Microsatellite Instability Analysis and NGS with Fragmented Sample Types

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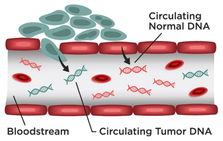
1. Introduction

A significant hurdle to using fragmented DNA for genomic studies is obtaining a sample of sufficient quantity and quality for rigorous downstream applications like NGS. Having effective tools to isolate, characterize, and analyze fragmented DNA containing samples, such as circulating cell free DNA (ccfDNA) and FFPE tissues, can prevent downstream failures, ultimately saving hours of work and precious samples. Here we present optimized methods for use with even highly fragmented DNA samples. Using this toolset, we demonstrate successful NGS and microsatellite instability (MSI) workflows using matched FFPE tissues and plasma samples.



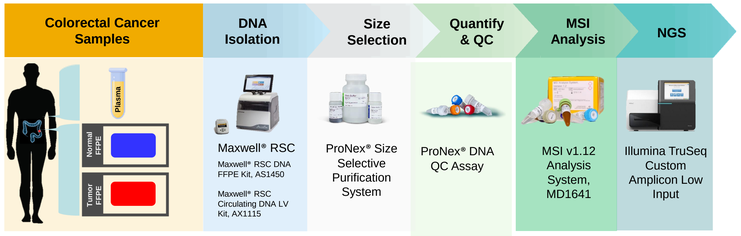
Objectives:

- *Demonstrate an effective workflow for matched tissue and plasma sample analysis*
  - *Including rigorous downstream applications: microsatellite instability, multiplex qPCR, and NGS variant detection*
- *Determine effect of ccfDNA size selection on variant detection*

2. Methods

Matched normal adjacent FFPE, tumor FFPE and plasma samples were obtained from three individuals (denoted A,B,C) with colorectal adenocarcinoma.

Experimental Work Flow

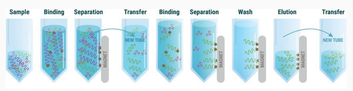


Automated DNA Isolation

- ccfDNA was purified from 4ml of plasma for each condition. DNA isolation was performed on the Maxwell ® RSC using a custom protocol and kit.
- DNA was purified from a single 10µm FFPE curl for normal and tumor tissues.

Size Selection

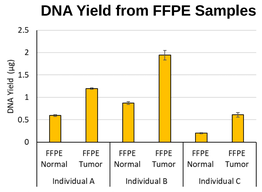
- Dual size selection performed on DNA from plasma to remove gDNA and enrich for ccfDNA



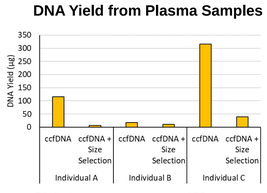
3. Amplification-Based DNA Quantitation

DNA concentration was measured using the ProNex® DNA QC Assay System. This system is a qPCR based method with 75, 150, and 300bp human DNA targets and an internal positive control. Quantitation of DNA was performed using the 150bp target.

DNA Yield from FFPE Samples



DNA Yield from Plasma Samples



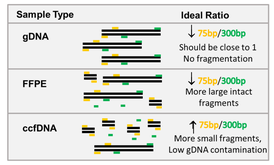
DNA concentration was calculated by linear regression analysis from a human DNA standard curve using a 150bp amplicon target. The + Size selection condition shows samples following ProNex size selection.

***Conclusions**: Efficient DNA isolation from FFPE and plasma samples. A sufficient amount of amplifiable,150bp or greater DNA was obtained from each sample to perform MSI and sequencing.*

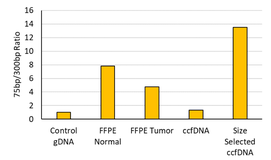
4. DNA Quality and Sizing

The ratio of 75bp to 300bp amplicon content in each sample was used to estimate DNA integrity. ccfDNA and FFPE samples types are highly fragmented sample types. Analysis of DNA integrity is important to accurately target the input template amount for downstream applications.

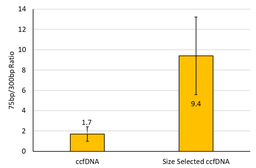
A. Using Quantitative PCR to Characterize DNA Integrity



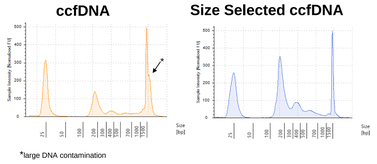
B. Amplification-Based DNA Size Characterization Using a 75bp/300bp Amplicon Ratio



C. Magnetic Bead-Based Size Selection Increases the 75bp/300bp Ratio of ccfDNA Samples



D. TapeStation Results Correspond with a qPCR-Based QC Method



A. Diagram of the DNA QC Assay used to characterize DNA integrity. **B.** Representative ratios of 75 to 300bp amplicon levels from a single individual as measured by multiplex quantitative real time PCR. **C.** Average 75/300bp ratios for ccfDNA with or without size selection. N=3 for each condition. **D.** Representative electropherogram images from samples run on an Agilent TapeStation with a D1000 High Sensitivity ScreenTape.

***Conclusions**: The 75/300bp amplicon ratio used by the DNA QC assay can estimate DNA integrity. Size selection decreases large DNA contamination of ccfDNA .*

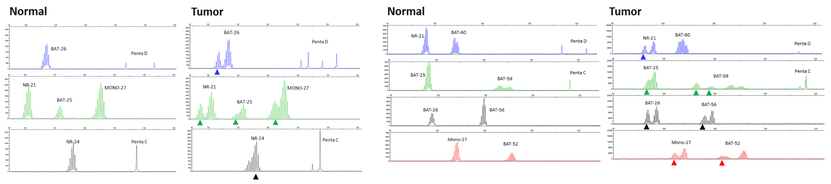
5. MSI Analysis

Microsatellite instability was examined using two MSI analysis systems with FFPE from tumor and normal adjacent tissue and ccfDNA .

MSI Analysis for FFPE Samples from Individual A

MSI v1.2 Analysis System
5 mononucleotide repeats

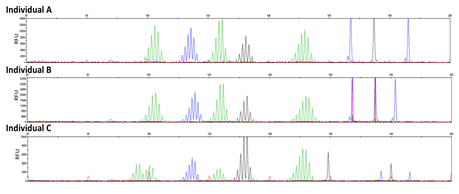
Prototype MSI Analysis System
4 mononucleotide repeats
+ 4 long mononucleotide repeats



**Arrow heads indicate a new mononucleotide peak (with max height >500bp) that is shifted greater than 2 nucleotides from the allele present in normal tissue.*

MSI with ccfDNA

- Full MSI profiles were obtained from all three individuals using size selected ccfDNA.
- No MSI was identified in ccfDNA, likely because these tumors were all regionally localized and may not have shed appreciable levels of DNA (stage IIA for individual B&C and stage IIIA for individual A).



***Conclusions**: MSI analysis was successful for FFPE and ccfDNA samples using two MSI analysis systems.*

6. NGS Quality

Next generation sequencing was performed using the TruSeq Custom Amplicon Low Input Kit by Illumina. Sequencing was run on a miSeq with v3 chemistry.

TruSeq Custom Amplicon Assay

PIK3CA, MAP2K4, EGFR, BRAF, PMS2, BRCA2, MSH6, MSH2, MLH1

**includes whole exon for each target*

- Successful sequencing was performed for each sample using a custom TruSeq Amplicon Assay
  - Whole exon sequencing with average of 89% coverage for 9 genes
  - 432 amplicons with 33,000bp total target
  - 150bp targeted amplicon size

Average Sequencing Quality Statistics by Sample Type

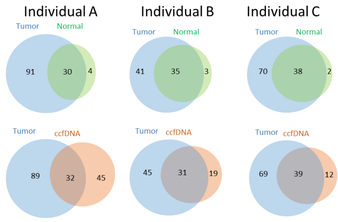
	Total Reads Passing Filter	Uniformity of Coverage	Mean Coverage Depth	Autosomal Call Rate
FFPE Normal	2,125,428	94.4%	2,880	93.2%
FFPE Tumor	2,188,240	92.8%	2,129	92.9%
ccfDNA	2,443,627	94.2%	3,748	91.4%
Size Selected ccfDNA	2,215,995	93.5%	2,866	90.3%

***Conclusions:** Successful targeted exome sequencing was performed with FFPE and ccfDNA samples.*

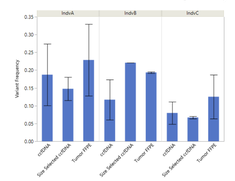
7. Variant Detection

Sequencing analysis was performed using the Illumina TruSeq Custom Amplicon Application on BaseSpace Sequencing Hub.

A. Pairwise Comparison of SNVs



B. Somatic Variant Read Frequency



C. Germline Variants Detected in Samples

	Sample	Gene	Position	Consequence	Variant Frequency	Total Read Depth
Individual A	FFPE NAT	MLH1	c.252delA	Frameshift Indel	0.6965	537
	FFPE Tumor				0.6911	1285
	ccfDNA+SizeSelection				0.5611	868
	ccfDNA				0.5156	1790
	FFPE NAT	PIK3CA	c.1634A>C	Missense	0.2864	405
	FFPE Tumor				0.6911	1285
	ccfDNA+SizeSelection				0.9705	305
	ccfDNA				0.1164	292
Individual B	FFPE NAT	PMS2	c.1864_1865 delAT	Frameshift Indel	0.4031	1826
	FFPE Tumor				0.4623	915
	ccfDNA+SizeSelection				0.4605	1998
	ccfDNA				0.4998	2565

MSI related variants detected:

- Individual A: germline MLH1 mutation
- Individual B: germline PMS2 mutation

A. Pairwise comparison of all SNV calls from each tissue. Shown is size selected ccfDNA. Analysis was performed using the Variant Calling Assessment Tool on BaseSpace. **B.** Somatic variants were defined as not occurring in matched normal tissue and having greater than 500 total read depth. Average somatic variant read frequency (reported as a fraction of total reads) for all somatic mutants normalized to total read depth. **C.** Germline variants were detected in normal and tumor FFPE samples at a frequency of greater than 15%. Listed variants were also filtered to have coding consequence.

***Conclusions:** Germline and tumor specific variants can be detected in ccfDNA.*

8. Conclusions

- ***Efficient DNA isolation from FFPE and plasma samples. Input for NGS library preparation and MSI analysis was calculated based on 150bp target quantities.***
- ***The 75/300bp amplicon ratio used by the DNA QC assay can estimate DNA integrity. Size selection decreases large fragment contamination of ccfDNA .***
- ***MSI analysis was successful for FFPE and ccfDNA samples using two MSI analysis systems.***
- ***Successful targeted exome sequencing was performed with FFPE and ccfDNA samples***
- ***Autosomal and tumor specific variants can be detected in tumor and ccfDNA.***

All chemistries and assays presented are For Research Use Only. Not for Use in Diagnostic Procedures.

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