A comparison of different formats of DNA reference standards commutable with solid and liquid biopsy samples for cancer genomic profiling.

Prabha Nagarajan, Bernice Freeman, Jesse Stombaugh, Sian Constantine, Julie Wickenden

Horizon Discovery, 8100 Cambridge Research Park, Waterbeach, Cambridge CB25 9TL, UK

Introduction

Comprehensive genetic testing using large gene NGS panels is used commonly in the detection of somatic variants in cancer genetic analysis. However, errors introduced during each step of the workflow can limit the interpretation of results. To ensure reliability of the process and mitigate internal biases, we have developed a family of oncology reference materials (**OncoSpan**), which is cell line derived, highly characterized and features more than 375 variants across more than 152 cancer genes. OncoSpan is commutable with patient samples and comprises the same genetic content but processed into three different configurations: formalin fixed paraffin embedded (FFPE), cell-free DNA (cfDNA) and genomic DNA (gDNA). The various steps in the process of generating clinical samples can introduce discriminatory biological variations that reflect complexities of variant calling with FFPE and cfDNA as compared to gDNA.

Method

Clonal wild type cell lines were genetically engineered for desired variants at specified genomic locations.

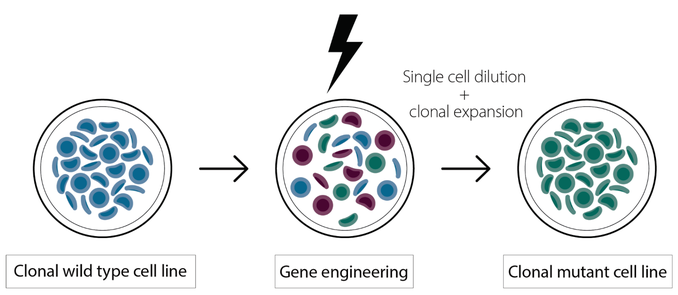

Fig 1. Engineering desired variants into cell lines

OncoSpan gDNA

Cell lines harbouring clinically relevant mutations were blended in defined ratios resulting in a broad range of allelic frequencies.

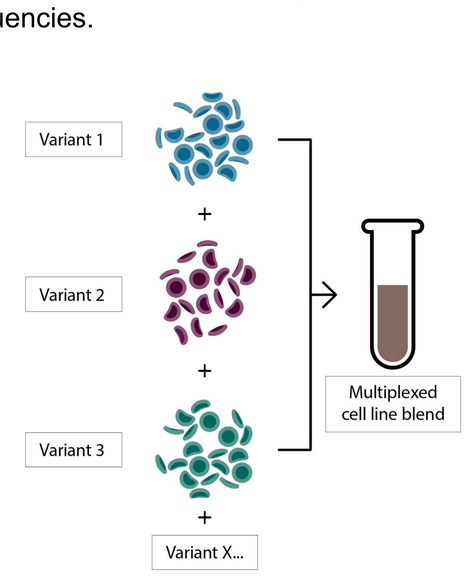

Fig 2. Making a multiplexed reference standard by blending different cell lines in defined proportions

Genomic DNA extracted from this blend was characterized as the gDNA format.

OncoSpan FFPE

FFPE material was prepared by fixation of the multiplex cell line blend and embedding in a paraffin block. The block was sectioned to yield 15 µm thick curls.

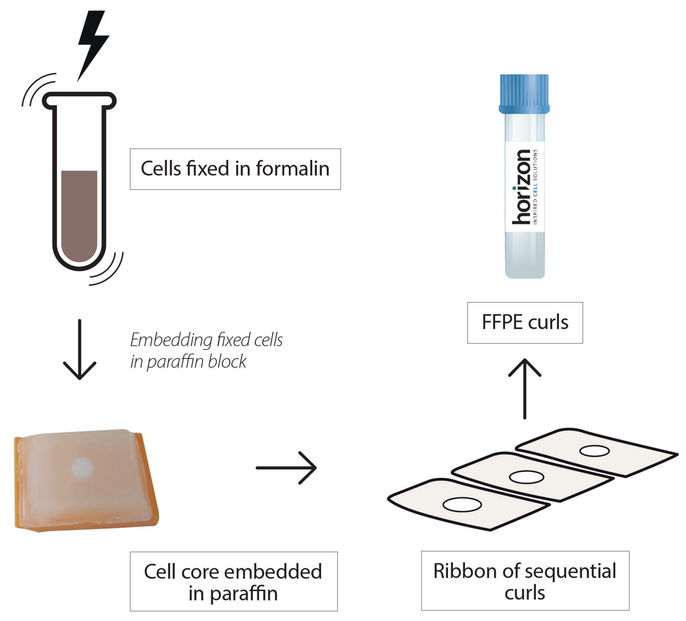

Fig 3. Generating FFPE block and curls from cell line blend

OncoSpan cfDNA

The cfDNA material was prepared by sonication of gDNA, yielding DNA molecules with an average fragment length of 160 bp

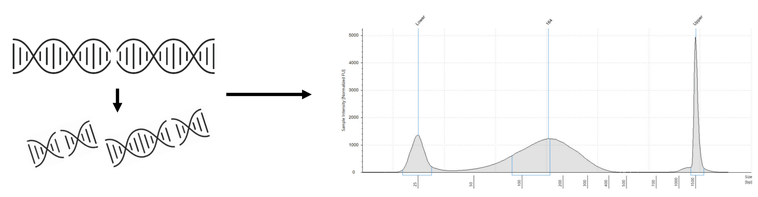

Fig 4. Generating OncoSpan cfDNA by mechanical shearing of gDNA. The average fragment length was measured using D1000 ScreenTape (Agilent) on a capillary based gel electrophoresis system,

Characterisation and Results

OncoSpan was characterized by various quality control measures to verify the allelic frequencies of claimed variants, average DNA yield, DNA integrity, and other features commutable with patient samples (Table 1).

Table 1. Quality testing steps for OncoSpan

	OncoSpan gDNA	OncoSpan cfDNA	OncoSpan FFPE
DNA integrity	Agarose gel electrophoresis	Tapestation	Agarose gel electrophoresis
DNA yield	Spectrophotometry (A260)	Qubit dsDNA BR Assay	Quantifluor™
Variant verification	Droplet Digital™ PCR	Droplet Digital™ PCR	Droplet Digital™ PCR
Secondary method for variant verification	NGS by whole exome sequencing (500x)	NGS by whole exome sequencing (500x)	NGS by whole exome sequencing (120 x)

Differences in characterisation of the OncoSpan gDNA, cfDNA and FFPE formats by droplet digital PCR

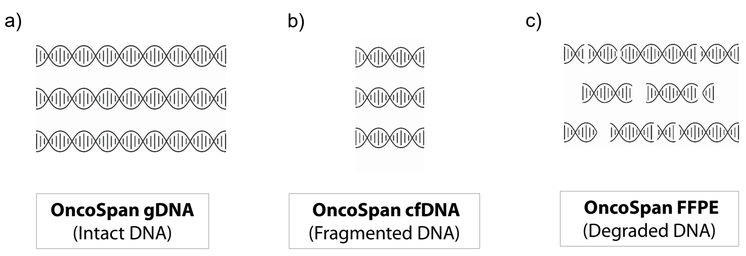

Fig 5. Three different formats of OncoSpan with various integrities of DNA.

Variant allelic frequencies (VAF) for 25 key cancer mutations ranging from 1% to >90% in OncoSpan were verified by droplet digital PCR. The observed VAFs had subtle differences among all three formats owing to the differences in the integrity of their DNA. The gDNA was the most intact, showing VAFs closest to the theoretically calculated values. Whereas, FFPE DNA suffers from degradation induced by the fixation process and showed a wider range of observed VAFs. The short DNA molecules in the cfDNA format showed amplification biases for some variants and resulted in underestimation of one VAF.

Differences in characterisation of the OncoSpan gDNA, cfDNA and FFPE formats by NGS

A high coverage WES was performed on the materials to confirm a larger set of variants. The NGS results were prejudicial only for the low VAFs and a few INDELS. In the gDNA and FFPE formats, >380 variants were verified across 152 genes, present between 1-100% VAF. This included >28 INDELS (>22 deletions and 6 insertions, ranging from 1-15 bp). NGS was not sensitive in calling out low VAFs, which was particularly problematic in the cfDNA format, where only 120 x coverage was achievable. We could verify >375 variants across 152 genes in the cfDNA reference material. This included 27 INDELS (>21 deletions and 6 insertions, ranging from 1-15 bp).

Conclusion

In summary, OncoSpan reference material is purely cell line derived and contains no synthetic DNA. The three OncoSpan reference standards are commutable with solid biopsy, liquid biopsy and extracted gDNA samples.

As they have the same genetic content, the differences in the ability to characterize them highlights the complexities of variant calling with FFPE and cfDNA as compared to gDNA. This family of controls can ensure this variability is controlled by selecting the format commutable to the patient sample used.