - IS variants assumed to be technical error.
  - Contingency tables used to identify significant VAF in sample compared to IS

See also Poster 3537/23-Craig et al, Targeted deep sequencing of driver mutations in airway epithelial cells from smokers High-throughput Sequencing April 2, 8:00 AM - 12:00 PM Section 36

Multiplex PCR Amplicon Library Prep for NGS



Results

Accugenomics Liquid Biopsy Lung SNAQ® Panel Calls in SEQC2 Samples CfAG, DfAG, and EfAG

Variant ID	Sample A VAF (ACG1 in Yellow)	Sample B VAF (ACG1 in Yellow)	Sample CfAG SEQC2/ACG1 Expected VAF	Sample CfAG ACG2 Measured VAF	Sample DfAG SEQC2/ACG1 Expected VAF	Sample DfAG ACG2 Expected VAF	Sample DfAG ACG2 Measured VAF	Sample EfAG SEQC2 Expected VAF	Sample EfAG ACG2 Expected VAF	Sample EfAG ACG2 Measured VAF	SEQC2 Variant Call	ACG1 Variant Call
EGFR_19_chr7:55242482_SNV_C>T	0.0093	0.0000	0.0047	0.0062	0.0019	0.0025	0.0022	0.0004	0.0005	0.0000	FP	TP
EGFR_20_chr7:55249063_SNV_G>A	0.6718	0.4891	0.5805	0.5791	0.5257	0.5366	0.5558	0.4964	0.5279	0.5255	TP	TP
ERBB2_chr17:37880987_SNV_C>T	0.0298	0.0000	0.0149	0.0159	0.0060	0.0064	0.0026	0.0012	0.0013	0.0000	TP	TP
MAP2K1_3_chr15:66729162_SNV_C>T	0.0351	0.0000	0.0176	0.0235	0.0070	0.0094	0.0047	0.0014	0.0019	0.0000	FP	TP
NOTCH1_26_chr9:139399344_SNV_A>G	0.0797	0.0000	0.0398	0.0430	0.0159	0.0172	0.0176	0.0032	0.0034	0.0051	TP	TP
NRAS_2_chr1:115258748_SNV_C>A	0.0428	0.0000	0.0214	0.0170	0.0086	0.0068	0.0128	0.0017	0.0014	0.0037	TP	TP
NRAS_3_chr1:115256529_SNV_T>A	0.0471	0.0000	0.0236	0.0146	0.0094	0.0058	0.0147	0.0019	0.0012	0.0000	TP	TP
PDGFRA_12_chr4:55141055_SNV_A>G	0.9971	0.9978	0.9974	0.9760	0.9976	0.9976	0.9724	0.9977	0.9976	0.9749	TP	TP
PIK3CA_10_chr3:178936091_SNV_G>A	0.0463	0.0000	0.0232	0.0279	0.0093	0.0112	0.0136	0.0019	0.0022	0.0031	FP	TP
TP53_7_chr17:7577085_SNV_C>T	0.0287	0.0000	0.0144	0.0039	0.0057	0.0016	0.0080	0.0011	0.0003	0.0000	TP	TP
TP53_7_chr17:7577118_SNV_C>A	10.4800	0.0000	0.0520	0.0449	0.0208	0.0180	0.0168	0.0042	0.0036	0.0000	FP	TP
CALR_chr19:13054678_SNV_A>G	N/A	N/A	N/A	0.0059	N/A	N/A	0.0000	N/A	N/A	0.0000	FP	FP
CALR_chr19:13054664_SNV_T>G	N/A	N/A	N/A	0.0052	N/A	N/A	0.0000	N/A	N/A	0.0000	FP	FP

Table 1. ACG SNAQ® LB Lung Panel. Eighteen actionable mutation targets assessed. Sample A (cancer cell line mixture, Agilent) was diluted into Sample B (normal). **All seven ACG1 TP somatic variants titrated appropriately.** Samples had same genotype at germ-line variants (EGFR20:55249063, PDGFRA:55141055), thus no titration. Four variants FP by SEQC2 validated TP through ACG1 titration experiment. Two FP variants were likely not called by ACG1 or SEQC2 due to low VAF, further studies pending. (Measured VAF Values: VAF >2.5%; VAF 0.5-2.5%, VAF 0.1-0.5%

Using ACG1 TP List			
	>2.5%	0.5%-2.5%	0.1%-0.5%
	N=8	N=14	N=10
Sensitivity	100%	100%	80%
Specificity	99.90%	99.90%	99.90%

Table 2. Summary of ACG SNAQ LB Lung panel performance.

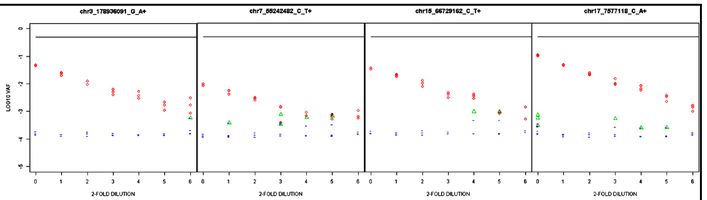


Figure 1. ACG1 serial 2-fold titration validation (Sample A into Sample B) of TP variants discordant with SEQC2.Green triangles: IS variant VAF. Blue dashes:1/seqdepth.

Summary of ACG SNAQ® Lung Panel Performance in Multiplex Amplicon Libraries for NGS

- Sensitivity to Detect SEQC2 Liquid Biopsy Reference material variants (SEQC2 or ACG1 true positives)
  - >2.5% VAF range: 100%
  - 0.5%-2.5% VAF range: 100%
  - 0.1-0.5% range: 80%
- Reproducibility across replicate library preparations and sites
  - In progress

- IS reliably identified base-substitution sequencing errors at each measured actionable mutation site.
  - Each IS variant (i.e., sequencing error) VAF <0.2%.
- Reduced Sequencing Reads for each sample/target
  - **SNAQ: 20,000 reads sufficient** for 0.5%-2.5% VAF range.
    - 10,000 reads for specimen, 10,000 reads for spike-in.
  - **Typical UMI method requires >200,000 reads** per target in each sample.

Conclusion

Preliminary data indicate that IS spike-in mixtures enable reliable analysis of ctDNA mutation fraction to LOD of 0.5% without need for UMI. This should enable 10-20 reduction in coast of liquid biopsy testing for actionable variants. **See also Poster 3537/23-Craig et al, Targeted deep sequencing of driver mutations in airway epithelial cells from smokers High-throughput Sequencing April 2, 8:00 AM - 12:00 PM Section 36**

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