Optimization Studies for the Development of Highly Multiplexed Reference Materials in FFPE Format for Solid Tumor Profiling

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INTRODUCTION

Next-generation sequencing (NGS) has revolutionized how IVD developers, laboratories, and clinicians are diagnosing, treating, and monitoring disease. NGS assays must be proven robust, accurate, and consistent through validation and verification studies. Sourcing individual FFPE samples (remnant patient specimens or cell line derived) for each of the somatic mutations of interest is expensive and time consuming. Materials with copy number variations (CNV) quantitatively characterized in a stable background have not been available. Therefore, highly multiplexed, engineered reference materials in FFPE format are needed. These FFPE reference materials must be consistently manufactured and must incorporate a wide variety of somatic variations including CNVs in addition to SNVs, INDELs, and structural variants.

MATERIALS AND METHODS

- Methods were optimized for embedding engineered cell line pellets in FFPE to minimize the variability between FFPE blocks and among sections cut from the same FFPE block (beginning, middle, and end sections- Figure 1).

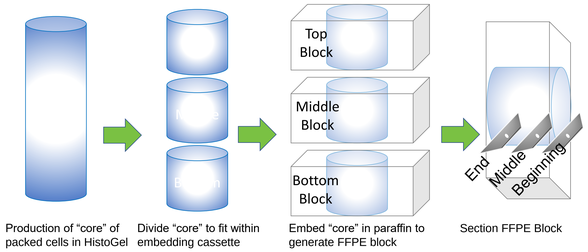

Figure 1: Scheme for embedding engineered cells in FFPE. The goal is to minimize variation between FFPE blocks and among curls cut from the beginning, middle or end of a block.

- Four conditions, varying the number of cells packed into the core and the ratio of cells to HistoGel™, were used for this optimization study.
- Once optimized conditions were standardized, nine different FFPE blocks were made on three independent days to demonstrate consistency.
- DNA was extracted from FFPE curls using Qiagen QIAamp® DNA FFPE Tissue kit, or Agencourt® Formapure® FFPE kit, and the consistency of DNA yield across blocks and within a single block were assessed using Qubit® dsDNA HS kit.
- Functional testing of variants important for tumor profiling used digital PCR assays run on the BioRad QX200™ system as well as NGS testing on Ion Ampliseq® Cancer Hotspot Panel v2 and Illumina TruSight® Tumor 15 panel.

RESULTS

Cell Embedding Optimization

Core	Number of Cells Embedded	HistoGel:Cells Ratio	% Difference in Yield Among FFPE blocks
Condition 1	70 million	~10	29%
Condition 2	70 million	~4.5	11%
Condition 3	100 million	~4.5	11%
Condition 4	100 million	~3	3%

Table 1: Optimization conditions and results.

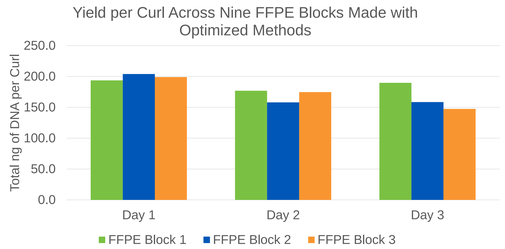

Figure 2: Consistency of DNA yield from Agencourt Formapure FFPE Kit extractions when using optimized conditions for FFPE block manufacture (condition 4 from Table 1).

- Four conditions for embedding were assessed that alter the cell pellet volume to Histogel volume.
- After two independent experiments, Condition 4 was selected for giving the most consistent distribution of cells within the core (Table 1).
- Using the optimized conditions, nine different FFPE blocks were produced. The average yield was 178 ng of DNA per curl and varied by less than 12% CV among blocks (Figure 2).

Prototype FFPE Block Preparation

Curl#	DNA Yield (ng)
10	218
11	216
12	260
31	246
32	283
33	245
39	297

Table 2: DNA yields from seven non-sequential curls from prototype FFPE block extracted using the Qiagen QIAamp DNA FFPE Tissue Kit.

- GM24385 cell line were engineered to contain 40 variants including SNVs, INDELs and gene fusions at an allele frequency of approximately 20%.
- Curls were extracted using Qiagen QIAamp FFPE kit. Yields from curls throughout the block were consistent (Table 2).
- Extracted DNA was tested for select variants by digital PCR, as well as NGS using Ion Ampliseq Cancer Hotspot Panel v2 (CHPv2) and Illumina TruSight Tumor 15 panel (TST15).
- Average allele frequency of assayed variants was 19.79% by digital PCR, 23.7% by Illumina TruSight Tumor 15 assay, and 20.7% by Ion Ampliseq Cancer Hotspot Panel v2 (Table 3).

Gene ID	COSMIC Identifier	Ion Ampliseq CHPv2	ILMN TST15
AKT1	COSM33765 (p.E17K)	25.25	22.05
APC	COSM13127 (p.R1450*)	18.40	
APC	COSM18561 (p.T1556fs*3)	Not reported	
ATM	COSM21924 (p.C353fs*5)	23.00	
BRAF	COSM476 (p.V600E)	17.25	15.05
CTNNB1	COSM5664 (p.T41A)	20.35	
EGFR	COSM6225 (p.E746_A750 delELREA)	30.00	27.60
EGFR	COSM12378 (p.D770_N771insG)	19.60	21.75
EGFR	COSM6224 (p.L858R)	27.30	25.75
EGFR	COSM6240 (p.T790M)	34.60	34.60
ERBB2	COSM20959 (p.A775_G776 insYVMA)	27.55	26.70
FGFR3	COSM715 (p.S249C)	14.60	
FLT3	COSM783 (p.D835Y)	20.95	
FOXL2	COSM33661 (p.C134W)		26.30
GNA11	COSM52969 (p.Q209L)	16.40	22.90
GNAQ	COSM28758 (p.Q209P)	7.45	
GNAS	COSM27887 (p.R201C)	17.25	
IDH1	COSM28747 (p.R132C)	18.85	
JAK2	COSM12600 (p.V617F)	14.60	
KIT	COSM1314 (p.D816V)	16.20	
KRAS	COSM521 (pG12D)	18.10	17.75
MPL	COSM18918 (p.W515L)	24.35	
NPM1	COSM17559 (p.W288fs*12)	16.40	
NRAS	COSM584 (p.Q61R)	23.55	22.75
PDGFRA	COSM736 (p.D842V)	26.65	
PDGFRA	COSM28053 (p.S566fs*6)	25.25	
PIK3CA	COSM763 (p.E545K)	16.00	12.40
PIK3CA	COSM775 (p.H1047R)	18.40	15.05
PIK3CA	COSM12464 (p.N1068fs*4)	14.05	
PTEN	COSM4986 (p.P248fs*5)	15.55	
PTEN	COSM30622 (p.K267fs*9)	Not reported	
RET	COSM965 (p.M918T)	24.45	26.50
SMAD4	COSM14105 (p.A466fs*28)	18.10	
TP53	COSM10660 (p.R273H)	24.55	22.85
TP53	COSM10662 (p.R248Q)	24.20	23.90
TP53	COSM6530 (p.C242fs*5)	24.50	23.65
TP53	COSM10648 (p.R175H)	36.75	33.75
TP53	COSM18610 (p.S90fs*33)	Not reported	25.40

Table 3: Allele frequencies reported by commercial tumor hotspot NGS panels. Grey shading indicates that the variant is not assayed by the panel. Three variants were detected in raw data, but not reported on CHPv2 assay due to bioinformatic filtering.

FFPE REFERENCE MATERIAL FOR CNV

- There is need for reference materials for CNV detection in FFPE format.
- GM24385 cells were engineered to carry copies of a large construct (>100Kb) containing the MYC gene.
- Three FFPE blocks were made targeting three total copies of MYC (near LOD for many NGS assays).
- Two curls were extracted from each block and assessed for MYC copy number by dPCR.

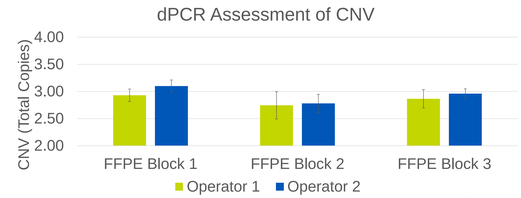

Figure 3: MYC CNV gains measured by dPCR in 3 FFPE blocks

- CNV constructs for MET, EGFR, ERBB2, MYCN and FGFR3 are also available and are being used to make more highly multiplexed CNV reference materials.

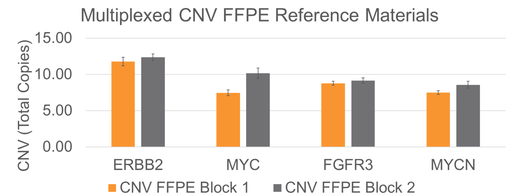

Figure 4: dPCR analysis of FFPE block made targeting >7 total copies of ERBB2, MYC, FGFR3, and MYCN

CONCLUSIONS

- Conditions were optimized for embedding engineered cells into FFPE blocks so that there is <12% CV in DNA yield extracted from curls from different FFPE blocks and ~12% CV in DNA yield extracted from curls throughout one FFPE block.
- Testing of prototype materials containing 40 somatic cancer variants on commercial hotspot NGS panels demonstrates how the technology can be useful for very highly multiplexed reference materials.
- Curls extracted and tested by different methods (digital PCR, Ion Ampliseq NGS, and Illumina NGS) all gave similar allele frequencies, indicating that the material can be useful for standardization, optimization, and benchmarking.
- Prototype FFPE blocks were also made to challenge lower limits of detection for copy number gains. Separate curls from the same block showed consistent CNV. All three blocks made were within 10% of the target CNV level.