Comparison of DNA and RNA from fresh-frozen vs. FFPE tissue samples

Key findings

- The Applied Biosystems™ MagMAX™ FFPE DNA/RNA Ultra Kit provides fast, reliable sequential nucleic acid isolation from formalin-fixed, paraffin-embedded (FFPE) samples.
- The quality, purity, and yield obtained from FFPE samples can be very comparable to their matched fresh-frozen (FF) counterparts, especially when the FFPE samples were controlled under standardized conditions, as outlined later.
- The process of fixing and embedding the FFPE samples does not render their nucleic acids useless for genomic analyses, and they can suitably serve as templates. Regardless of the sample type, i.e., FFPE or FF, the same conclusions could be reached in terms of variant calling and gene expression.

Introduction

Extraction of nucleic acids from FF and FFPE tissues is a critical step in routine workflows for biomedical researchers. Extraction of both DNA and RNA from a single section of a tissue sample in a single workflow can now be achieved with the MagMAX FFPE DNA/RNA Ultra Kit using magnetic separation techniques. To demonstrate that the extracted nucleic acids are pure and highly functional, they were compared to those obtained using other kits that are designed and optimized to only extract DNA or RNA from FF samples. All samples were processed on the Thermo Scientific™ HM 355S Automatic Microtome, the Thermo Scientific™ CryoStar™ NX70 cryostat, and the Thermo Scientific™ KingFisher™ Flex Purification System, which enables high-throughput, robust, and repeatable nucleic acid extraction from FF and FFPE tissues. In addition to quantitating the samples, we also analyzed RNA integrity, performed real-time PCR to assess functionality of the nucleic acids, and sequenced samples to evaluate hotspot regions in commonly mutated human cancer genes and their corresponding RNA transcripts.

Materials and methods

Tissue selection and FFPE preparation

Nucleic acids were extracted from cancerous human tissues purchased from Asterand Bioscience (Detroit, MI). All tissues used conformed to the following criteria: de-identified patient information, >2 g of sample with >95% tumor volume, and RNA integrity number (RIN) >9.5. Two different tissue types with significant clinical relevance were selected for this study: lung cancer samples from non-small cell lung carcinoma (LCa) of adenocarcinoma subtype from three different patients, and breast cancer (BCa) samples from infiltrating ductal adenocarcinoma tissues from three different patients. All frozen tissues were kept at –80°C except during grossing and sectioning, and immediately returned to –80°C when possible. One section was saved from each tissue sample to serve as a FF control sample, and the rest were fixed and embedded. Tissues were fixed in 10% Thermo Scientific™ Richard-Allan Scientific™ Neutral Buffered Formalin (NBF) for 24 hours, sectioned into 300 mm^3 pieces, then processed for 8 hours on a Thermo Scientific™ Excelsior™ ES Tissue Processor, and embedded in Thermo Scientific™ Richard-Allan Scientific™ Histoplast Paraffin at a Thermo Scientific™ HistoStar™ Embedding Workstation.

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Fixation, processing, and embedding took place under RNase-minimized conditions using tools and surfaces sterilized with 75% ethanol in nuclease-free water, followed by rinsing with Invitrogen™ RNase *AWAY*™ Decontamination Reagent. All of the blocks were prepared in 2016.

Instrumentation and kits for tissue sectioning and nucleic acid extraction

The FF tissues were sectioned at –20°C on a CryoStar NX70 cryostat, cutting 7 µm curls. A single curl was placed into 50 µL of respective lysis buffer with cold forceps. Tubes were kept inverted on ice until all sectioning was complete. After sectioning, all tubes were centrifuged at 12,000 rpm at 4°C for 1 minute to collect the tissue and buffer at the bottom of the Thermo Scientific™ Sorvall™ Legend™ Micro 21 microcentrifuge tube. Then the remaining lysis buffer was added. The FFPE tissues were sectioned on an HM 355S Automatic Microtome set at a 7 µm cutting thickness using Thermo Scientific™ MX35 Premier Disposable Low-Profile Microtome Blades. A single curl was placed directly into an empty sterile 1.5 or 2.0 mL microcentrifuge tube. The FFPE samples were then deparaffinized using a standard xylene protocol as outlined in the MagMAX FFPE DNA/RNA Ultra Kit.

The KingFisher Flex Purification System was used with the following compatible kits for lysate preparation and nucleic acid extraction: Applied Biosystems™ MagMAX™ *mir*Vana™ Total RNA Isolation Kit (for RNA from FF tissue), MagMAX™ DNA Multi-Sample Kit (for DNA from FF tissue), and MagMAX FFPE DNA/RNA Ultra Kit (for both DNA and RNA from FFPE tissue). Methods for these kits were carried out according to the protocols listed in their manuals, using Thermo Scientific™ KingFisher™ Flex™ sterile microtiter 96 deep-well plates.

Nucleic acid quantitation and RNA analysis

Extracted RNA and DNA were quantified using the Invitrogen™ Qubit™ 3.0 Fluorometer using the Qubit™ dsDNA and RNA HS Assay Kits, and the Thermo Scientific™ NanoDrop™ 2000c Spectrophotometer. Nucleic acid purity was measured on the NanoDrop spectrophotometer, focusing on A_{260}/A_{230} and A_{260}/A_{280} absorbance ratios. RNA fragment sizes were measured using an Agilent Technologies 2100 Bioanalyzer™ system. One sample from each group was run on the Bioanalyzer system.

Real-time PCR assays

For the DNA samples, DNA inputs of equal mass were used in 10 µL PCR reactions with the Applied Biosystems™ TaqMan® Universal II Master Mix, no UNG, and run on the Applied Biosystems™ 7900HT Fast Real-Time PCR System with 384-well block under standard cycling conditions. Biological duplicates from each of the three donors and for each tissue type were processed.

For the RNA samples, RNA inputs of equal mass were used in 20 µL reverse-transcription reactions. The Invitrogen™ SuperScript™ VILO™ cDNA Synthesis Kit was used for the generation of first-strand cDNA under standard cycling conditions. Two microliter from the reverse transcription reaction was used in a 10 µL PCR reaction with the TaqMan Universal II Master Mix, no UNG, and run on the 7900HT Fast Real-time PCR System with 384-well block under standard cycling conditions. Biological duplicates from each of the three donors and for each tissue type were processed.

Next-generation targeted sequencing

Ten nanograms of each DNA and RNA was used in the Ion AmpliSeq™ Library Kit 2.0 with the Ion AmpliSeq™ Cancer Hotspot Panel v2 and the Ion AmpliSeq™ RNA Cancer Panel, with the appropriate number of cycles. Libraries were then quantitated with the Ion Library TaqMan® Quantitation Kit. Based on the quantitation, libraries were pooled at equimolar concentration and diluted to 60 pM for the Ion Chef™ instrument, and samples were then sequenced on an Ion 530™ chip using the Ion S5 sequencing system. Data were analyzed with the Torrent Suite™ Coverage Analysis plugin, Torrent Variant Caller plugin, and the AmpliSeqRNA plugin.

Results and discussion

The Qubit fluorometer and the NanoDrop spectrophotometer are instruments for nucleic acid quantitation, with their own advantages and disadvantages. Due to specific dye-binding chemistry, the Qubit assays are sensitive enough to distinguish between RNA and DNA species, whereas the NanoDrop instrument measures all absorbance at 260 nm. Since RNA, ssDNA, and dsDNA all absorb at 260 nm, the NanoDrop measurements tend to overestimate nucleic acid concentrations.

The nucleic acid yields varied from donor to donor, as expected. On average, the FF BCa samples yielded over 125 ng of DNA and 200 ng of RNA. For FFPE BCa samples, average yields were about 100 ng of DNA and 700 ng of RNA. For FF LCa samples, 100 ng of DNA and 150 ng of RNA were obtained. For FFPE LCa samples, average yields were about 100 ng of DNA and 570 ng of RNA (Figure 1). Although the FFPE RNA is fragmented, sufficient yield was obtained, but the template may not be as pristine. Additionally, through the fixation process RNases are inactivated in FFPE samples, whereas frozen tissue still contains RNases, which become active as they thaw, which may be contributing to lower yield in the FF RNA samples. The MagMAX FFPE Ultra kit was able to retrieve a quantity of DNA from FFPE samples similar to or greater than the amount of DNA obtained from FF sections using the MagMAX DNA Multi-Sample Kit.

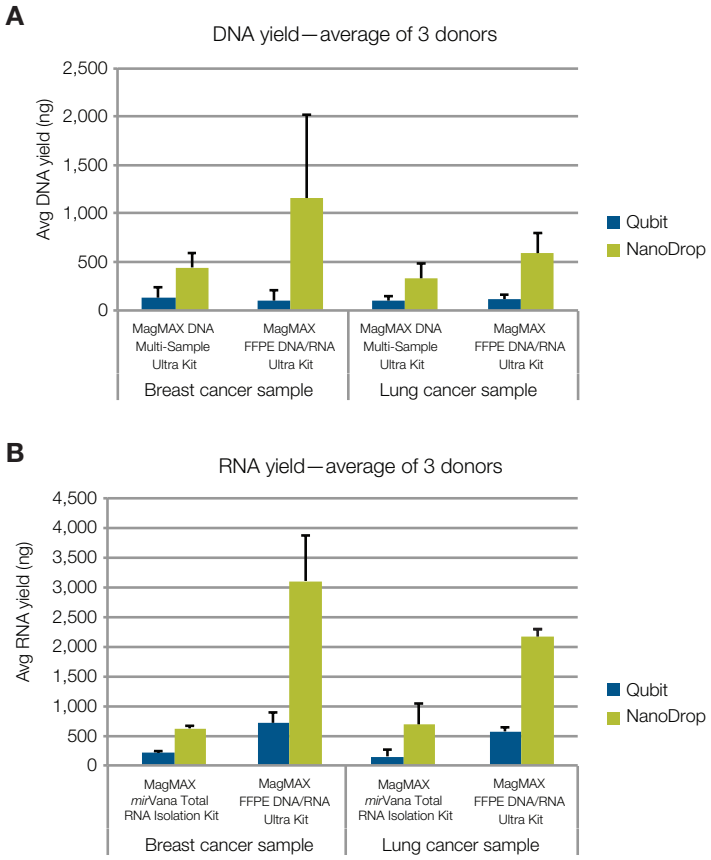


Figure 1. Average DNA and RNA yields from 3 donor samples. **(A)** DNA and **(B)** RNA from FF samples were extracted using the MagMAX DNA Multi-Sample Ultra Kit and MagMAX *mir*Vana Total RNA Isolation Kit, respectively; the MagMAX FFPE DNA/RNA Ultra Kit was used to extract both DNA and RNA from FFPE samples. For each sample type, one 7 µm section was used. The FF and FFPE samples were quantified using the Qubit and NanoDrop instruments, respectively.

The ratio of absorbance at 260 nm and 280 nm (A_{260}/A_{280}) is used to assess the purity of DNA and RNA. In cases where there are low readings using the NanoDrop spectrophotometer (<10 ng/µL), ratio readings are skewed and less accurate. FFPE RNA A_{260}/A_{280} values were at the optimal ratio of 2.0, but the FF samples were slightly outside the range. A_{260}/A_{280} values for DNA were close to the optimal range for FFPE sections extracted using the MagMAX FFPE Ultra kit, but the values for FF DNA were slightly higher than optimal (Figure 2). A secondary purity measurement is the A_{260}/A_{230} ratio. The A_{260}/A_{230} ratios for DNA were low, which is attributable to the low nucleic acid concentrations, so accurate A_{260}/A_{230} ratios could not be obtained for DNA, but for RNA these ratios were higher. Although purity ratios and spectral profiles can be indicators of sample quality, the best indicator of quality is the appropriate function of the nucleic acid samples in your desired downstream applications. With the achieved purity ratios, there were no issues with any of our downstream applications, which included real-time PCR and targeted sequencing.

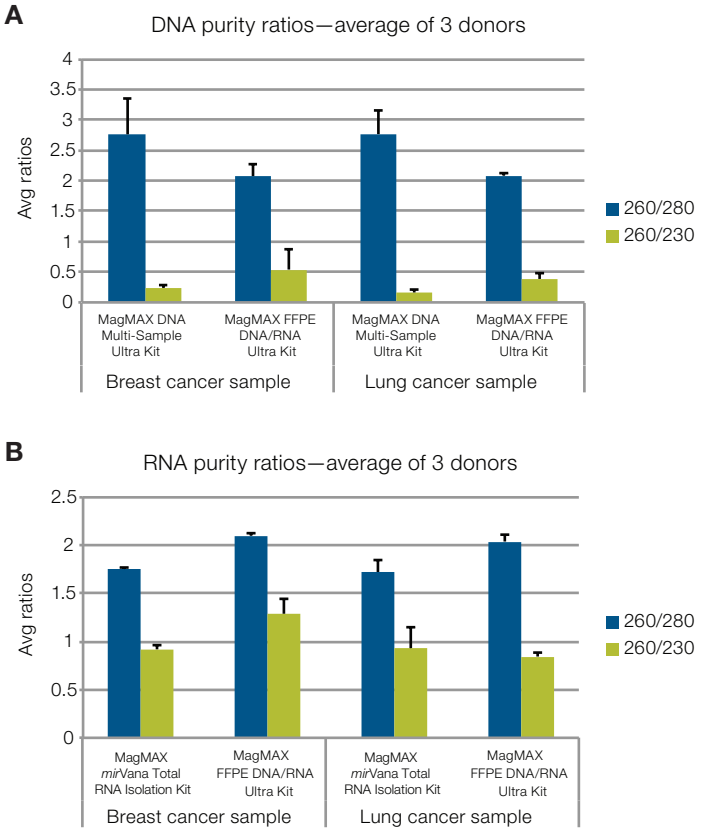


Figure 2. Average purity ratios of 3 donor samples, obtained using the NanoDrop instrument. **(A)** DNA and **(B)** RNA from FF samples were extracted using the MagMAX DNA Multi-Sample Ultra Kit and MagMAX *mir*Vana Total RNA Isolation Kit, respectively; the MagMAX FFPE DNA/RNA Ultra Kit was used to extract both DNA and RNA from FFPE samples. The FF and FFPE samples were quantified using the Qubit and NanoDrop instruments, respectively.

The 2100 Bioanalyzer system was used to assess RNA integrity and size. The RNA integrity number (RIN) represents the condition of assayed RNA relative to the intact total RNA on a scale of 1–10, with 10 indicating completely intact 18S and 28S ribosomal RNA (rRNA). As expected, with the FF samples, nice ribosomal RNA peaks are present. A clearly identifiable small-RNA peak is also present since the MagMAX *mir*Vana kit can isolate small RNAs as well. A clear distinction between FFPE and FF samples is that ribosomal peaks are not usually present in FFPE samples, leading to a very low RIN, usually <2; however, the size of RNA fragments can still be very large. This is observed for both FFPE BCa and LCa samples processed—the percentage of fragments >200 bp was over 65%. With FFPE samples, RIN numbers hold little value since the samples are inherently degraded, whereas overall fragment sizes play a bigger role (Figure 3).

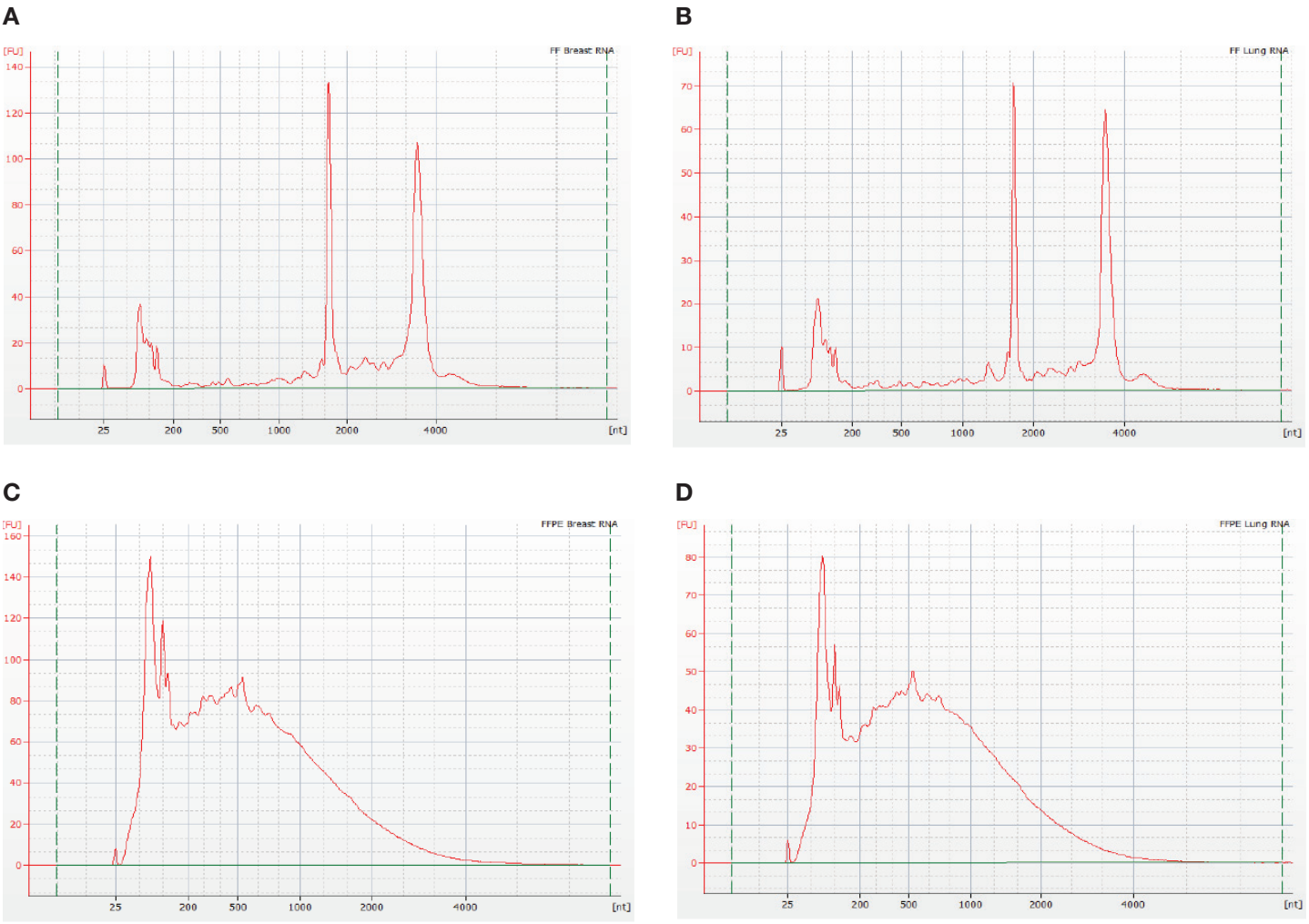


Figure 3. RNA analysis with the Agilent RNA 6000 Pico kit. RNA from **(A)** an FF breast cancer sample and **(B)** an FF lung cancer sample had distinct ribosomal peaks with RIN values of 9 and 8.7, respectively. RNA from **(C)** an FFPE breast cancer sample and **(D)** an FFPE lung cancer sample still had large RNA fragments but without clear ribosomal peaks, with RIN values of 1.8 for both.

Real-time PCR was performed to assess the functionality of the extracted nucleic acid templates. Both DNA and RNA from FFPE samples are fragmented and chemically modified, but the degree of fragmentation and chemical modification vary widely from sample to sample. The fixation process alone does not necessarily lead to fragmentation, but it does in combination with different factors. For example, during the embedding process, the high temperatures required for paraffin infiltration can accelerate chemical reactions that may modify the RNA and DNA. During storage afterward, these modifications can cause nucleic acid fragmentation and degradation, especially for RNA. DNA tends not to fragment as easily as RNA, so it was not surprising to see equivalent or mostly better C_t values for the FFPE samples compared to the FF samples (Figure 4A), especially for younger blocks. However, since RNA is more susceptible to degradation, diminished function as a template for polymerases is expected. We see a difference of 0.7–5 cycles in the C_t values (Figure 4B). Values from older blocks of samples may be more variable, due to a number of factors such as storage conditions or more uncontrolled practices such as fixing the samples for too long. This could lead to more issues with the template being less functional. Nowadays most protocols are more standardized, leading to better handling of the samples.

DNA and RNA libraries were made using 10 ng of template. The number of cycles to be used for the first amplification of target was selected based on the number of primer pairs in the panels and on the sample type (FF or FFPE). Basic sequencing run metrics include >93% loading, <15% low-quality reads, and 0% adapter dimer.

For DNA samples, we tracked the following metrics: mean read lengths, percent mapped, percent reads on target, and percent uniformity. In addition to these basic metrics, we also checked variant calling and compared the results. For mean read lengths the average was >115 bp for either sample type, with very high mapping percentage (Figure 5). The data quality was good, with minimum filtering of short reads. The percent reads on target was overall good but lower for the FFPE samples, which might have been affected by the modified FFPE DNA. On average the percent uniformity was good for both sample types, with values >96%, with the exception of 1 sample at 94%. Figure 5 outlines that the same hotspot mutations were called with similar allele frequencies between FF and FFPE samples for both tissue types, allowing the same conclusions to be drawn.

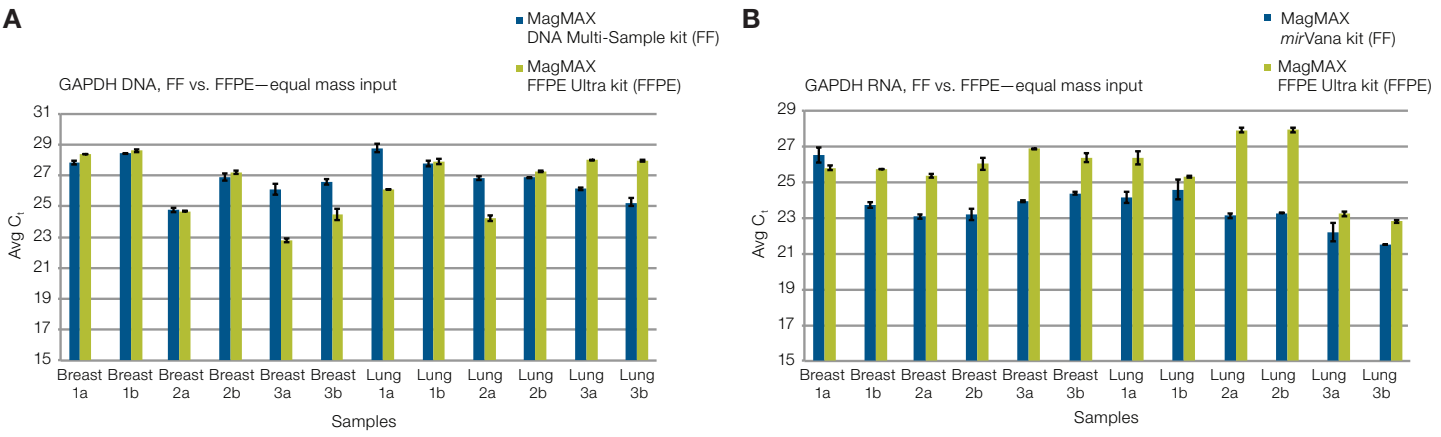
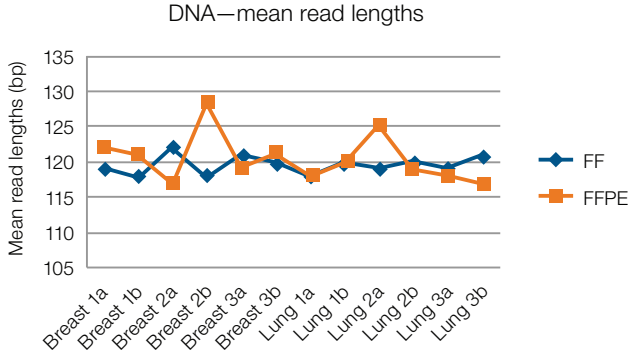


Figure 4. Real-time PCR analysis of DNA and RNA samples. (A) Equivalent or mostly better C_t values were achieved with the FFPE DNA samples compared to the FF samples. **(B)** A difference of 0.7–5 cycles in the C_t values was observed in the RNA samples.

For RNA samples, the following metrics were tracked: mean read lengths, percent mapped, percent valid reads, and percent on target. Also, we correlated the normalized reads per kilobase million (RPKM) counts for the 50 genes in the RNA cancer panel between biological replicates for a given sample and tissue, or between sample types and tissue (FF vs. FFPE). The mean read lengths for these samples were very good, which led to high mapping (>99%) for both sample types, with an average of 127 bp for FF; the mean read lengths for the FFPE samples, as expected, were shorter, with an average of 116 bp, possibly due to the presence of more degradation and fragmentation in the samples. High-quality reads were also obtained, as determined by the percent valid reads with high on-target reads to the 50 genes in the panel. We obtained high correlations to normalized RNA counts for all parameters, including between biological sample replicates and, most importantly, between FF and FFPE samples for the same tissue type. This gives high confidence that the same genes are being expressed at similar levels (Figure 6).

A



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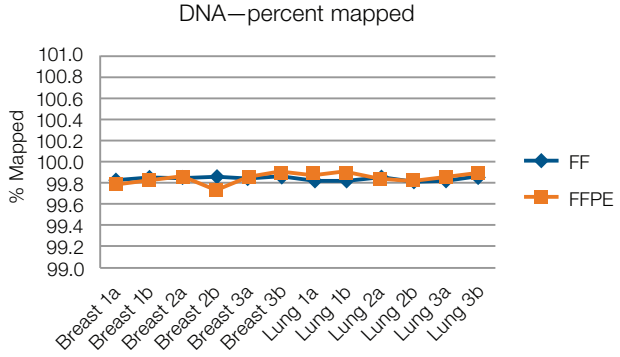

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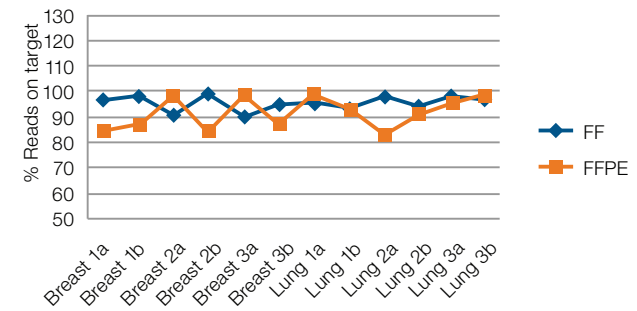

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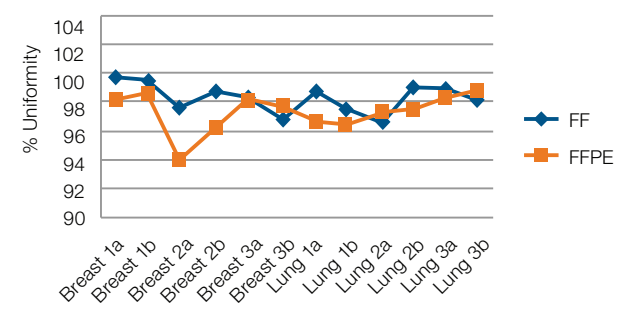

E

Sample	Gene	Allelic frequency (replicate 1)	Allelic frequency (replicate 2)	Sample	Allelic frequency (replicate 1)	Allelic frequency (replicate 2)	Ref to variant
FF breast cancer	*PIK3CA*	33.0	41.1	FFPE breast cancer	41.6	46.5	G→A
	KIT	49.4	48.0		41.6	42.6	A→C
	TP53	69.6	68.7		64.6	66.3	T→A
FF lung cancer	*CTNNB1*	27.7	21.7	FFPE lung cancer	18.5	18.0	C→G
	HRAS	52.4	51.2		44.4	41.1	A→G

Figure 5. Targeted DNA sequencing metrics. (A) Comparable mean read lengths and **(B)** high mapping percentages for both sample types (FF and FFPE breast and lung cancer samples) are shown. **(C)** On-target percentages were comparable, with better results for FF samples. On average, 95.5% of the sequences were on target for FF and 91.7% for FFPE samples. **(D)** Percent uniformity was also very comparable across the samples types. On average, it was 98.3% for FF and 97.4% for FFPE samples. **(E)** Variant calling—the same hotspot mutations were called with similar frequencies.

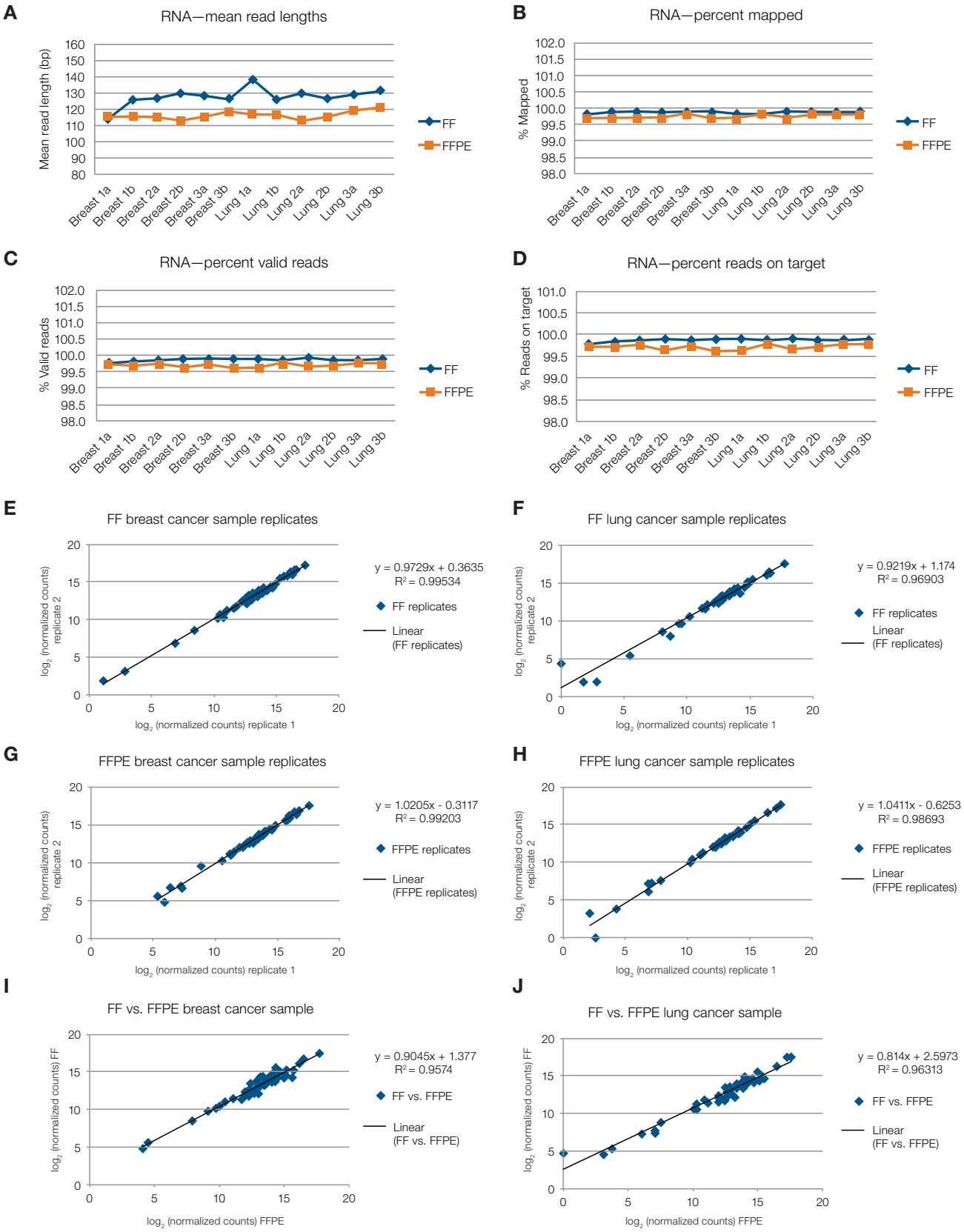

Figure 6. Targeted RNA sequencing metrics. (A) Mean read lengths for FFPE samples were shorter, as expected, than the FF samples but still were >110 bp. **(B)** Percent mapped was high, yielding comparable results of >99% mapped. **(C)** Percent valid reads was high, yielding comparable results of >99.5% valid reads. **(D)** Percent reads on target was high, yielding comparable results of >99.5% reads on target. **(E)** Correlation between FF breast cancer sample replicates; $R^2 = 0.995$. **(F)** Correlation between FF lung cancer sample replicates; $R^2 = 0.969$. **(G)** Correlation between FFPE breast cancer sample replicates; $R^2 = 0.992$. **(H)** Correlation between FFPE lung cancer sample replicates; $R^2 = 0.987$. **(I)** Correlation between FF and FFPE breast cancer samples; $R^2 = 0.957$. **(J)** Correlation between FF and FFPE lung cancer samples; $R^2 = 0.963$.

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Conclusion

Using the workflow described, biomedical researchers can extract RNA and DNA from FF and FFPE samples with sufficient yield, purity, and quality for many downstream genomic and transcriptomic analyses. When researchers aim to obtain both RNA and DNA from the same sections of FFPE tissues, high-quality nucleic acids can be obtained with the MagMAX FFPE DNA/RNA Ultra Kit. With the samples we outlined here, regardless of the sample-processing techniques—either frozen, or fixed and embedded—extraction of high-quality nucleic acids was achieved, allowing the same conclusions to be made with confidence. Older sample blocks may be more problematic due to uncontrolled practices, but older FFPE samples can still be suitable and be used as functional templates for many applications.

Ordering information

Product	Cat. No.
Richard-Allan Scientific 10% Neutral Buffered Formalin	5701TS
Excelsior ES Tissue Processor	A78400006
HistoStar Embedding Workstation	A81000001
Richard-Allan Scientific Histoplast Paraffin	8332
RNase *AWAY* Decontamination Reagent	10328011
CryoStar NX70 Cryostat	957030H
HM 355S Automatic Microtome	905200
MX35 Premier Disposable Low-Profile Microtome Blades	3052835
1.5 or 2.0 mL microcentrifuge tubes	02-681-271 or 05-408-129
KingFisher Flex Purification System	5400630
KingFisher Flex Microtiter Deepwell 96 Plates, sterile	95040460
MagMAX FFPE DNA/RNA Ultra Kit	A31881
Qubit 3.0 Fluorometer	Q33216
Qubit dsDNA HS Assay Kit	Q32854
Qubit RNA HS Assay Kit	Q32855
NanoDrop 2000c Spectrophotometer	ND2000C

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