Purification Strategies, Quantitation, and QC Measurements for Predicting Downstream NGS Success with FFPE and Circulating Cell-Free DNA Plasma Samples

Doug Wieczorek, PhD, Spencer Hermanson, Curtis Knox, Jennifer Mook, Douglas Horejsh, PhD, Eric Vincent, PhD, Douglas Storts, PhD, Trista Schagat, PhD,
Promega Corporation, 2800 Woods Hollow Rd. Madison, WI

1. Introduction

Formalin fixed, paraffin embedded (FFPE) tumor tissue samples have long been an important source of genetic material for mutational analysis. However, the quality of DNA from FFPE samples is often highly variable, and the resulting degradation and crosslinking due to the fixation process can lead to issues with amplifiability and difficulty in NGS analysis. An alternative to FFPE is obtaining circulating cell-free DNA (ccfDNA) from plasma or other biological fluids. Collecting ccfDNA samples from plasma is non-invasive and can be used when no tumor is accessible or detectable. Samples can be collected quickly and frequently and allows for the ability to monitor the disease or response to treatment over time. The drawbacks are that yields of ccfDNA are often very low and circulating tumor DNA is typically present at low frequencies.

We have developed novel nucleic acid purification chemistries that improve upon current manual and automated methods for the purification of DNA from FFPE and plasma and demonstrate their use in NGS applications. DNA was purified from multiple FFPE tumor tissue types and matching plasma as well as normal FFPE tissue samples using multiple methods. DNA quantity and quality was measured by two separate strategies to study degradation levels of the nucleic acid obtained. Libraries were constructed using a commercially available 56 gene oncology panel for targeted NGS and sequencing quality was evaluated. The overall quality of the sequencing data correlates with measured quality metrics derived from a prototype DNA QC assay currently in development.

2. Methods

Sample types and DNA purification:

- Matched breast, lung, and colon derived tumor and normal tissue FFPE samples were purified for DNA using the ReliaPrep™ FFPE gDNA Miniprep System[1], the Maxwell® RSC DNA FFPE Kit[1], and the QIAamp DNA FFPE Tissue Kit.
- In addition, purported matched plasma and rectal tumor and normal tissue FFPE samples were purified using the Maxwell RSC ccfDNA plasma kit[1], the Maxwell RSC DNA FFPE Kit, and the ReliaPrep FFPE gDNA Miniprep System.
- For each FFPE sample, 3 or 4 individual scrolls were extracted from adjacent tissue
- For plasma, 4 replicates of 1 mL was extracted

DNA quantitation:

- DNA extracts were quantified with the Quantus™ Fluorometer[1] and QuantiFluor® dsDNA dye system[1] as well as with an 84 bp target qPCR assay
- qPCR results were used to normalize and proceed with downstream NGS library prep

NGS library preparation and sequencing:

- NGS libraries were generated from a selected set of the purified samples using the Swift Biosciences Accel-Amplicon 56G Oncology Panel
- Sequencing was performed on the Illumina MiSeq instrument with v3 chemistry and 2x300bp reads.

DNA quality assessment:

- After sequencing, samples with remaining volume were retroactively assayed for DNA quality and quantity using a prototype multiplexed, probe-based qPCR system (in development) designed to assess the quantity and quality of human genomic DNA (see section 6 for more detail).

3. Comparative Quantitation Results – Tumor Tissue

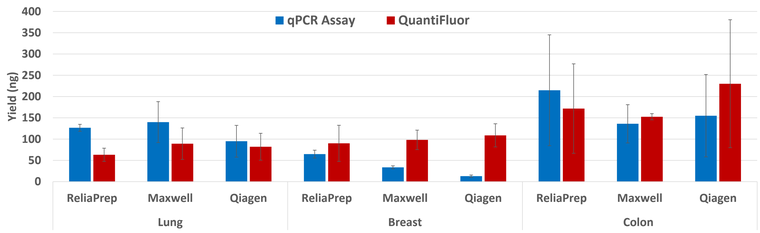

Fig. 1: Comparative FFPE tumor tissue sample purification between three different methods and two different quantitation strategies.

The QuantiFluor® dsDNA dye system is designed to measure total double-stranded DNA concentration without regard for species, size, or amplifiability of the DNA, whereas a qPCR assay (84 bp target) is a human-specific qPCR test designed to measure amplifiable DNA. Samples with significantly lower qPCR quantitation results vs. fluorescent dye-based quantitation are indicative of degraded DNA.

4. Comparative Quantitation Results – Normal Tissue

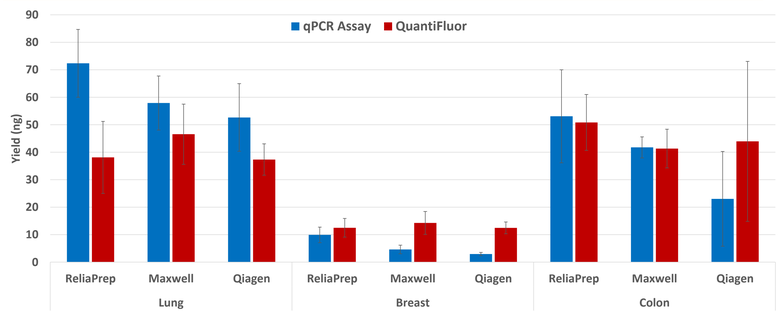

Fig. 2: Comparative FFPE normal tissue sample purification between three different methods and two different quantitation strategies.

In each sample, Qiagen provided the lowest amount of amplifiable DNA for downstream analysis.

5. Comparative Quantitation Results – Rectal Normal and Tumor Tissue and Plasma

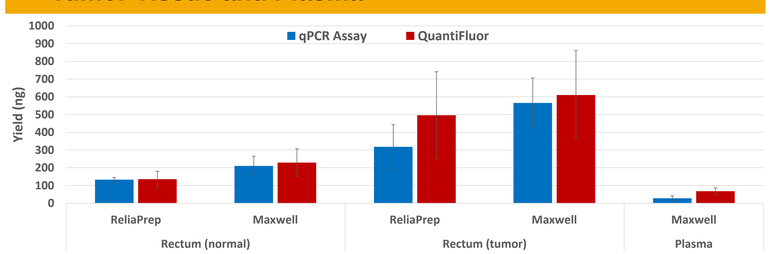

Fig. 3: Rectal cancer FFPE tumor and normal adjacent tissue samples were purified and quantified with two different methods along with a matched plasma sample for the same anonymous donor.

Due to limited sample access, Qiagen was not included as a purification method for the rectal FFPE samples, and only the Maxwell RSC ccfDNA plasma kit was used for the plasma purification.

6. DNA QC Assay Predicts Sequencing Quality

After sequencing, samples with remaining DNA were retroactively amplified with a prototype multiplexed, probe-based qPCR system (in development) designed to assess the quantity and quality of human genomic DNA derived from FFPE samples. The prototype may also be used to evaluate the ratio of the quantity of desired circulating cell free DNA (ccfDNA) to higher molecular weight genomic DNA from plasma samples. The multiplex assay detects 75bp, 150bp and 300bp human genomic targets, and includes an internal positive control (IPC) to test for false-negative results that may occur in the presence of PCR inhibitors.

TUMOR SAMPLES

	DNA QC Assay	Library	NGS Metrics		
Samples	ratio (75/300)	Yield (nM)	% bases On-Target*	Average Coverage	Coverage Uniformity**
MBT1	117810	0.961	100%	4223	67%
MBT2	16312	0.615	100%	4154	69%
MBT3	17969	0.654	99%	2323	67%
MCT1	168	21.5	99%	8203	86%
MCT2	211	18.4	99%	6918	89%
MCT3	191	13.8	99%	8326	91%
MLT1	44	14.3	99%	4945	95%
MLT2	47	9.88	99%	4851	96%
MLT3	40	9.82	99%	8648	95%
MRT1	2.2	23.2	97%	5805	96%
MRT2	2.4	37.3	97%	3487	95%
MRT3	2.9	16	98%	7841	96%
MRT4	2.6	23.5	97%	5650	95%
QCT1	127	6.99	99%	5789	90%
QCT2	130	4.82	99%	4272	88%
QCT3	161	8.62	99%	4328	90%
QLT1	35	11.3	99%	5375	96%
QLT2	30	9.79	99%	6381	95%
QLT3	38	7.39	99%	7513	95%
RBT1	>200000	0.696	100%	4920	69%
RBT2	>200000	0.593	100%	4280	62%
RBT3	>200000	1.19	100%	4427	68%
RCT1	161	9.42	99%	6577	91%
RCT2	170	20.9	99%	6197	88%
RCT3	125	18.8	99%	6034	90%
RLT1	47	20.4	99%	6522	94%
RLT2	39	6.02	99%	6912	95%
RLT3	42	21.4	99%	5281	95%
RRT2	2.4	52.9	97%	5229	96%
RRT3	2.7	33.5	97%	5546	95%
RRT4	2.3	22.5	97%	5681	96%

NORMAL SAMPLES

	DNA QC Assay	Library	NGS Metrics		
Samples	ratio (75/300)	Yield (nM)	% bases On-Target*	Average Coverage	Coverage Uniformity**
MBN1	NA	0.7	100%	1221	75%
MBN2	NA				
MCN1	544	4.1	99%	1238	90%
MLN1	44.2	9.8	99%	1234	95%
MRN1	2.1	24.6	97%	1308	95%
QCN3	766	1.4	99%	1064	88%
QLN3	29.1	7.6	98%	1365	94%
RBN1	NA	0.9	99%	786	84%
RBN2					
RBN3					
RCN3	513	3.6	99%	1302	92%
RLN1	43	9.8	99%	1918	96%
RRN1	2.5	59.6	98%	2275	97%

NA = Not attempted

PLASMA SAMPLES

	DNA QC Assay	Library	NGS Metrics		
Samples	ratio (150/300)	Yield (nM)	% bases On-Target*	Average Coverage	Coverage Uniformity**
Mplasma1	NA	51.7	99%	7372	93%
Mplasma2	2.5	41.7	98%	11485	97%
Mplasma3	2.1	24.1	97%	7191	97%
Mplasma4	2.4	54.1	98%	7489	97%

* % Bases On-taget: # bases aligned to the target / bases aligned to the human genome
** Coverage uniformity: Percentage of the bases covered at least 0.2x of the average depth

Table 1. Ratios of small to large amplicons as measured in the DNA QC assay are predictive of coverage uniformity and sequencing quality. For FFPE samples, a lower ratio of small (75 bp) to large (300 bp) amplicon target is indicative of less degradation of the DNA.

- **Green** = Amplicon ratios ≤47, coverage uniformity ≥93%
- **Yellow** = Amplicon ratios 125-766, coverage uniformity 85-92%
- **Red** = Amplicon ratios ≥ 1000, coverage uniformity ≤84%

Plasma samples show good coverage uniformity, but an unexpectedly low ratio of medium (150 bp) to large (300 bp) amplicon, indicating the presence of genomic DNA. Treatment of the plasma samples prior to purification is unknown and may affect these results. Further, initial mutation analysis of the plasma sample indicated that the source of this sample and that of the rectal FFPE samples did not come from the same donor.

7. Conclusions

- Levels of DNA obtained from FFPE samples vary greatly by tissue type, individual sample, and purification method.
- qPCR-based methods are more appropriate for determining quality of sample when degradation is of concern, and fluorescent quantitation methods may overestimate the amount of amplifiable materials present.
- High ratios of small amplicons to larger amplicons is a strong indicator for DNA degradation, with notable correlation to final coverage uniformity. Low coverage uniformity is indicative of low confidence for mutation detection.
- Therefore, qPCR-based QC assays may be a useful method for prediction of downstream NGS success and could be used to triage precious samples for less complex testing when QC measures indicate low probabilities of success for highly multiplexed sequencing.

1. For Research Use Only. Not for use in diagnostic procedures.