Streamlined next generation sequencing assay development using a highly multiplexed FFPE quality control technology based on the Genome in a Bottle

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ABSTRACT

Introduction: Next generation sequencing (NGS) assay development for solid tumor sequencing requires characterization of variant calling directly from formalin-fixed paraffin embedded (FFPE) tissue samples. However, cell line based FFPE and human FFPE samples only contain 2 to 20 variants, which require laboratories to invest significant resources in sample sourcing and preparation when developing assays to detect 100+ variants. This challenge is further compounded by the inherent heterogeneity of human FFPE samples. In this study, we demonstrate the development of a standardized full process (controlling from extraction to result) FastFFPE™ quality control (QC) material that contains more than 500 variants commonly found in lung, breast, colon, and other solid tumors. Notably, all 500+ variants are contained within a single FFPE section. To ensure predictable performance and enable accuracy applications, all variants were Sanger sequenced and cells from the Genome in a Bottle (GIAB) Consortium were used as a background genome. The performance of the FastFFPE™ samples was evaluated for variant allelic frequency (AF) using next-generation sequencing (NGS) and droplet digital PCR platforms. The extraction yield was also determined.

Methods: FastFFPE™ quality control material was generated to contain 500+ cancer hotspot variants (including SNP, MNP, INDEL) at various AF in the background of the GIAB GM24385 cells. Blocks of FastFFPE™ material were sectioned into 10 micron scrolls and stored at 2-8° C. The DNA was extracted in triplicate from each block using the QIAamp® DNA FFPE tissue kit, and concentration was determined using the Qubit 3.0® dsDNA HS assay kit. The Ion AmpliSeq™ Cancer Hotspot Panel v2 was used for library preparation and tested on Ion Torrent™ Personal Genome Machine™ (PGM™). AF results determined using the PGM™ instrument were compared to the AF by Bio-Rad® droplet digital PCR (ddPCR) platform.

Results: The FastFFPE™ QC samples showed an average AF of 70%, 13.5%, 6.5% and 1.2% by ddPCR. The AF obtained using NGS was similar to ddPCR results, except for the lowest AF% level, which suggests that the performance of FastFFPE™ samples is platform agnostic. Each section at the respective AF (except 1%) showed a greater than 96% hotspot mutation detection rate by NGS, indicating that each scroll has consistent performance. DNA extraction yields were ~80 ng/section, indicating that each section provides sufficient material for multiple replicate testing.

Conclusions: A highly multiplexed FastFFPE™ QC material has been generated and tested on both NGS and ddPCR platforms. This external quality control material could provide reliable and consistent testing results for NGS solid tumor assay development, from DNA extraction through to variant calling.

INTRODUCTION

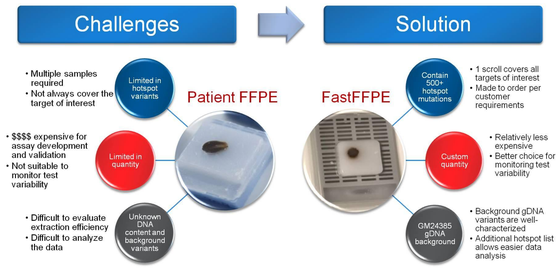

Figure 1. Challenges and solutions to using patient FFPE samples in assay development and validation. The use of patient FFPE for assay development could be problematic due to the limited amount of samples and target variants, heterogeneity of background DNA, increased number of runs, and high costs per sample. One possible solution is to use a FastFFPE™ QC material.

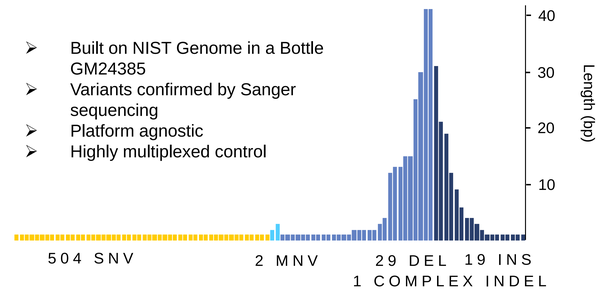

Figure 2. MegaMix™ Technology. The use of AcroMetrix™ MegaMix™ Technology allows for 550+ cancer hotspot variants in the background of NIST Genome in a Bottle. All of the variants are confirmed by Sanger sequencing.

ABL1	CDH1	ERBB4	FLT3	HRAS	KIT	MSH6	PTEN	SMO
AKT1	CDKN2A	EZH2	FOXL2	IDH1	KRAS	NOTCH1	PTPN11	SRC
ALK	CSF1R	FBXW7	GNA11	IDH2	MAP2K1	NPM1	RM1	STK11
APC	CTNNB1	FGFR1	GNAQ	JAK2	MET	NRAS	RET	TP5
ATM	EGFR	FGFR2	GNAS	JAK3	MLH1	PDGFRA	SMAD4	VHL
BRAF	ERBB2	FGFR3	HNF1A	KDR	MPL	PIK3CA	SMARCB1	

Table 1. Core Targeted Genes: 555 mutations including 504 SNV, 2 MNV, 29 DEL, 19 INS and 1 complex indel all in one control sample.

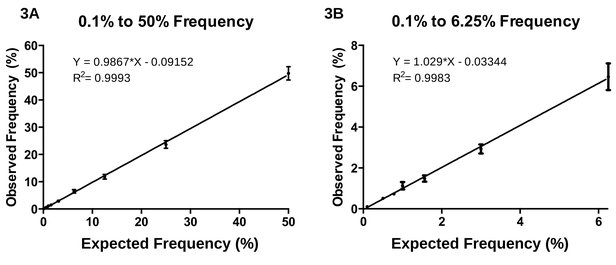

Figure 3. Dilution of 555-Hotspot Frequency Ladder. (A-B) All 555 variants were targeted to a defined frequency range around 50%. Samples were then diluted with target frequencies of 50%, 25%, 12.5%, 6.3%, 3.1%, 1.5%, 0.5%, 0.1%. Dilutions were made in GM24385 Genome in a Bottle gDNA.

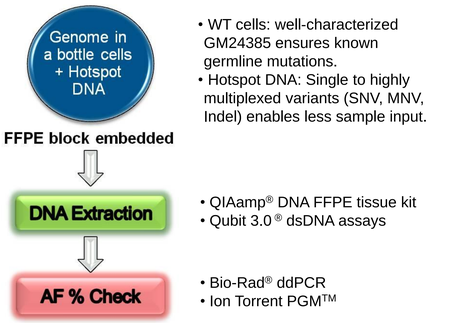

Figure 4. Testing Scheme. Blocks of FastFFPE™ material with highly multiplex Oncology Hotspot mutations at 4 target frequencies in the background of NIST GIAB GM24385 cells were generated and sectioned into 10 micron scrolls. DNA from each scroll was extracted and the concentration was checked. Bio-Rad® ddPCR and Ion Torrent PGM™ were used to determine AF%.

RESULTS

Ladder Level	Average DNA yield (ng)/scroll	SD	N
Level 1	84.5	14.9	5
Level 2	152.4	40.9	5
Level 3	105.8	19.7	5
Level 4	190.3	39.2	5

Table 2. Each scroll of FastFFPE™ sample yielded ~80 ng/scroll. FastFFPE™ samples were extracted using QIAamp® DNA FFPE tissue kit and the concentration was determined by Qubit 3.0 ® instrument using dsDNA HS assay kit. Five scrolls from each level were extracted, and the average DNA yield was determined.

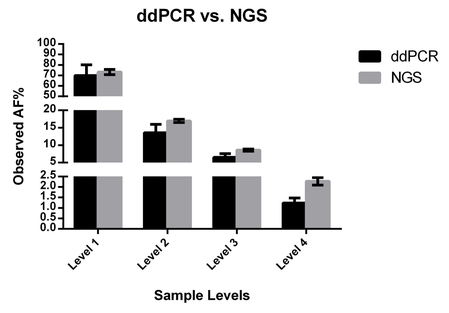

Figure 5. Similar AF% of each scroll was observed using Bio-Rad ® ddPCR and Ion Torrent PGM™ platforms. DNA from FastFFPE™ samples at 4 different frequency levels was extracted and tested. Sample 1-4 showed 70%, 13.5%, 6.5%, and 1.2% on ddPCR, and 73%, 16.9%, 8.6% and 2.2% on NGS, respectively (N=3). The results showed similar AF% between the two platforms except for the lowest level sample, suggesting that the FastFFPE™ QC material is platform agnostic.

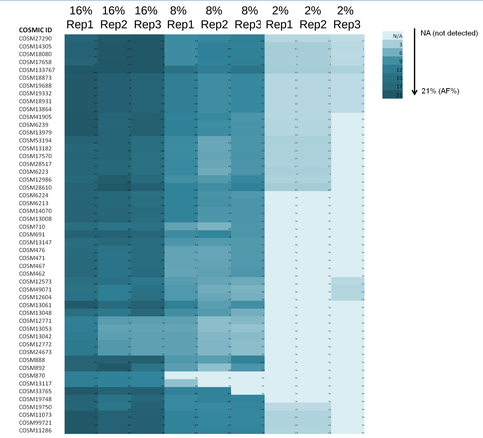

Figure 6. Use of FastFFPE™ QC materials to determine assay sensitivity. FastFFPE™ samples at 16%, 8% and 2% target AF% were extracted and tested in triplicate on Ion Torrent PGM™ using the Ion AmpliSeq™ Cancer Hotspot Panel v2 for library preparation. Fifty cancer hotspot mutations were used as representative variants to demonstrate assay sensitivities. The allelic frequency AF% ranges from NA (not detected) to 21% as shown as a blue.

FastFFPE Ladder Member	Replicates	Expected Hotspot Variants	Observed Hotspot Variants	Detection Rate (%)
Level 1	1	358	356	99%
	2	358	353	99%
	3	358	354	99%
Level 2	1	358	345	96%
	2	358	349	97%
	3	358	348	97%
Level 3	1	358	351	98%
	2	358	350	98%
	3	358	346	97%
Level 4	1	358	158	44%
	2	358	158	44%
	3	358	35	10%

Table 3. More than 96% detection of hotspot mutations on three FastFFPE™ panel levels. The detection rate of cancer hotspot mutations of each FastFFPE scroll was compared with the expected hotspot mutations. Except for the level 4 samples, all other samples showed >96% detection rate.

CONCLUSIONS

- A full-process FastFFPE™ QC material that is highly multiplexed with cancer hotspot variants in each scroll was developed in the background of NIST GIAB GM24385 cell line.
- Each scroll provides at least 80 ng total DNA yield after extraction for downstream analytical testing.
- The allelic frequency determined by Bio-Rad droplet digital PCR and Ion Torrent PGM™ platforms showed similar AF% results, suggesting the FastFFPE™ QC material is platform agnostic.

ACKNOWLEDGEMENTS

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TRADEMARKS

The control is currently in development and not available for use.