Comparing Mutation Detection Sensitivity from Matched FFPE Tissue and Liquid Biopsy Plasma Samples Using Optimized High-Throughput Sample Preparation Workflows

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INTRODUCTION

Cancer researchers are avidly working to enable circulating cell free DNA (cfDNA) profiling as a new more sensitive tool to detect and screen for the presence of solid tumors before detection through clinical methods. Despite the high level of interest in cfDNA, researchers still have reservations until enough data has demonstrated complementarity between methodologies. In this study, we examined the data quality and concordance of mutations called for a small number of matched formalin fixed paraffin embedded (FFPE) tissue and plasma samples.

MATERIALS AND METHODS

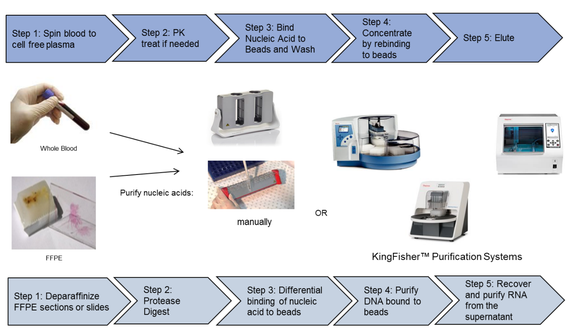
18 total matching FFPE tissue blocks and plasma from the same patients were acquired from Conversant Biologics. Age of samples ranged from 2015-2017. For each FFPE tissue block, one 5µM section was deparaffinized with a standard xylene protocol or with the use of spin cups followed by protease digestion. DNA and RNA were then isolated from the same sample following Figure 1 using the MagMAX™ FFPE DNA/RNA Ultra Kit (A31881) on the KingFisher™ Presto integrated with the Hamilton NIMBUS automation system allowing for hands-free automation. The obtained nucleic acids were quantified with the Qubit™ dsDNA and RNA assay respectively for DNA and RNA quantification. RNA was also assessed using the Agilent 2100 Bioanalyzer® instrument. A nano chip was run to obtain RNA integrity information. As expected, samples were degraded with RIN values of <2.5 however RNA fragments obtained were relatively large.

Corresponding matching cell-free plasma (2-4mL) was isolated with the MagMAX™ Cell-Free DNA Isolation Kit (A29319) or the MagMAX™ Cell-Free Total Nucleic Acid Isolation Kit (A36716), which allows for isolation of both cfDNA and cfRNA. Samples were eluted in a low elution volume of 15µl so that the majority of the sample can go straight into library prep. Conversant Biologics pre-spun the plasma prior to shipping samples to make them cell-free. All samples were run on the Agilent 2100 Bioanalyzer® instrument with the High Sensitivity kit to assess the cfDNA peak versus gDNA contamination.

gDNA or cfDNA from the FFPE tissue samples and plasma samples were made into libraries with the Oncomine™ Breast and Colon cfDNA Assay using 10ng of DNA from the FFPE tissue samples or the total volume of cfDNA from plasma. For a subset of samples where cfTNA was isolated, libraries were made with the Oncomine™ Lung cfTNA Research Assay. Final libraries were quantified by qPCR using the Ion Library Quantitation kit. We used the Ion Chef™ instrument for template preparation and chip loading. Samples were sequenced on the Ion S5 Sequencer on 530 or 540 chip. After runs were completed, data were analyzed with the Variant Caller on the Torrent Suite using the appropriate Oncomine Liquid Biopsy or Oncomine Tumor analysis plugin.

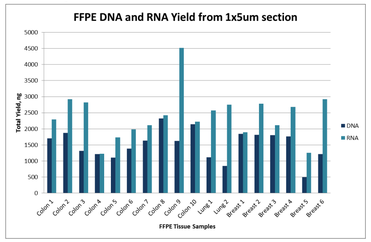
For some of the mutations detected by sequencing, there were validated TaqMan Liquid Biopsy dPCR Assay. dPCR reactions were set up with the QuantStudio 3D Digital PCR 20K Chip Kit v2 using the QuantStudio 3D Digital PCR Master Mix v2. Chips were then read with the QuantStudio 3D Digital PCR System. Analysis was performed in the Thermo Fisher Scientific Cloud analysis suite.

Figure 1. MagMAX™ Sample Prep Workflow



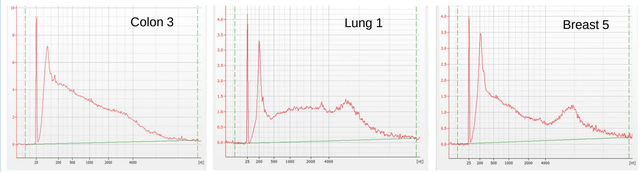
RESULTS

Figure 2. DNA and RNA yield from FFPE tissue



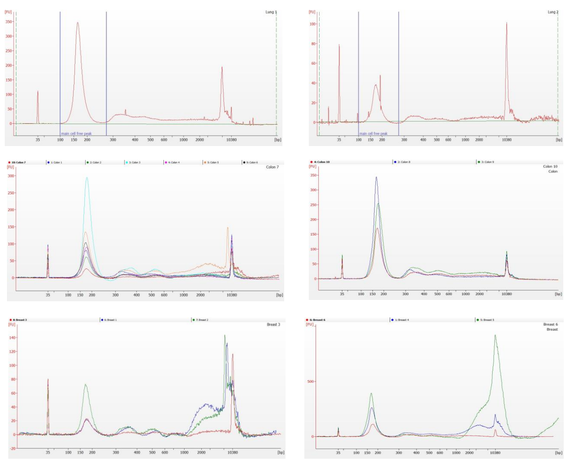
Both DNA and RNA were extracted from 1x5um FFPE section with the KingFisher™ Presto Purification system integrated with the Hamilton NIMBUS. Nucleic acids were quantified with the Qubit™ dsDNA and RNA assay

Figure 3. Example RNA traces from FFPE tissue samples



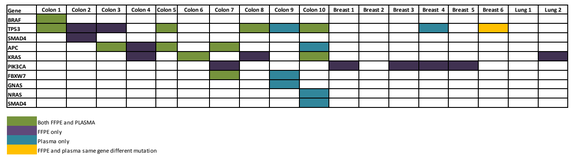
1ul of RNA was loaded on an Agilent RNA Nano chip. In some samples, remnant ribosomal peaks were visible. RIN values are <2.5 but on average 85% of the sample had fragments >200nt.

Figure 4. Example cfDNA traces from plasma samples



1ul of cfNA was loaded on an Agilent HS DNA chip. The main cell-free peak is highlighted between 100-225bp. Fragments larger than ~1000bp are considered contaminating gDNA.

Figure 5. Concordance Heatmap



Overview of all 18 samples analyzed by sequencing. True concordant positive mutations are highlighted in green. Mutations detected only in FFPE samples are highlighted in purple. Mutations detected only in plasma samples are highlighted in blue. Partially concordant mutations were highlighted in yellow.

Figure 6. Detailed Concordance Chart

Samples	AA change	Gene	Seq. Allelic Freq in FFPE	Seq. Allelic Freq in Plasma	dPCR Allelic Freq in FFPE	dPCR Allelic Freq in Plasma
* Colon 1	pV600E	BRAF	12.69	13.59	7.99	10.02
	p.R273C	TP53	2.33	0.59		
	p.G244D	TP53		8.35		
Colon 2	p.R213Q	TP53	29.3	-		
	p.R361H	SMAD4	32.66	-		
Colon 3	p.R1114Ter	APC	17.89	-		
	p.E1309fs	APC	35.57	0.5		
	p.G244D	TP53	51.88	-		
	p.R273H	TP53	0.57	-	<LOD	
	P.G245S	TP53	0.4	-	<LOD	
# Colon 4	pE1309fs	APC	31.78	-		
	pG12D	KRAS	26.41	-	13.27	
Colon 5	p.E1379Ter	APC	58.6	0.16		
	pG245S	TP53	46.88	0.05	38.97	< LOD
Colon 6	p.G12V	KRAS	34.06	0.09	29.098	<LOD
# Colon 7	p.S45K	PIK3CA	4.63	-	<LOD	
	p.Q546K	PIK3CA	11.45	-		
	p.R479Q	FBXW7	15.08	0.34		
	p.Q1291Ter	APC	16.41	0.09		
# Colon 8	p.R248W	TP53	50.15	0.054		
	p.G12V	KRAS	38.15	-		
# Colon 9	p.R465C	FBXW7	-	0.053		
	p.H179Y	TP53	-	0.051		
	p.R175H	TP53	-	0.127		
	p.R201H	GNAS	-	1.461		
# Colon 10	p.G13D	KRAS	23.729	0.281		
	p.R248W	TP53	38.667	0.348		
	p.Q61K	NRAS	-	0.064		
	p.E1306Ter	APC	-	0.125		
	p.R273L	TP53	-	0.066		
	p.R213L	TP53	-	0.066		
	p.R175L	TP53	-	0.052		
	p.G510V	SMAD4	-	0.055		
Breast 1	p.E542K	PIK3CA	3.12	-	<LOD	
	p.H1047R	PIK3CA	1.51	-	1.48	
# Breast 2	No Mutations					
Breast 3	p.H1047R	PIC3CA	52.09	-	<LOD	
Breast 4	p.C238Y	TP53	-	0.088		
	p.H1047R	PIK3CA	20.912	-		
Breast 5	p.H1047R	PIK3CA	44.248	-		
Breast 6	p.R273H	TP53	-	0.298		
	p.L194R	TP53	-	0.186		
	p.C176Y	TP53	40.319	-		
# Lung 1	NA	NKX2-1	NA	NA		
# Lung 2	p.Gly12Cys	KRAS	8.99	-	7.9	

Not Tested
- = not detected
True positive concordance @ nucleotide change

All detected hotspot mutations are listed by gene and amino acid change. Observed allelic frequencies from FFPE tissue and plasma are recorded. Some mutations were confirmed by digital PCR. All plasma samples were 2mL except Colon 8, 9,10 and Breast 4, 5, 6 which were 4mL.

Figure 7. Sample Characteristics

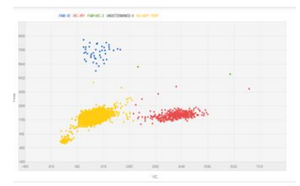
Mean Age (years) of samples	n=18	62.6
Sex		
Male	n=3	16.70%
Female	n=15	83.30%
Type of Cancer		
Colon	n=10	55.60%
Breast	n=6	33.30%
Lung	n=2	11.10%
Cancer Stage		
<III	n=9	50.00%
III #	n=8	44.40%
IV *	n=1	5.60%
Smoking History		
current/ former	n=2	11.10%
never	n=16	88.90%
Alcohol History		
current/former	n=1	5.60%
never	n=17	94.40%

Figure 8. Composite NGS data comparing cfNA vs. FFPE Tissue

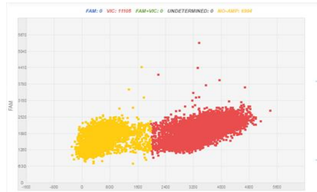
Total number of alterations dected in the cfNA samples	25
Total number of alterations dected in the FFPE tissue samples	29
Mean allele frequency in cfNA samples	1.08% (0.050%-13.59%)
Mean allele frequency in FFPE tissue samples	23.7% (0.15%-58.6%)
Number of unique cfDNA mutations not detected in tissue	15
Number of unique FFPE tissue mutations not detected in cfNA	19
Number of true positive concordant* mutations * same exact gene and nucleotide change dectected in both sample types n=44 total alterations detected for both sample types	n=10; 22.70%

Figure 9. Example digital PCR Plots-Colon 1 BRAF pV600E

Wild-type Control-plasma sample



Colon 1-plasma sample



Wild-type Control-FFPE sample

Colon 1-FFPE sample

CONCLUSIONS

Overall, our small study demonstrates that data from FFPE tissue and plasma samples are complementary. Although concordance was low with low allelic frequencies in some of the plasma samples, confidence is high due to the same alteration being detected in the matching FFPE tissue. Sensitivity of alterations detected in plasma could presumably be increased with more plasma i.e. >4mL. The sequencing data revealed that each sample type-FFPE tissue or plasma, independently detected alterations not found in the other sample demonstrating that additional information can be provided from the other sample type. Concordance rates will vary from study to study depending on the samples evaluated i.e. certain cancer types will have more detectable cfNA, cancer stage as well as the treatment status of the patients. Understanding the sample source material paired with optimized sample preparation and mutation detection methods is an important first step for developing molecular profiling research tools and ultimately to develop diagnostics and personalized cancer treatments.

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ACKNOWLEDGEMENTS

We would like to thank Conversant Biologics Inc. for kindly collaborating with us to procure and provide high quality FFPE tissue blocks and cell-free plasma.

TRADEMARKS/LICENSING