Formalin Fixation Delivers Quality Biomarker Results by NGS and IHC

A case study of three non–small cell lung carcinomas (NSCLC), Adenocarcinoma (ADC) subtype

Application Note

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Abstract

This study demonstrates that high quality and well-correlated biomarker analysis with IHC and NGS are possible with formalin-fixed paraffin embedded tissue samples. Here, three non-small cell lung carcinoma (NSCLC) cases were utilized to study DNA, RNA, and protein biomarkers with NGS and IHC. Quality control results post-extraction and post NGS are highlighted.

Introduction

Correlation between biomarkers at various cellular levels (DNA, RNA, protein) is an important facet of biomedical research that may help elucidate neoplastic cell development. In some clinical settings, biomarker correlation can help guide treatment decisions (1, 2,3). Immunohistochemistry (IHC) continues to be a popular method to study protein expression of target biomolecules in tissue sections while next generation sequencing (NGS) has become the leading technology to accurately assess RNA expression and pinpoint critical mutations in DNA. IHC and NGS are often utilized to study and characterize a tumor, and in some cases, serve as aids in diagnostic testing. In this study, we show that tissues under certain pre-analytical conditions yield reliable biomarker results with both IHC and NGS. Three non-small cell lung carcinoma (NSCLC) tissues of the adenocarcinoma (ADC) subtype were studied.

Formalin-fixed paraffin embedded (FFPE) tissues comprise a plentiful biospecimen archive that are often well-annotated with histopathologic findings and linked with clinical information and outcomes. Formalin fixation has been shown to preserve antigens well for detection with IHC and is recommended for preservation of breast tissues and lung tissues for IHC testing (4, 5, 6, 7, 8, 9). Though prolonged formalin fixation has been shown to degrade nucleic acids, under the right preanalytical conditions, the effects of formalin fixation on nucleic acids does not appear to negatively impact DNA and RNA assessment with NGS (10, 11, 20).

Proper biospecimen preservation, processing, and handling of FFPE tissues is key to successful correlation of biomarkers. Formalin fixation is considered the gold standard for specimen preparation ahead of IHC (4, 5, 6, 7, 8, 9). Guidance documents are available that recommend between 6 and 72 hours of fixation in 10% NBF for breast cancers (4, 5, 6, 7, 8, 9). Independent research articles corroborate these pre-analytical conditions for other cancers, including lung cancers (6, 12). For the size of the tissue specimens in this study, 24 hours of formalin fixation was sufficient for appropriate IHC reaction and did not appear to hinder NGS test results. During NSG Library preparation, DNA or cDNA sequences are physically fragmented into small strands prior to adaptor ligation (13). The generated data are aligned to a reference genome and variants are called (13, 14). The nature of induced nucleic acid fragmentation and digital data alignment may make some allowances for pre-fragmented nucleic acid sequences that occur in FFPE tissues.

In this study, IHC biomarkers were selected to differentiate between NSCLC adenocarcinoma and squamous cell carcinoma subtypes. Common IHC markers for squamous cell carcinoma (SCC) subtype

include cytokeratin 5, p63, and p40 (2, 15). Common IHC markers for adenocarcinoma subtype include Napsin A and thyroid transcription factor-1 (TTF-1) (2, 15). If taking a minimalist approach to conserve biopsy material, such may be the case in needle core biopsies or cytology cases, p40 and TTF1 appear to be the recommended IHC antibodies for use on NSCLC to help distinguish between SCC and ADC (2, 3). In the selected NSCLC ADC cases, antibodies were selected based on the information provided above. In all cases the histopathology was previously known and samples were tested and annotated by the biobank.

Materials & Methods

Tissue Procurement and Fixation

Three different annotated, flash-frozen lung carcinoma cases were selected from a commercial biobank (BioIVT). These cases conformed to the following selection criteria: de-identified patient information, 2+ grams of >95% tumor volume and an RNA integrity number (RIN) of > 9.0). The cases were NSCLC, ADC subtype. These tissues had a delay to fixation (DTF) of 1 hour or less. All tissues were handled and prepared using RNAse-minimized conditions. The tissues were grossed to 10 x 10 x 3 mm, placed in a nuclease-minimized tissue cassette, and fixed in 10% neutral buffered formalin (NBF) (Epredia) for 24 hours.

Tissue Processing, Embedding, and Microtomy

The tissues were processed using the Excelsior AS tissue processor (Epredia). The processing protocol can be found in Table 1; note all fixation was conducted offline. The tissues were embedded in Histoplast LP paraffin (Epredia) using the HistoStar embedding station (Epredia) and sectioned on the HM 355S microtome (Epredia) using a nuclease minimized technique. For the nuclease minimized technique, RNAse-Away (MB Bio, Fisher Scientific) was utilized to treat Nitrile-gloved hands, implements, utensils, tools, and surfaces. Molecular biology grade deionized (MBDI) water (Fisher Scientific) was used in a nuclease-minimized water bath for FFPE microtome sections. Each block was sectioned at 4 microns: FFPE ribbons were floated onto the MBDI water bath and positively charged slides were used to collect sections for IHC. The slides were air dried overnight and baked in a 60ºC oven for 30 minutes. Each block was additionally sectioned at 7 microns to create FFPE curls. The curls were placed into sterile 2 mL microcentrifuge tubes for biomolecule extraction.

Table 1. Tissue Processing Protocol, Excelsior AS tissue processor. Note that formalin fixation occured offline, for 24 hours prior to processing.

Step	Reagent	Time (min)	Temperature (ºC)
1	10% NBF	0	Ambient
2	10% NBF	0	Ambient
3	75% Dehydrant	30	Ambient
4	90% Dehydrant	30	Ambient
5	95% Dehydrant	40	Ambient
6	100% Dehydrant	30	Ambient
7	100% Dehydrant	30	Ambient
8	100% Dehydrant	40	Ambient
9	Xylene	30	Ambient
10	Xylene	30	Ambient
11	Xylene	40	Ambient
12	Paraffin (Histoplast LP)	30	62
13	Paraffin (Histoplast LP)	30	62
14	Paraffin (Histoplast LP)	40	62

Table 2. IHC staining protocol

Step	Procedure
1	Deparaffinize and rehydrate tissue section.
2	Buffer wash step.
3	If required, incubate tissue in appropriate pretreatment or digestive enzyme.
4	Buffer wash step.
5	To reduce nonspecific background staining due to endogenous peroxidase, incubate slide in UltraVision Hydrogen Peroxide Block for 10 minutes.
6	Buffer wash step.
7	Apply UltraVision Protein Block and incubate for 5 minutes to block nonspecific background staining. NOTE: Do not exceed 10 minutes or there may be a reduction in desired stain. (May be omitted if primary antibodies are diluted in buffers containing 5-10% normal goat serum.)
8	Blow step.
9	Apply primary antibody and incubate according to manufacturer's recommended protocol.
10	Buffer wash step.
11	Apply Primary Antibody Amplifier Quanto and incubate for 10 min.
12	Buffer wash step.
13	Apply HRP Polymer Quanto and incubate for 10 min. (NOTE: HRP Polymer Quanto is light sensitive. Please avoid unnecessary light exposure and store in opaque vial.)
14	Buffer wash step, followed by wash steps with DI water and buffer.
15	Add 30 µl (1 drop) DAB Quanto Chromogen to 1 ml of DAB Quanto Substrate, mix by swirling and apply to tissue. Incubate for 5 minutes.
16	DI water wash step.
17	Counterstain and coverslip using a permanent mounting media.

Table 3. Primary antibodies and pretreatment methods

Antibody	Clone	Vendor	Antibody Cat. No.	Pretreatment	Vendor	Pretreatment Cat. No.
Keratin 5/6	D5/16/B4	Epredia	MS-1814-RQ	HIER Buffer M	Epredia	TA-135-HBM
Keratin 7	OV-TL	Epredia	MS-1352-RQ	HIER Buffer M	Epredia	TA-135-HBM
Keratin 20	Ks20.8	Epredia	MS-377-RQ	HIER Buffer M	Epredia	TA-135-HBM
TTF-1	8G73/1	Epredia	MS-699-P	HIER Buffer L	Epredia	TA-135-HBL
Napsin-A	EPR6252	Epredia	RM-2121-S	Dewax and HIER Buffer H	Epredia	TA-999-DHBH
EGFR	EP384	Epredia	RM-2111-S	HIER Buffer M	Epredia	TA-135-HBM
Her2	SP3	Epredia	RM-9103-RQ	HIER Buffer L	Epredia	TA-135-HBL

Immunohistochemistry

IHC slides were deparaffinized and rehydrated to water through manual exchanges of xylene, reagent grade alcohols, graded alcohols, and DI water. All IHC slides received heat induced epitope retrieval (HIER) by being placed in either HIER Buffer L (citrate-based), HIER Buffer M (EDTA-based), or Dewax and HIER Buffer H (Tris-EDTA based) inside the PT Module (Epredia) and processed to 98ºC for 20 minutes, followed by a 20-minute cool down period. All slides were immuno stained on the IHC Autostainer 360 (Epredia) using the IHC staining protocol in Table 2. Primary antibodies utilized in this study include and the pretreatment methodology can be found in Table 3. This study utilized the UltraVision Quanto HRP/DAB Detection System (Epredia). All slides were evaluated by a pathologist using a digitized scoring system (Table 4).

Biomolecule Extraction

The MagMax™ Total Nucleic Acid Isolation Kit (Thermo Scientific) was utilized to extract RNA and DNA from FFPE samples. FFPE curls were deparaffinized and rehydrated to MBDI water according to the MagMax™ instructions for use. Heated steps were performed using a ThermoMixer™ (Eppendorf) and centrifugation steps were performed using the Sorvall™ Legend™ 17R Microcentrifuge (Thermo Scientific). Extracted nucleic acids were quality tested for quantity using the Qubit™ 3.0 Fluorometer (Thermo Scientific), the Qubit dsDNA BR Assay Kit (Cat. No. Q32850), and the Qubit RNA BR Assay Kit (Cat. No. Q10210). The extracted nucleic acids were also quality tested for purity using the 2100 BioAnalyzer (Agilent Technologies) and the Nanodrop 2000c (Thermo Scientific). Quality control data is important ahead of NGS Library preparation.

Next Generation Sequencing (NGS)

Nucleic acids were processed through the Ion Ampliseq workflow using the Thermo Scientific Ion Ampliseq RNA and DNA Cancer 50 gene panels upon the Thermo Scientific Ion Personal Genome Machine (PGM). The goal is to prepare an NGS library from the sample that has reads that are evenly distributed across the entire region of interest (that would be "100%", however, 100% is not a realistic target with actual NGS data). Some regions of the target sequence will be over-represented, and some will be under-represented. Some sources note that a score of 90% or greater is considered good quality; others indicate that a score of 80% or greater is considered good quality (16, 17, 18). Read Alignment is another quality metric. This measurement describes the base reads on target. Bases generated from the sample are considered of target if they align with the targeted region. Alignment of 90% or greater is considered good quality (16, 17, 18).

Table 4. Pathologist scoring system

DB Digitize Staining	
Pathologist Score	Digitized Score
3+	3.50
3+/-	3.25
3+/3-	3.25
3	3.00
3-/3	2.85
3-	2.75
2+/3-	2.63
2+	2.50
2/3-	2.50
2/2-	2.25
2	2.00
2-/2	1.85
2-	1.75
1+/2-	1.63
1+	1.50
1+/-	1.25
1	1.00
0	0.00

Results and Discussion

Quality Control

Quality control testing of the resulting nucleic acids was performed after biomolecule extraction and after NGS. Extracted DNA was tested for concentration (yield) and purity using the Nanodrop 2000c spectrophotometer (A260/230 and A260/280 ratio analysis). Extracted RNA was tested for the concentration (yield) via the Qubit 3.0 Fluorometer and purity was tested using the Nanodrop 2000c spectrophotometer (A260/230 and A260/280 ratio analysis) and Agilent Bioanalyzer (RIN value analysis).

The average DNA yield of each of the lung carcinoma samples was above the 10 ng minimum yield required by the Ion Ampliseq DNA Cancer 50 gene panel (10). The samples averaged 22.1, 15.5, 12.2 ng/µl in DNA yield (Figure 1). Nanodrop spectrophotometry ratios were used to measure the relative purity of nucleic acids absorbing at 260 nm compared to protein that absorbs at 280 nm, a measure indicative of nucleic acid concentration. Nanodrop spectrophotometry ratios were also used to measure the relative purity of nucleic acids absorbing at 260 nm compared and salt impurities that absorb at 230 nm. The FFPE samples showed a DNA absorbance ratio of 1.6 at 260/280 nm with an average absorbance ratio of 0.6 at 260/230 nm (Figure 2).

The average RNA yield of each of the lung carcinoma samples was above the 5 ng minimum yield required by the Ion Ampliseq RNA Cancer 50 gene panel (10). The samples averaged 16.4, 12.7, and 10.2 ng/µl in RNA yield (Figure 3). Nanodrop spectrophotometry ratios were used to measure the relative purity of nucleic acids absorbing at 260 nm compared to protein that absorbs at 280 nm and salt impurities that absorb at 230 nm. The FFPE lung carcinoma samples showed an RNA absorbance ratio of 1.8 at 260/280 nm with an average absorbance ratio of 1.0 at 260/230 nm (Figure 5).

RIN was determined using the Agilent Bioanalyzer and represents the condition of the assayed RNA relative to intact total RNA on a scale from 1 to 10. The higher the RIN value, the more completely intact the RNA strand is, compared to intact 18s and 28s rRNA. Good quality fresh or frozen tissue sample should have a RIN value of 8 or higher; however, formalin fixation and processing to paraffin sections is known to cause RNA strand

Figure 1. Average DNA yield from three lung carcinoma cases. Each case contained 10 replicates. The minimum required yield for the Ion Ampliseq Cancer Hotspot Panel is 10 ng/µl.

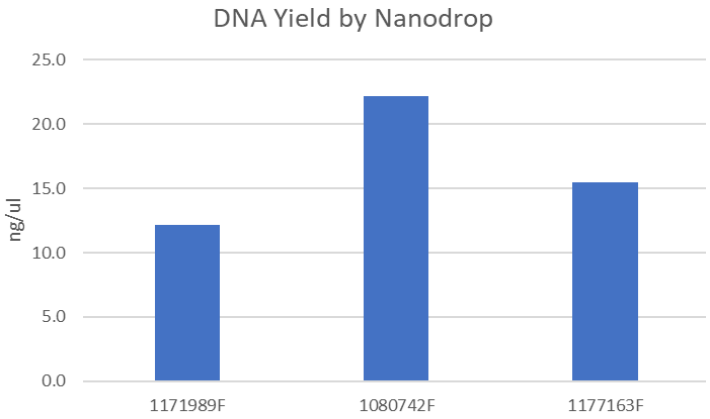


Figure 2. Average DNA purity from three lung carcinoma cases. Each case contained 10 replicates.

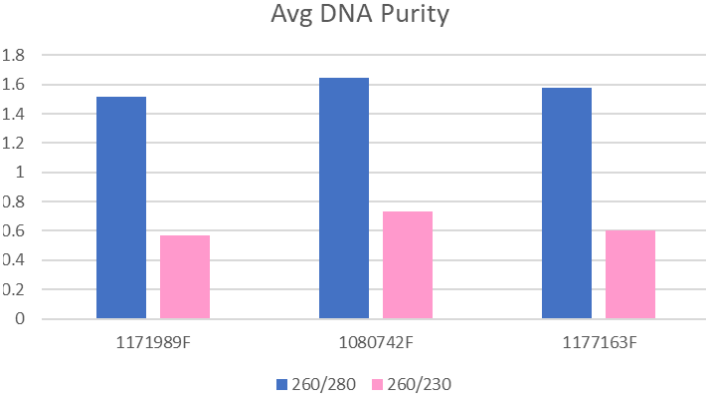


Figure 3. Average RNA yield from three lung carcinoma cases. Each case contained 10 replicates. The minimum required yield for the Ion Ampliseq RNA Cancer Panel is 5 ng/µl.

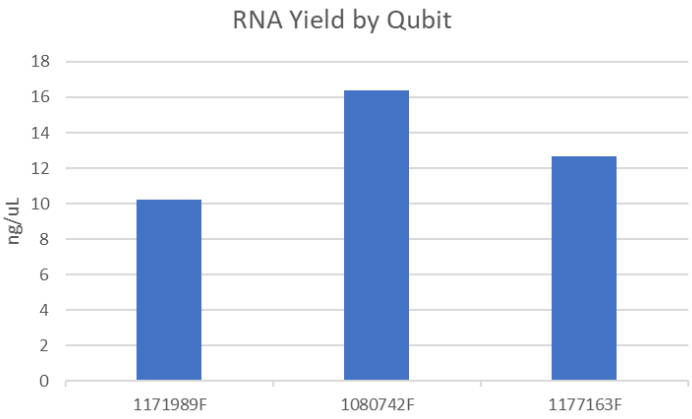
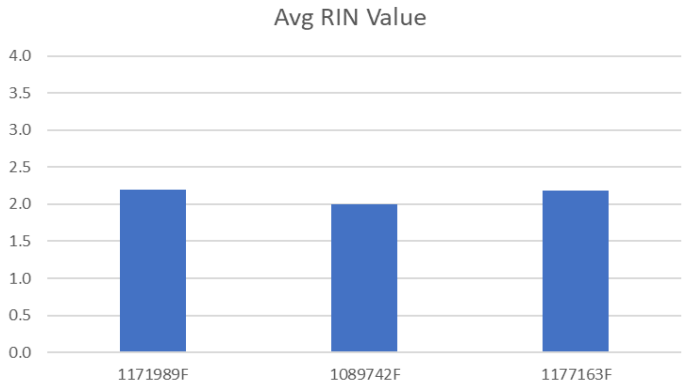


Figure 4. Average RNA purity from three lung carcinoma cases. Each case contained 10 replicates. The average RIN value for most FFPE processed tissues is ~ 2.



degradation (21). This can be problematic for PCR methods, but for next generation sequencing smaller strands can still be assessed. The average RIN value of formalin-fixed paraffin embedded tissues is typically around 2; and although a low score, generally a minimum RIN value of 1.4 is necessary to be useful (14). The RIN values for all FFPE lung carcinoma samples averaged to 2 or greater (Figure 4).

NGS run metrics were also reviewed for DNA and RNA. Images in Figures 6 and 8 are from the Ion Torrent run reports and detail several metrics of the NGS runs. ISP loading refers to the percent of chips that contain an Ion Sphere Particle (ISP), and the ISP Loading density displays the loading distribution inside the wells (16). The coloration ranges from red (high loading) to blue (low loading) (16). An ISP minimum of 30% has been noted as a good quality run (17). The samples in this study delivered between 55% and 87% for ISP loading (Figures 6 and 8). Total reads include filtered and trimmed reads (16). Useable reads are those that pass internal quality filters; a good run should also have a minimum of 30% useable reads (16, 17). The samples in this study delivered useable reads between 51% and 72% (Figures 6 and 8).

The final library percent is an indicator of the percentage of reads that pass all filters (16) in which a 100% would indicate perfectly even distribution across the entire region of interest. With NGS some regions of the target sequence are over-represented and other areas have little or no coverage A score of 90% or greater is often marked as good quality; although other sources note that a score of 80% also deliver good results (16, 17, 18). Sample 1171980F delivered a final library percent of 88% for DNA

Figure 5. Average RNA purity from three lung carcinoma cases. Each case contained 10 replicates.

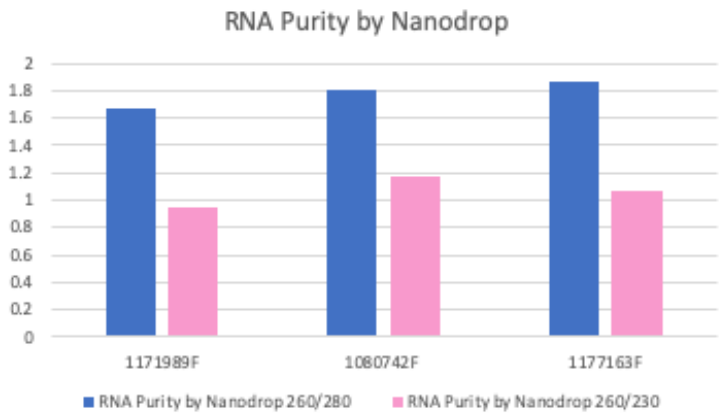
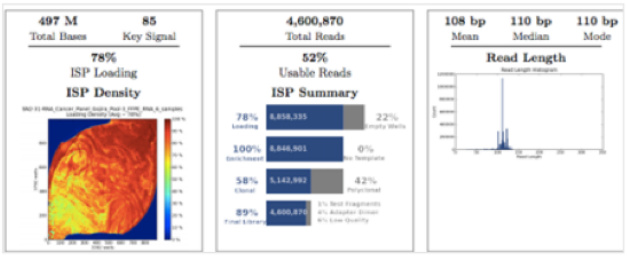
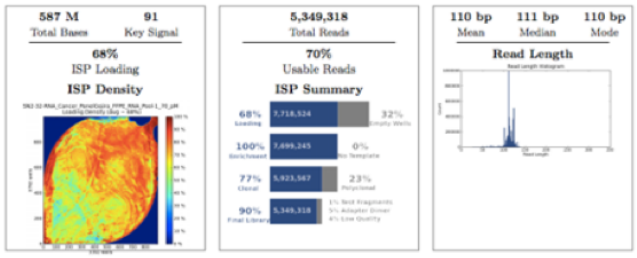


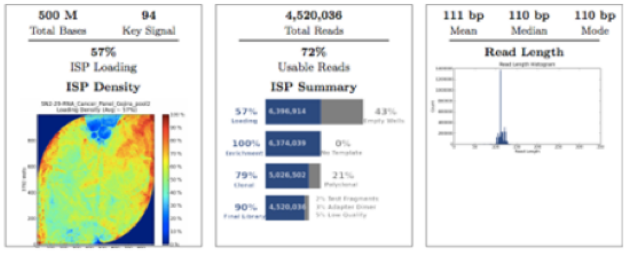
Figure 6. The NGS run statistics for the RNA assessment of three lung carcinomas. Note the high percentage of reads aligned to target for all three formalin-fixed paraffin embedded lung carcinoma samples 1171989F, 1080742F, and 1177163F.



1171989F – RNA Run Summary



1080742F – RNA Run Summary



1177163F – RNA Run Summary

Sample ID	Sample Type	Biomarker	Final Library %	Total Reads	Aligned Total Reads	% Reads Aligned to hg 19
1171989F	Lung CA	RNA	89%	4,443,216	4,414,839	99.4%
1080742F	Lung CA	RNA	90%	5,230,883	5,198,022	99.4%
1177163F	Lung CA	RNA	90%	4,404,145	4,395,891	99.8%

Figure 7. The NGS Library preparation data of the three lung carcinoma samples. Each of the samples were prepared for DNA and RNA analysis by NGS. A final library percent of 80%-90% is good quality.

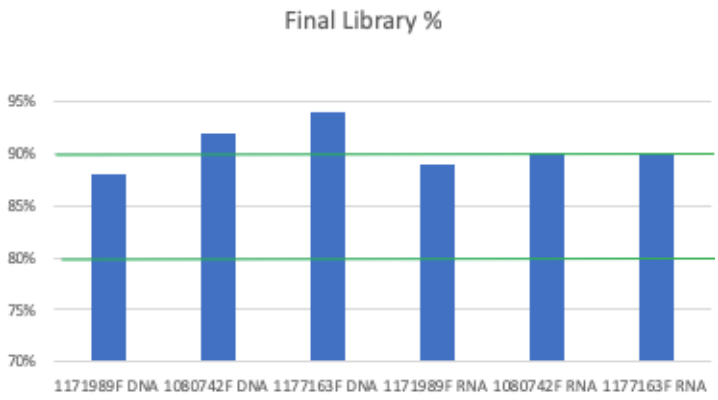


Figure 9. The NGS Read Alignment data of the three lung carcinoma samples. Each of the samples were prepared for DNA and RNA analysis by NGS. A read Alignment percent above 98% is good quality.

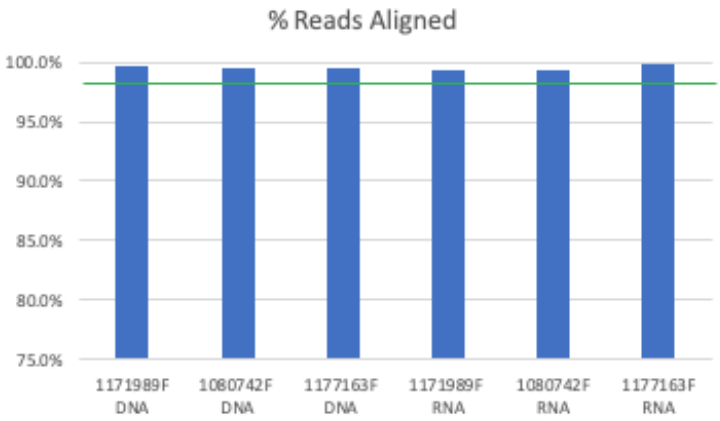
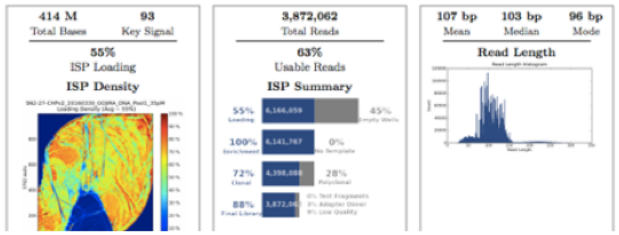
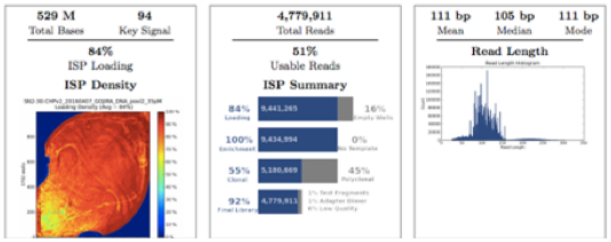


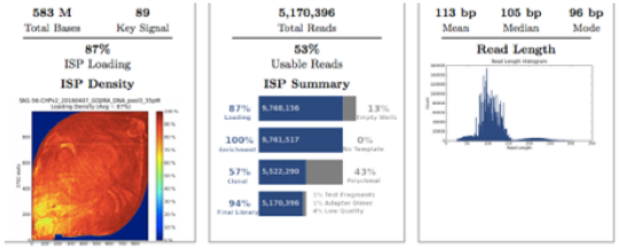
Figure 8. The NGS run statistics for the DNA assessment of three lung carcinomas. Note the high percentage of reads aligned to target for all three formalin-fixed paraffin embedded lung carcinoma samples 1171989F, 1080742F, and 1177163F.



1171989F – DNA Run Summary



1080742F – DNA Run Summary



1177163F – DNA Run Summary

Sample ID	Sample Type	Biomarker	Final Library %	Total Reads	Aligned Total Reads	% Reads Aligned to hg 19
1171989F	Lung CA	DNA	88%	3,796,248	3,783,283	99.7%
1080742F	Lung CA	DNA	92%	4,638,366	4,661,848	99.5%
1177163F	Lung CA	DNA	94%	5,012,135	4,989,996	99.6%

and 89% for RNA; samples 1177163F and 1080742 delivered a final library percent score of at least 90% (Figures 6-9). Percent reads aligned is another NGS metric. Bases generated from the sample are considered "on target" if they align with the targeted region of the reference genome (16). A score of 98% or greater is considered good quality (16, 18). All samples delivered percent reads aligned scores of at least 99.4% (Figures 6-9).

Immunohistochemistry

All immunohistochemistry slides were read by a pathologist using the scoring system shown in Table 4. Stain results (Table 5) were consistent with expectations for non-small cell lung carcinoma, adenocarcinoma subtype (15, 19). Case 1171989F biomarker status includes positive staining for cytokeratin 7, TTF1, Napsin A, EGFR overexpression, MET and KRAS. The tumor does not express proteins for cytokeratin 5, cytokeratin 6, or cytokeratin 20. Case 1080742F is also a known NSCLC of the ADC subtype. Its biomarker status includes positive staining for all protein-based markers by IHC, except for Napsin A. Case 1177603F is the third NCSLC of the ADC subtype in the study. Its biomarker status includes positive staining for cytokeratin 7, cytokeratin 20, TTF1, Napsin A, and MET. The tumor does not express proteins for cytokeratin 5, cytokeratin 6, or overexpress Her2. Expression of EGFR was classified at a 2+ staining intensity, which is equivocal by IHC. A summary of the immunohistochemistry results for lung samples is also in Table 5, select micrographs are displayed in Figure 10.

Figure 10. IHC Images (a) Lung CA 171989F6 CK7, 200X, (b) Lung CA 171989F6 EGFR, 200X (c) Lung CA 171989F6 TTF1, 200X (d) Lung CA 171989F6 Napsin A, 200X.

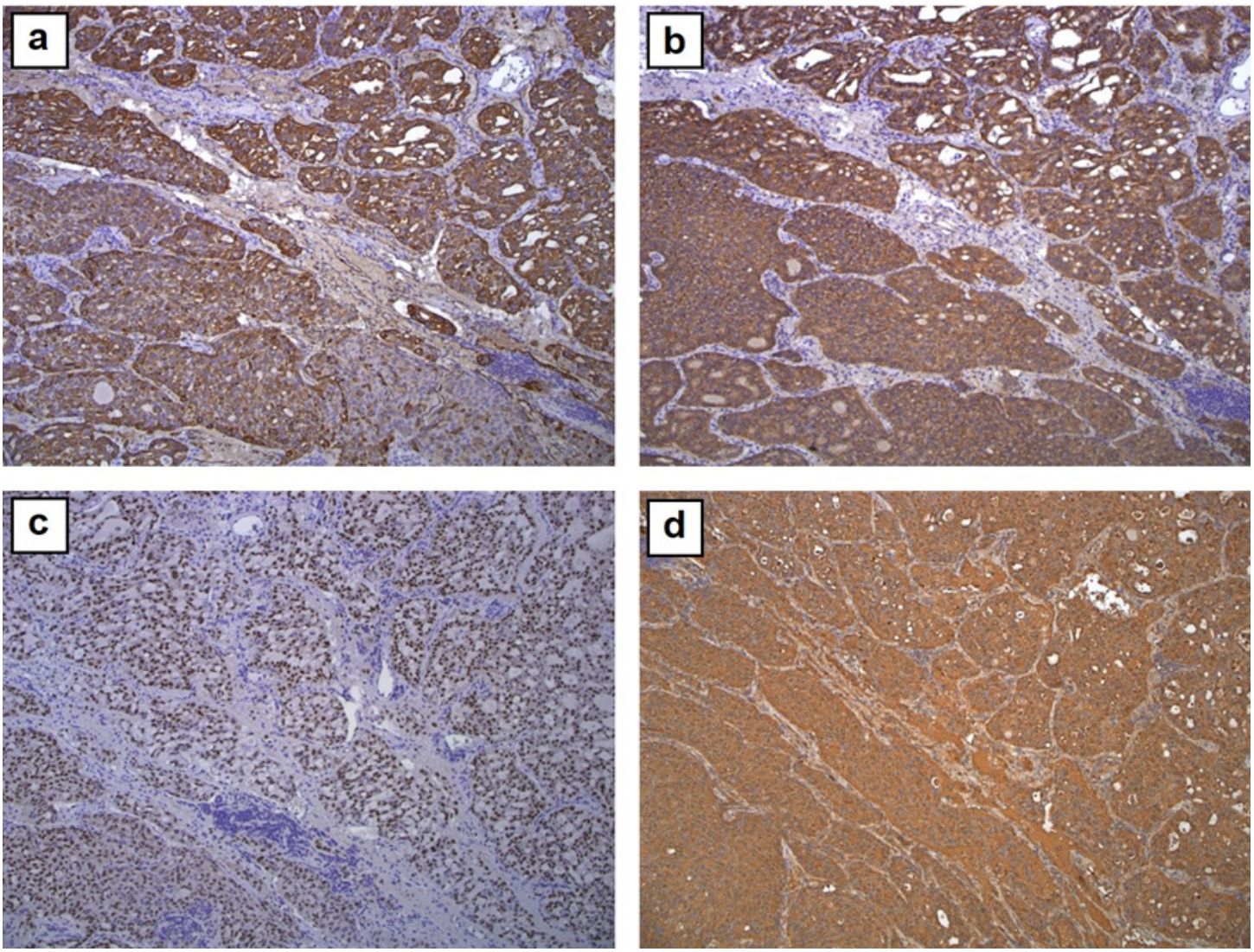


Table 5. Summary of biomarker data from three different lung adenocarcinoma cases. Green indicates a positive result. Orange indicates a negative result. NA is not applicable (not tested).

Lung CA 1171989F (ADC)			
Lung CA	NGS DNA	NGS RNA	FFPE Actual IHC
Ker5	NA	NA	Neg
Ker 6	NA	NA	Neg
Ker 7	NA	NA	Pos (3-4)
Ker 20	NA	NA	Neg
TTF1	NA	NA	Pos (3-4)
Napsin A	NA	NA	Pos (3-4)
EGFR	5 Mutations	Over-Expressed (+)	Pos (3-4)
Her2	3 Mutations	Not Over-Expressed (-)	Neg
MET	1 Mutation	Over-Expressed (+)	Pos (3)
Kras	Not Mutated	Over-Expressed (+)	Pos (3)
ABL1	3 Mutations	Over-Expressed (+)	NA
AKT1	2 Mutations	Over-Expressed (+)	NA
BRAF	Not Mutated	Over-Expressed (+)	NA
HRAS	1 Mutation	Over-Expressed (+)	NA
NRAS	Not Mutated	Over-Expressed (+)	NA
PDGFR	9 Mutations	Over-Expressed (+)	NA
PIK3CA	Not Mutated	Over-Expressed (+)	NA
PTEN	Not Mutated	Not Over-Expressed (-)	NA
RET	2 Mutations	Not Over-Expressed (-)	NA
TP53	7 Mutations	Over-Expressed (+)	NA

Lung CA 1080742F (ADC)			
Lung CA	NGS DNA	NGS RNA	FFPE Actual IHC
Ker5	NA	NA	Pos (3-4)
Ker 6	NA	NA	Pos (3-4)
Ker 7	NA	NA	Pos (3-4)
Ker 20	NA	NA	Pos (3-4)
TTF1	NA	NA	Pos (3-4)
Napsin A	NA	NA	Neg
EGFR	7 Mutations	Over expressed (+)	Pos (3-4)
Her2	Not Mutated	Over expressed (+)	Pos (3-4)
MET	Not Mutated	Over expressed (+)	Pos (3-4)
Kras	1 Mutation	Over expressed (+)	Pos (3-4)
ABL1	1 Mutation	Over expressed (+)	NA
AKT1	Not Mutated	Over expressed (+)	NA
BRAF	Not Mutated	Over expressed (+)	NA
HRAS	Not Mutated	Over expressed (+)	NA
NRAS	Not Mutated	Over expressed (+)	NA
PDGFR	4 Mutations	Over expressed (+)	NA
PIK3CA	Not Mutated	Over expressed (+)	NA
PTEN	1 Mutation	Over expressed (+)	NA
RET	1 Mutation	Not Overexpressed (-)	NA
TP53	2 Mutations	Over expressed (+)	NA

Lung CA 1177163F (ADC)			
Lung CA	NGS DNA	NGS RNA	FFPE Actual IHC
Ker5	NA	NA	Neg
Ker 6	NA	NA	Neg
Ker 7	NA	NA	Pos (3-4)
Ker 20	NA	NA	Pos (3-4)
TTF1	NA	NA	Pos (3-4)
Napsin A	NA	NA	Pos (3-4)
EGFR	4 Mutations	Over Expressed +	(Eq)2+
Her2	1 Mutation	Over Expressed +	Neg
MET	1 Mutation	Not Over expressed (-)	Pos (3-4)
Kras	1 Mutation	Over Expressed +	(Eq)2+
ABL1	1 Mutation	Over Expressed +	NA
AKT1	Not Mutated	Over Expressed +	NA
BRAF	Not Mutated	Over Expressed +	NA
HRAS	1 Mutation	Over Expressed +	NA
NRAS	Not Mutated	Over Expressed +	NA
PDGFR	2 Mutations	Over Expressed +	NA
PIK3CA	Not Mutated	Over Expressed +	NA
PTEN	Not Mutated	Over Expressed +	NA
RET	1 Mutation	Not Over expressed (-)	NA
TP53	Not Mutated	Over Expressed +	NA

Next Generation Sequencing

Quality control testing of the RNA and DNA ahead of library preparation, as measured by Qubit, Nanodrop, and Bioanalyzer methodology showed passing results for the FFPE tissues. Run data indicate that the biomarker quality from the FFPE samples passed quality specifications. Fourteen mutations of clinical significance were studied in the lung carcinoma samples (Table 5).

Biomarkers

Case 1171989F is a known NSCLC of the ADC subtype. Its biomarker status includes positive staining for cytokeratin 7, TTF1, Napsin A, EGFR overexpression, MET and KRAS. The tumor does not express proteins for cytokeratin 5, cytokeratin 6, or cytokeratin 20. RNA overexpression is occurring in EGFR, MET, KRAS, ABL1, AKT1, BRAF, HRAS, NRAS, PDGFR, and PiK3CA. The tumor has mutations at the gene level for EGFR, Her2, MET, ABL1, AKT1, HRAS, PDGFR, RET, and TP53. Four biomarkers were tested at all three levels: RNA, DNA, and protein levels: EGFR, Her2, MET and KRAS. Overexpression of RNA and protein is observed with EGFR along with several mutations in the gene. Her2 does not show overexpression at the RNA and protein levels, but there are 3 mutations present at the gene level. MET is overexpressed at the RNA and protein level and harbors one mutation at the gene level. KRAS shows overexpression at the RNA and protein level but lacks any mutation.

Case 108742F is also a known NSCLC of the ADC subtype. Its biomarker status includes positive staining for all protein-based markers by IHC, except for Napsin A. Protein overexpression was observed with EGFR, Her2, MET and KRAS. RNA overexpression was observed in all biomarkers excepting RET. Several mutations were observed at the gene level, including those for EGFR, KRAS, ABL1, PDGFR, PTEN, RET, and TP53.

Case 1177603F is the third NCSLC of the ADC subtype in the study. Its biomarker status includes positive staining for cytokeratin 7, cytokeratin 20, TTF1, Napsin A, and MET. The tumor does not express proteins for cytokeratin 5, cytokeratin 6, or overexpress Her2. Expression of EGFR was classified at a 2+ staining intensity, which is equivocal by IHC. By RNA analysis, all biomarkers were overexpressed except MET ad RET. Gene analysis shows that EGFR, Her2, MET, KRAS, ABL1, HRAS, PDGFR, and RET all harbor mutations. As the protein expression was classified as equivocal for EGFR and the RNA expression by NGS shows that EGFR is overexpressed, it is observed that NGS provides clarification on the expression level for this biomarker. With Her2, it is observed that there are some non-concordant results between IHC and NGS. While by IHC the staining was negative, there is overexpression of the Her2 RNA. Although this could be attributed to technical error, the IHC testing was repeated to confirm the result. Non-concordant results may also be attributed to differences in the biology of Her2: it was noted in the result that the Her2 gene harbored a mutation. It is conceivable that the wild-type Her2 is not being over-expressed, as the Her2 antibody detects normal Her2 proteins, while the mutated Her2 may be overexpressed. FISH/CISH testing in future studies may be useful to understand whether the amplified gene was wildtype Her2 or mutated Her2.

Information provided by NGS has become important in some clinical treatment decisions. Several different treatment options are available to patients with non-small cell lung carcinoma (NSCLC); but different treatments are only safe and effective for tumors with specific biomarkers. Subtyping NSCLC has become an important part in histological testing. For example, antifolate pemetrexed shows improved efficacy in NSCLC adenocarcinoma (ADC) subtype when compared to platinum chemotherapy but is contraindicated for NSCLC squamous cell carcinoma (SCC) subtype (2). Two tyrosine kinase inhibitors, erlotinib and gefitinib, show efficacy in tumors with somatic EGFR mutations (which are frequently identified in adenocarcinomas) (2). Gefitinib can be detrimental to patients with wild type EGFR (2). Other cytokeratin markers such as cytokeratin 7 and cytokeratin 20 have also been used to distinguish between ADC and SCC (3). However, cytokeratin 7 cannot always differentiate between ADC and SCC, as CK7 can be positive in both (3).

Over expression of EGFR can be observed in about 60% of NSCLC cases (13). Protein expression data is tied to whether a patient is a candidate for anti-EGFR monoclonal antibody therapy such as cetuximab and necitumumab (13). Some studies have shown that over expression of normal EGFR and Her2 proteins may play a role in understanding how well tumors will respond to EGFR-specific TKI therapies (1). Co-overexpression of EGFR and Her2 in a tumor appear to indicate that the tumor will respond well to EGFR-TKI treatment (1). Biomarker correlation studies are useful in understanding the full picture of NSCLC at the genetic, transcriptomic, and protein expression levels. Determination of treatment options may further be informed by such studies. Formalin fixed paraffin embedded tissues can be used to study genotype, phenotype, and the full interconnected biology of NSCLC.

Conclusion

Correlated biomarker data for three lung carcinoma cases (NSCLC, ADC subtype) have been illustrated in this study. DNA (gene level, mutations), RNA (transcriptomics), and protein expression by IHC are relevant in understanding the underpinnings of cancer genotypes and phenotypes. Biomarker testing on both protein and nucleic acid levels can contribute to insights in NSCLC behavior and response to different treatment options. FFPE specimens are often well-annotated with rich clinical and histopathologic information, making them excellent source materials for study. It is fortunate that proper formalin fixation preserves tissues well for downstream biomarker assays.

The data in this study illustrate that formalin fixation provides biospecimen stability for biomarker analysis by IHC and NGS. Formalin fixation is known to fragment nucleic acids into small fragments that can contribute to poor test results via traditional sequencing methods (10). However, the presented data show this fragmentation does not appear to negatively impact the performance of NGS testing. This may partly be attributed to the nature of sample preparation ahead of NGS and the nature of data alignment and variant calling. Although guarantees cannot be made about biospecimen preparation ensuring performance at the NGS level, this data provides some confidence about formalin's ability to deliver high quality results.

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