Seraseq™ Circulating Tumor DNA-I Reference Materials for characterizing, developing and validating plasma-based assays

Using both digital PCR and next-generation sequencing based assays, a demonstration of allele frequency linearity and reliable NGS-based assay performance

There is great interest in developing assays to detect circulating tumor DNA (ctDNA) 'driver mutations' for personalized medicine in oncology, whether for early detection, prognosis or monitoring of disease progression or relapse after treatment. This interest is driven by its potential to supplement or perhaps replace invasive biopsy procedures as a diagnostic or screening tool, described as an 'important paradigm shift in precision medicine.'[1] One challenge has been the typically low amounts of circulating DNA (in healthy individuals on the order of 1-10 ng per mL, but reported as high as >1000 ng per mL).[2] In addition, ctDNA is understood to not be associated with exosomes or other particles, but rather associated with histones[1] and on the order of 170 base pairs in length.

SeraCare has developed a set of commutable process reference materials from biosynthetic constructs of actionable mutations that include single-nucleotide variants, and insertion and deletion mutations across commonly mutated cancer genes such as BRAF, EGFR, and NRAS.

A collection of nine mutations in Table 1 were synthetically created and formulated to equimolar concentrations then spiked into GM24385 genomic DNA which was chosen as the wild-type background (Corielle Institute).

Table 1: *List of gene names, specific mutations and their mutation types in the Seraseq Circulating Tumor DNA-I Reference Materials*

Gene	Mutation	Type of Variant
BRAF	V600E	SNV
EGFR	T790M	SNV
EGFR	p.D770_N771insG	INDEL
EGFR	p.E746_A750delELREA	INDEL
PIK3CA	p.H1047R	SNV
PIK3CA	p.N1068fs*4	INDEL
NRAS	p.Q61R	SNV
KRAS	G12D	SNV
KIT	D816V	SNV

The blended material is then fragmented to approximately 170 base-pairs, and the mutant-to-wildtype ratio determined by digital PCR (Bio-Rad QX200™ Droplet Reader); after which it is formulated into a nucleosome mimetic, and diluted into a commutable, proprietary, plasma-like matrix.[3] The nucleosome mimetic stabilizes the fragmented DNA; without it, the DNA becomes unusable for PCR or NGS library preparation (data not shown). The DNA concentration in the commutable matrix is 12 ng per mL (the amount is quantified as 12 ng per mL of extractable nucleic acid, using the QIAGEN® QIAamp® Circulating Nucleic Acids Kit, although other methods certainly apply). This process is illustrated in Figure 1.

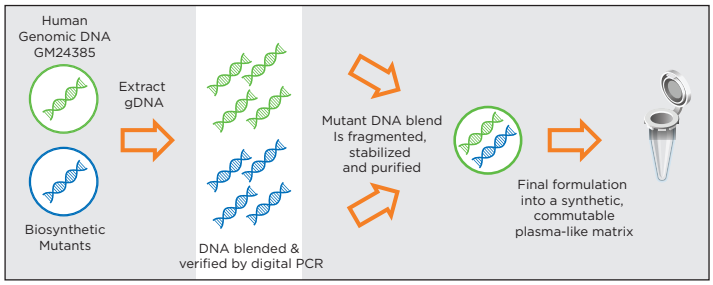

Figure 1: *SeraCare's preparation of Seraseq Circulating Tumor DNA Reference Material from extracted genomic DNA and biosynthetic mutant DNA into a commutable matrix.*

Digital PCR Linearity

The linearity of the digital PCR data for five sampled mutations is illustrated in Figure 2, with very good agreement between the target allele frequencies (AF) and the measured allele frequency of the fragmented intermediate.

This artifact of disproportionate allele frequency for one of the targets is an EGFR mutation (COSMIC ID 6225), which is a 15 base pair in-frame deletion in exon 19. The wild-type amplicon is under-represented in the digital PCR results in the fragmented sample compared to the unfragmented one. This is due to the longer wild-type amplicon (used to measure the relative allele frequency in the digital PCR assay) which is at a lower level compared to the mutated amplification (that is 15 base pairs shorter), giving a higher overall measured allele frequency.[4] In other words, since the mutant PCR amplicon has a shorter target sequence, in the fragmented material a larger number of these shorter targets are present, while the wild-type sequence has a larger target sequence, the fragmented material has a smaller number of these larger targets.

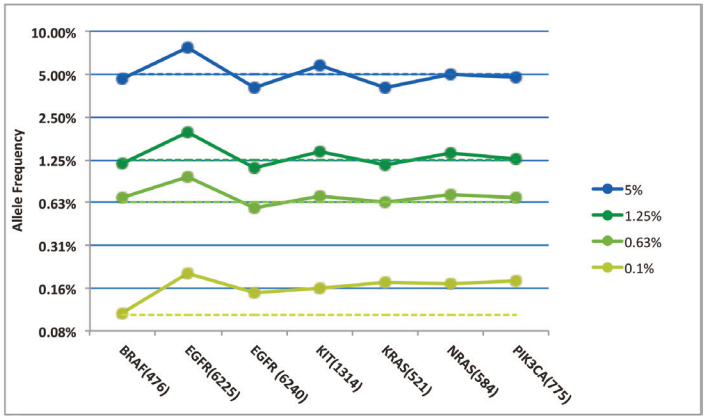

Figure 2: *Linearity of 0.1% to 5% allele frequency by digital PCR. Highly reproducible results obtained by digital PCR are illustrated by a comparison between fragmented and non-fragmented DNA shown in Figure 3. There is a negligible effect of fragmentation upon the quantification of allele frequency, except in the case at a large 15 base-pair deletion, indicated by the asterisk (see figure).*

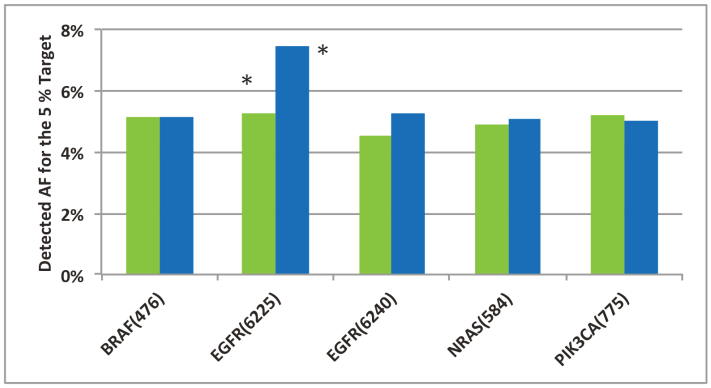

Figure 3: *Consistent 10% allele frequency by digital PCR for either sheared DNA (green) or non-sheared DNA (blue) samples. Asterisk indicates INDEL-specific bias for EGFR COSMIC ID 6225, a 15 base-pair deletion in the sheared sample (see text for details).*

NGS Testing

The Seraseq Circulating Tumor DNA-I Reference Material was then assayed with the Swift Biosciences Accel-Amplicon™ 56G Oncology Panel, that features a 263-amplicon multiplex for 56 clinically relevant oncology-related genes. It is one of the first commercially-available kits on the market for generating NGS libraries from circulating cell-free DNA. All nine mutations in the Seraseq product were detected, although variation between targets was observed (Figures 4 and 5).

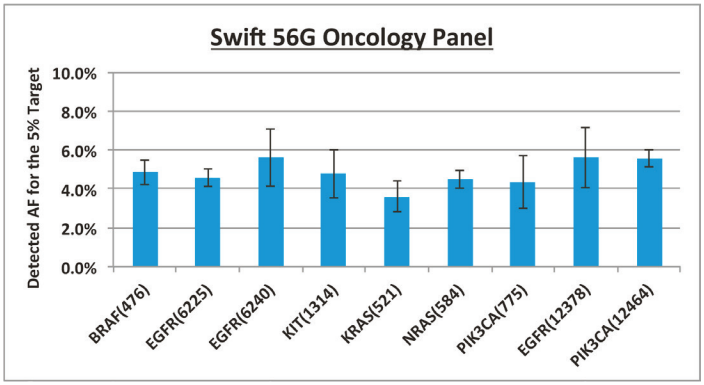

Figure 4: *Variation at a 5% allele frequency across nine mutations by the Swift Biosciences Accel-Amplicon™ 56G Oncology Panel*

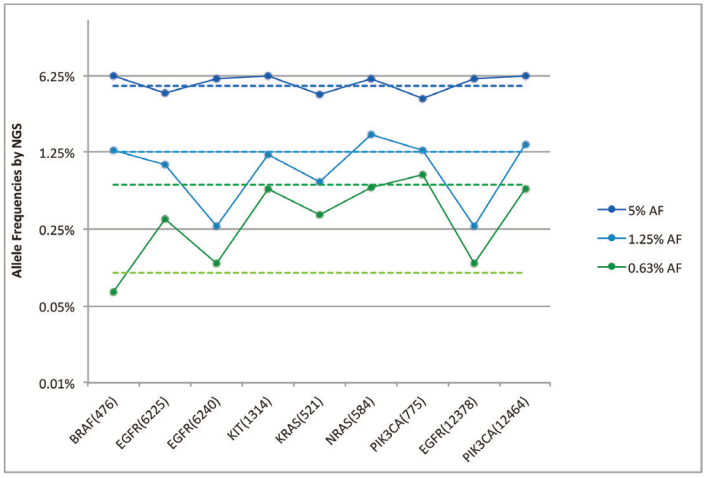

Figure 5: *1.25% to 5% allele frequency detected by the Swift Biosciences Accel-Amplicon 56G Oncology Panel. Swift reports confidence in their assay at allele frequencies >1%. Dataset shows the CV doubling at <5% allele frequencies.*

Conclusions

Seraseq Circulating Tumor DNA-I Reference Material offers a linear, flexible, and commutable reference material for oncology-based digital PCR or next-generation sequencing assays. The biosynthetic nature of this product allows for customization to any variant, as well as providing a full process reagent that behaves similarly to a patient sample.

SeraCare also offers a kit of purified nucleic acid (without formulation into the nucleosome mimetic nor blending with the plasma-like matrix) called the Seraseq Circulating Tumor DNA-I Mutation Mix Kit (AF5-WT) for assay development purposes.

Ordering Information

Product	Item Number
Seraseq Circulating Tumor DNA-I (AF5) Reference Material	0710-0012
Seraseq Circulating Tumor DNA-I (AF1.2) Reference Material	0710-0014
Seraseq Circulating Tumor DNA-I (AF0.6) Reference Material	0710-0015
Seraseq Circulating Tumor DNA-I (AF0.1) Reference Material	0710-0016
Seraseq Circulating Tumor DNA-I (WT) Reference Material	0710-0017
Seraseq Circulating Tumor DNA-I Mutation Mix Kit (AF5-WT)	0710-0018

References

1. Gold B, Cankovic M, Furtado LV, Meier F, Gocke CD. Do circulating tumor cells, exosomes, and circulating tumor nucleic acids have clinical utility? A report of the association for molecular pathology. J Mol Diagn. 2015 May;17(3):209-24. doi: 10.1016/j.jmoldx.2015.02.001. Review. PubMed PMID: 25908243; PubMed Central PMCID: PMC4411248.

2. Mussolin L, Burnelli R, Pillon M, Carraro E, Farruggia P, Todesco A, Mascarin M, Rosolen A. Plasma cell-free DNA in paediatric lymphomas. J Cancer. 2013 Apr 16;4(4):323-9. doi: 10.7150/jca.6226. Print 2013. PubMed PMID: 23678368; PubMed Central PMCID: PMC3654488.

3. Patent Pending

4. Best K, Oakes T, Heather JM, Shawe-Taylor J, Chain B. Computational analysis of stochastic heterogeneity in PCR amplification efficiency revealed by single molecule barcoding. Sci Rep. 2015 Oct 13;5:14629. doi: 10.1038/srep14629. PubMed PMID: 26459131; PubMed Central PMCID: PMC4602216.

seracare™ Passion. Innovation. Precision.

800.676.1881
508.244.6400
info@seracare.com
SeraCare.com