New Technology to Generate Commutable and Comprehensive Circulating Tumor DNA (ctDNA) Reference Materials for NGS

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INTRODUCTION

The need for commutable and comprehensive ctDNA reference materials is evident from the increasing number of liquid biopsy diagnostics and comprehensive panels on the market that are accompanied by reports of discordant results. Assay development, validation, verification, and QC testing benefit from consistent ctDNA reference materials. Commutable, patient-like materials formulated with single variants are required for diagnostics while comprehensive panels must be validated with complex, multianalyte reference materials containing mutations, translocations, and copy number variation (CNV). In addition to the Seraseq® ctDNA Complete™, Seraseq ctDNA EGFR Panel, Seraseq ctDNA EGFR T790M, and Seraseq ctDNA EGFR ex19del reference materials, we also have developed a custom VariantFlex™ library making highly customizable materials for any ctDNA profiling workflow possible.

MATERIALS AND METHODS

- Biosynthetic DNA was generated for each variant of interest (Table 1). Sequences containing portions of various genes with SNVs or INDELs were constructed. Translocations were simulated by combining sequences from two genes. Larger gene segments were used to replicate CNVs.
- The biosynthetic DNA was precisely mixed with genomic DNA from a well-characterized cell line (GM24385) targeting various allele frequencies (AF) for somatic mutations and translocations and distinct copy numbers for each gene amplification.
- The AF of each mutation and the CNV of each amplified gene was quantified by digital PCR.
- The mixed DNA was fragmented and novel purification and processing steps were used to mimic the size distribution of native ctDNA, which was confirmed by Agilent Bioanalyzer® analysis. Wild-type GM24385 was fragmented similarly and subsequently not carried through the novel workflow for comparison.
- The manufactured ctDNA was encapsulated and diluted in synthetic plasma to provide stability and allow it to be processed like a typical patient plasma sample.
- Extraction of ctDNA from the encapsulated plasma-like matrix was carried out using the Qiagen QIAamp® Circulating Nucleic Acid kit, and variant AF and CNVs were verified by digital PCR.
- 50 ng of extracted ctDNA was analyzed by next-generation sequencing (NGS) using the Archer® Reveal ctDNA™ 28 Kit and sequencing on an Illumina MiSeq® using v2 (2x150 bp) PE chemistry reagents. Multiplexing was performed to achieve 4-5 million reads per sample.
- Archer Analysis (version 5.1.7) was used for data analysis using default settings with error correction turned on.

RESULTS & DISCUSSION

VARIANTS INCLUDED

Gene ID	COSMIC Identifier	HGVS	Amino Acid Change	Variant Type
AKT1	33765	c.49 G>A	p.E17K	SNV
ALK	28055	c.3522 C>A	p.F1174L	SNV
ALK	144250	c.3604 G>A	p.G1202R	SNV
BRAF	476	c.1799 T>A	p.V600E	SNV
BRCA1	1383519	c.1961 delA	p.K654fs*47	Deletion
BRCA2	1738242	c.7934 delG	p.R2645fs*3	Deletion
EGFR	6240	c.2369 C>T	p.T790M	SNV
EGFR	6223	c.2235_2249 del	p.E746_A750delELREA	Deletion
EGFR	6224	c.2573 T>G	p.L858R	SNV
EGFR	6256	c.2254_2277 del	p.S752_I759delSPKANKEI	Deletion
EGFR	12370	c.2240_2257 del	p.L747_P753>S	Deletion
ERBB2	682/20959	c.2324_2325 ins	p.A775_G776insYVMA	Insertion
KIT	1314	c.2447 A>T	p.D816V	SNV
KRAS	516	c.34 G>T	p.G12C	SNV
KRAS	521	c.35 G>A	p.G12D	SNV
KRAS	554	c.183 A>C	p.Q61H	SNV
NRAS	584	c.182A>G	p.Q61R	SNV
PIK3CA	775	c.3140 A>G	p.H1047R	SNV
PIK3CA	12646	c.3204_3205insA	p.N1068fs*4	Insertion

Translocations	Gene Amplifications
CD74-ROS1	ERBB2
EML4-ALKv1	MET
NCOA4/RET	MYC

Table 1: Variants in the Seraseq ctDNA Complete reference materials with associated COSMIC ID, HGVS nomenclature, amino acid change, and variant type.

SIZE DISTRIBUTION

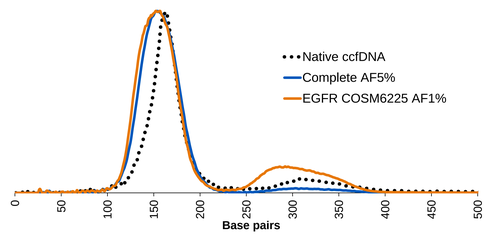

Figure 1: Size profiles of purified Seraseq ctDNA Complete AF5% (blue) and Seraseq ctDNA EGFR ex19del AF1% (orange) reference materials compared to circulating cell free DNA (ccfDNA) (black).

LIBRARY COMPLEXITY

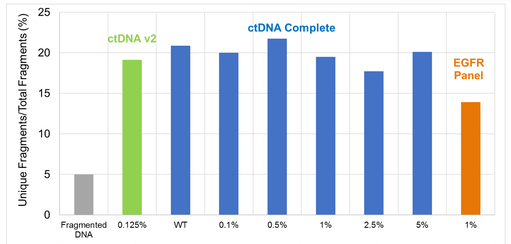

Figure 2: The percentage of unique versus total fragments from 50 ng of DNA input in NGS libraries made from Seraseq ctDNA materials improves with novel manufacturing processes compared to unprocessed fragmented GM24385 WT gDNA.

AVERAGE AF% MEASURED BY dPCR

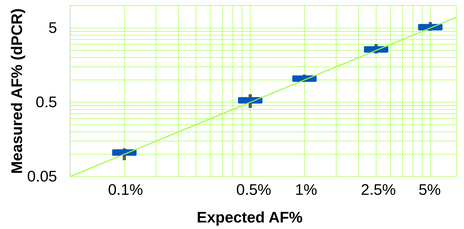

Figure 3: The average AF% for the Seraseq ctDNA Complete series of materials as measured by dPCR are shown in blue (4-7 replicates). The range of AF% measured across all 24 variants is shown by vertical black bars.

OBSERVED NGS VS. dPCR AF%

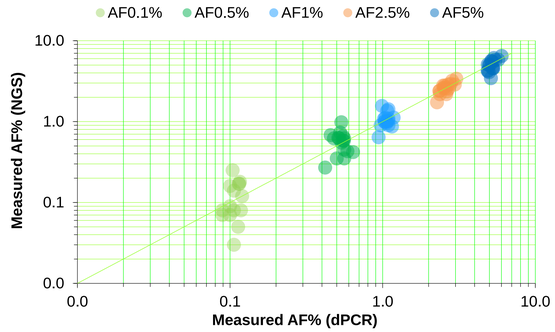

Figure 4: Comparison of variant AF% measured by NGS versus AF% measured by dPCR for the Seraseq ctDNA Complete reference materials (log scale), showing concordance between both platforms.

OBSERVED NGS VS. dPCR AF%

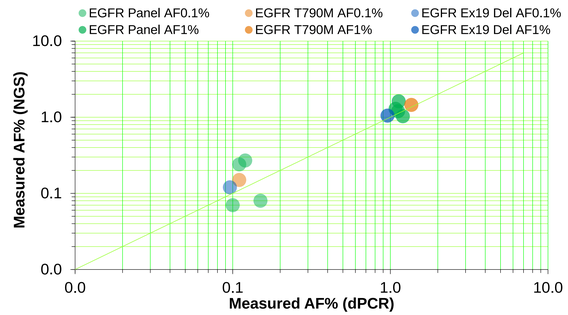

Figure 5: Comparison of variant AF% measured by NGS versus AF% measured by dPCR for the Seraseq EGFR Panel, Seraseq ctDNA EGFR T790M, and Seraseq ctDNA EGFR ex19del reference materials (log scale).

CNVs MULTIPLEXED WITH SNVs AND INDELS

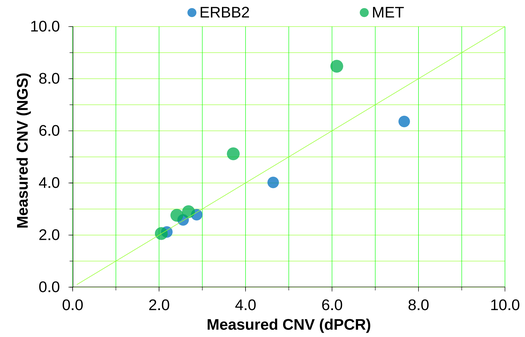

Figure 5: : The average number of total copies per cell (CNV) as measured by dPCR compared to the CNV as measured by NGS in the Seraseq ctDNA Complete series of reference materials.

CONCLUSIONS

- Greater than 70% of the synthetic ctDNA fragments were between 100 and 225 bp, exhibiting a profile more like native ccfDNA than sheared gDNA.
- Our novel approach to creating ctDNA reference materials improves unique fragments in NGS libraries by 3- to 5-fold for a given mass of input compared to sheared gDNA.
- The average AF of each variant in the Seraseq ctDNA Complete materials measured by NGS was within 24% (percent difference) of the AF measured by dPCR for all mixes targeted above 1%. More variability was seen when approaching the lower limit of detection. A similar pattern was seen with the EGFR panel and individual EGFR variant mixes, indicating broad assay compatibility.
- ERBB2 CNVs were detected by NGS within 17% (percent difference) of CNVs detected by dPCR in all mixes. Incorporation of the ERBB2 CNV was challenging due to the ERBB2 insertion variant also present in the mix. The amount of the insertion fragment was optimized after addition of the full length ERBB2 gene sequence.
- MET CNVs were more variable between dPCR and NGS (up to 39% difference), perhaps due to the method used to increase the representation of this larger gene.
- Both single variant-containing reference materials and complex multianalyte Seraseq controls are moving towards increased commutability compared to materials composed only of fragmented gDNA. They also perform consistently across several platforms, highlighting the flexibility and value of SeraCare technology.

ACKNOWLEDGEMENTS

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