Functional DNA Quantification Guides Accurate Next-Generation Sequencing Mutation Detection in Formalin-Fixed, Paraffin-Embedded Tumor Biopsies

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SUMMARY

- Chemical modifications and degradation of DNA from formalin-fixation and paraffin-embedded (FFPE) biopsies can undermine interpretations of targeted next-generation sequencing (NGS) data.
- In this poster, we evaluated the impact of functional FFPE DNA quality and library complexity upon variant quantification and detection.
- FFPE DNA quantification was compared across spectrophotometry (Nanodrop), fluorescent dye-binding assay (Qubit®), and a novel quantitative PCR assay (QFI™-PCR) that measures the absolute copy number of amplifiable DNA.
- The results demonstrate the value of an integrated workflow using QFI-PCR to increase the accuracy of NGS mutation detection and guide changes in input that can improve the analysis of low quality FFPE DNA.

INTRODUCTION

Formalin-fixed, paraffin-embedded (FFPE) biopsies are indispensable samples for cancer diagnostics. These samples, however, are fraught with chemical modifications and degradation that can compromise molecular assays, such as targeted enrichment upstream of next-generation sequencing (NGS). A rigorous and quantitative understanding of FFPE DNA quality is needed to ensure accurate mutation calls from NGS-based assays, especially in clinical settings.

MATERIALS AND METHODS

Three methods for FFPE DNA quantification were compared, including a novel quantitative PCR assay (Quantitative Functional Index PCR, or QFI™-PCR) that measures the absolute copy number of amplifiable DNA. QFI-PCR was validated across 43 FFPE samples, and then applied to a broader set of 165 residual clinical FFPE samples. The impact of FFPE DNA copy number and library complexity on variant quantification and detection was evaluated by PCR-based targeted NGS using two different platforms, the Ion Personal Genome Machine® and Illumina MiSeq®.

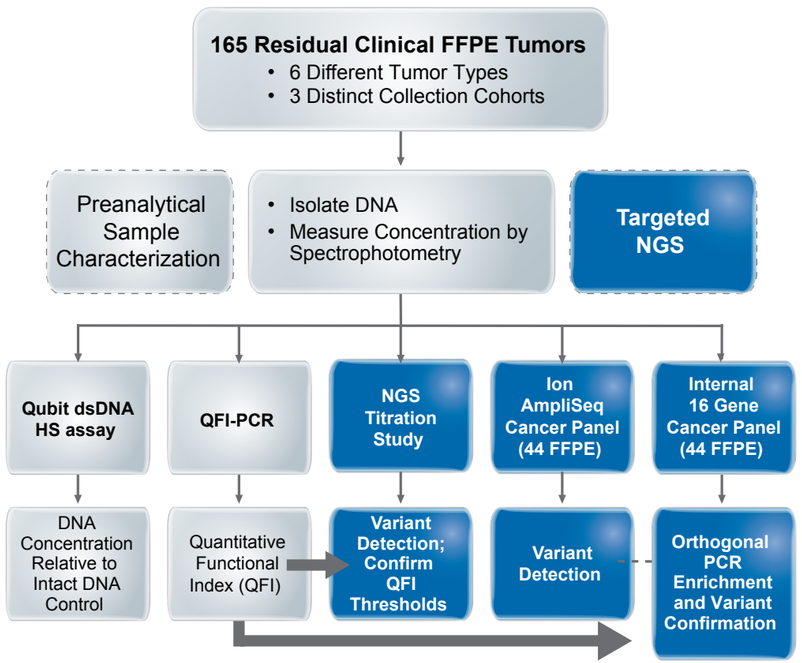

Figure 1. Study Design. The study design coupled pre-analytical FFPE DNA characterization using three different quantification methods with variant calling results from targeted NGS and confirmation assays to assess the impact of template quality.

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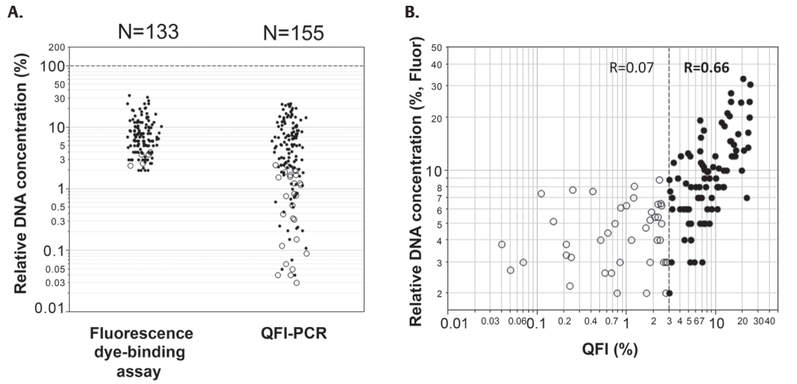

Figure 2. NanoDrop reports a >15-fold higher DNA concentration than Qubit or QFI-PCR. DNA concentrations measured by Qubit and QFI-PCR track with functional quality, but only QFI-PCR can differentiate lower quality FFPE DNA. Filled circles in Figure 2A represent values detected above background by both quantification methods, whereas open circles represent values that were only detected using the designated assay. In contrast, the filled circles in Figure 2B highlight data that followed a linear trendline between Qubit and QFI-PCR measurements, whereas the open circles designate those FFPE DNA samples that could only be differentiated using QFI-PCR.

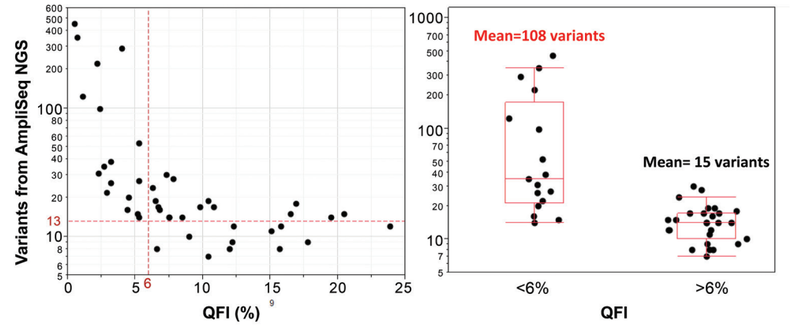

Figure 3. The sample QFI is inversely related to the number of variants detected by targeted NGS. Cancer gene loci were enriched using the AmpliSeq® Cancer Panel and sequenced on the PGM. The number of detected variants increased by >7-fold for low quality (<6% QFI, <200 cp into enrichment) compared to higher quality (>6% QFI) DNA.

Table 1. A false positive rate of up to 89% was observed following NGS of FFPE DNA with the lowest QFI. AmpliSeq NGS variant calls were confirmed using SuraSeq® 500, a 17 oncogene enrichment panel (Hadd et al. J Mol Diagn 2013, 15:234-247)

Sample	Quality by QFI-PCR	QFI (%)	Qubit (%)	Total # of mutations from AmpliSeq	Overlapping Variants in 16 Gene Panel	Overlapping Variants in 16 Gene Panel (PIK3CA FP removed**)	Overlapping Variants in 16 Gene Panel (PIK3CA FP removed and >5%**)	Variant Confirmed (17 Gene Panel)	True Positives	# of Germline SNPs Annotated in dbSNP	Confirmed COSMIC Mutation
RS00863	Low	0.5	4	451	48	44	28	3	10.7%	2	BRAF V600E 23.8%
RS00856	Low	0.7	5	352	42	36	26	3	11.5%	2	KRAS G13C 29.0%
RS01279	Low	1.1	4	123	23	19	8	3	37.5%	3	None
RS00865	Low	2.2	4	222	33	30	21	3	14.3%	2	NRAS G12R 23.0%
RS01283	Low	2.3	0	31	9	6	3	3	100.0%	3	None
RS01282	Low	2.4	5	98	21	17	10	3	30.0%	3	None
RS01289	Low	2.7	2	35	11	8	5	4	80.0%	3	None
RS01274	Low	2.9	3	22	12	9	6	4	66.7%	3	NRAS Q61R 42.6%
RS00866	Medium	6.3	7	24	11	8	5	5	100.0%	3	NRAS Q61H 72.1% PIK3CA H1047R 12.4%
RS00860	Medium	6.5	7	19	7	4	3	3	100.0%	3	None
RS01294	Medium	6.6	8	8	3	3	3	3	100.0%	3	None
RS00875	Medium	6.7	5	17	6	3	2	1	50.0%	1	None
RS00855	Medium	6.8	11	16	8	5	3	3	100.0%	2	KRAS G12D 37.8%
RS00876	High	16.5	12	15	6	4	4	3	75.0%	2	PIK3CA H1047R 43.6%
RS01291	High	16.9	13	18	9	6	3	3	100.0%	3	None
RS00871	High	17.8	16	9	4	2	2	2	100.0%	2	None
RS00873	High	19.5	10	14	6	3	3	3	100.0%	3	None
RS00857	High	20.5	13	15	5	4	4	4	100.0%	3	KRAS G12V 29.8%
RS00877	High	23.9	20	12	7	4	4	4	100.0%	3	KRAS G12S 48.0%

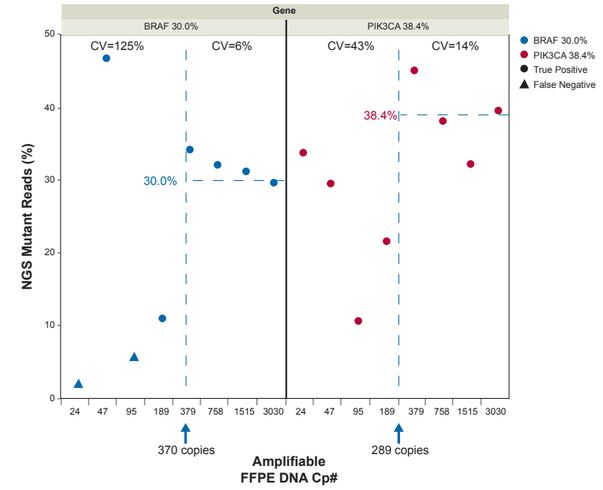

Figure 4. Titration of QFI-determined FFPE DNA copy numbers demonstrates unreliable variant quantification at less than 10 mutant copies into targeted NGS. Variant calls were stable at ≥370 cp of the BRAF V600E sample (30% mutant, 9% QFI) and ≥289 cp of the PIK3CA H1047R sample (38.4% mutant, 9% QFI).

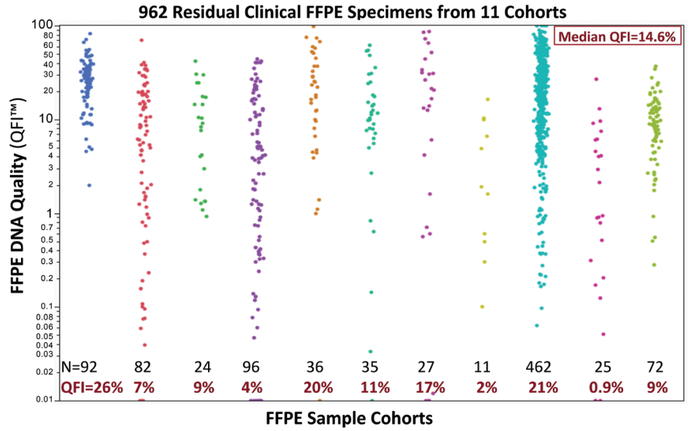

Figure 5. Median residual clinical FFPE DNA template quality is 4-100x lower than intact cell line DNA. The median QFI across 962 clinical FFPE tumors ranged from a low of 0.9% and a high of 26% depending on the sample cohort.

CONCLUSION

- Reliance on DNA quantification assays that underestimate or ignore FFPE sample quality can produce both false positive and negative variant calls.
- Qubit can segregate the highest and lowest quality DNA samples, but it cannot discriminate among lower quality samples as QFI-PCR can.
- QFI-PCR can guide corrections in sample DNA input to help “rescue” low quality samples, improve the accuracy of variant detection, and minimize the burden of confirmation in heterogeneous cancer samples.
- We recommend the routine integration of quantitative, pre-analytical QC thresholds for FFPE tumor DNA into targeted NGS procedures, particularly for clinical testing.