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A High-Throughput Workflow for Large Scale Multi-omic Biomedical Studies Involving FFPE Tissue Samples

Jonathan Chen, Emily Chen, Jerry Liu, Matthew Decker, Faith Holloway, Yi Xu, Standa Forman, Xiaojing Yang, and Keith Booher

Zymo Research Corporation, 17062 Murphy Ave. Irvine, CA 92614
Email: kbooher@zymoresearch.com

Highlights

- This workflow enables high-throughput DNA/RNA purification and sequencing of FFPE samples.
- Unique NGS library prep chemistry improves library complexity and read depth across the entire genome.
- The end-to-end workflow described here presents a robust and affordable way to generate multi-omic data sets from a ubiquitous and highly relevant sample source, supporting large-scale biomedical studies powered by AI/ML analysis techniques.

Introduction

The field of genomics has entered a renaissance. Cutting-edge next generation sequencing (NGS) technology platforms have dramatically reduced the cost and increased the speed of generating multi-omic DNA or RNA sequence data. Similarly, mature cloud computing and information technology (IT) infrastructure means that large datasets can now be securely and easily accessed, transferred, or stored across multi-site or inter-institute research endeavors. As the demand, complexity, and availability of NGS data continue to grow, recent advancements in Artificial Intelligence (AI) and Machine Learning (ML) algorithms have expanded the bioinformatics analysis toolbox, enabling the identification of patterns and extraction of meaningful insights from large datasets in ways that were previously unattainable. To capitalize on the unprecedented technological advancements in the field of genomics, researchers are seeking out the most informative biological samples with which to design large-scale studies for biomarker or therapeutic discovery. Often overlooked due to perceived insurmountable technical challenges associated with processing and NGS preparation, formalin-fixed paraffin-embedded (FFPE) tissue represents one such promising sample source.

FFPE tissue collection has long been a staple method of sample preparation and long-term storage for pathology labs around the world. The fixation procedure maintains the native tissue architecture, allowing FFPE samples to be used primarily for gross histology, which is accomplished by slicing the preserved tissue into micron-thick slices, staining, and affixing them onto slides for microscopy. FFPE tissue often represents clinically relevant specimens originating from biopsies and surgical resections. Thanks to the widespread practice of FFPE sample preparation, numerous research institutions and biobanks can maintain vast collections of tissue specimens representative of diverse disease states and outcomes. Access to such collections could facilitate the large-scale genomic studies best suited to utilize the highly powerful NGS instruments and sophisticated AI/ML tools now available for biomedical research. Moreover, the existing supply of such samples removes the high costs and time investment associated with prospective study design, research subject enrollment, and eventual fresh frozen tissue sample collection.

Although FFPE samples offer clear advantages for biomedical studies, technical challenges have limited their widespread adoption for sequencing applications. For example, although the fixation process preserves cellular structures within tissues, it does little to preserve DNA and RNA integrity. Instead, the fixative agent simply crosslinks the nucleic acids to proteins or other cellular structures, resulting in significant DNA and RNA fragmentation. Furthermore, fixation and embedding can contaminate tissues with formalin, paraffin, or other impurities that must be removed during nucleic acid extraction. The resulting DNA and RNA are typically low in both quantity and quality and may contain inhibitors that adversely impact downstream sequencing steps. Consequently, whole genome sequencing (WGS) applications have not been widely adopted given the difficulty of producing quality sequencing data (Bruinsma et al., 2018). In turn, the primary DNA sequencing applications currently used for FFPE tissue samples rely on enrichment methods, using either exome sequencing or other targeted gene panel approaches. However, enrichment approaches limit the scope of investigation to a small portion of the genome and offer limited opportunities for discovery.

Many library prep kits on the market involve lengthy protocols or biased chemistries that allow users to produce libraries from FFPE DNA, but the resulting sequence data is of poor quality (Cappello et al., 2022; Steiert et al., 2023). Here, we describe an end-to-end method developed by Zymo Research that generates high-complexity WGS libraries from purified FFPE DNA. The workflow is automatable, robust, and straight-forward, and lends itself to high-throughput studies that call for processing and sequencing many samples. Furthermore, we extend the method development to include RNA isolation from FFPE tissue followed by ribosomal RNA depletion and RNA-seq library prep. The combined genetic and transcriptomic profiling facilitates multi-omic research from previously intractable sample types, which represent a rich source of highly valuable clinical metadata for large-scale, population- or indication-specific translational studies powered by AI or ML analysis tools.

Materials and Methods

Samples and Nucleic Acid Isolation

A total of 16 archived brain tumor (meningioma) scroll samples were processed using the Zymo Research high-throughput FFPE tissue DNA/RNA extraction workflow (Figure 1). Duplicates of each sample were processed in parallel — one set of 16 scrolls for DNA extraction and WGS library prep, and another set of 16 samples for RNA extraction and RNA-seq library prep. The tissue was prepared for nucleic acid purification using the *Quick*-DNA/RNA™ MagBead protocol (R2130, Appendices, DNA/RNA Purification from FFPE Samples). Scrolls for both DNA and RNA extraction were heated at 55°C for 2 minutes using Deparaffinization Solution to melt the paraffin. After spinning down to pellet the liberated tissue, Deparaffinization Solution was removed. 100 µL of a Proteinase K reaction mixture was added to help further break down the sample. Tissue for RNA analysis was digested for 1 hour at 55°C while tissue for DNA analysis was digested for 3 hours. Digested DNA lysate was decrosslinked at 94°C for 20 minutes while RNA lysate was decrosslinked at 65°C for 15 minutes. DNA/RNA Lysis Buffer was then added to all lysates at a 3:1 ratio (300 µL to 100 µL).

For the following nucleic acid purification steps, samples were processed using either the DNA or RNA MagBead protocol on the KingFisher Apex (Thermo Fisher). DNA was purified from prepared lysates using the *Quick*-DNA/RNA MagBead protocol (R2130) for total nucleic acids. RNA was purified using the same protocol, but incorporating DNase treatment to remove DNA contamination. DNA and RNA were eluted in 25 µL of DNA Elution Buffer or DNase/RNase-Free Water, respectively. Purified DNA and RNA were quantified using the HS dsDNA Qubit and HS RNA Qubit assays, respectively. Purity was assessed using the Nanodrop 2000. Nucleic acid quality was imaged using the Genomic DNA and RNA TapeStation assays on the TapeStation 4200.

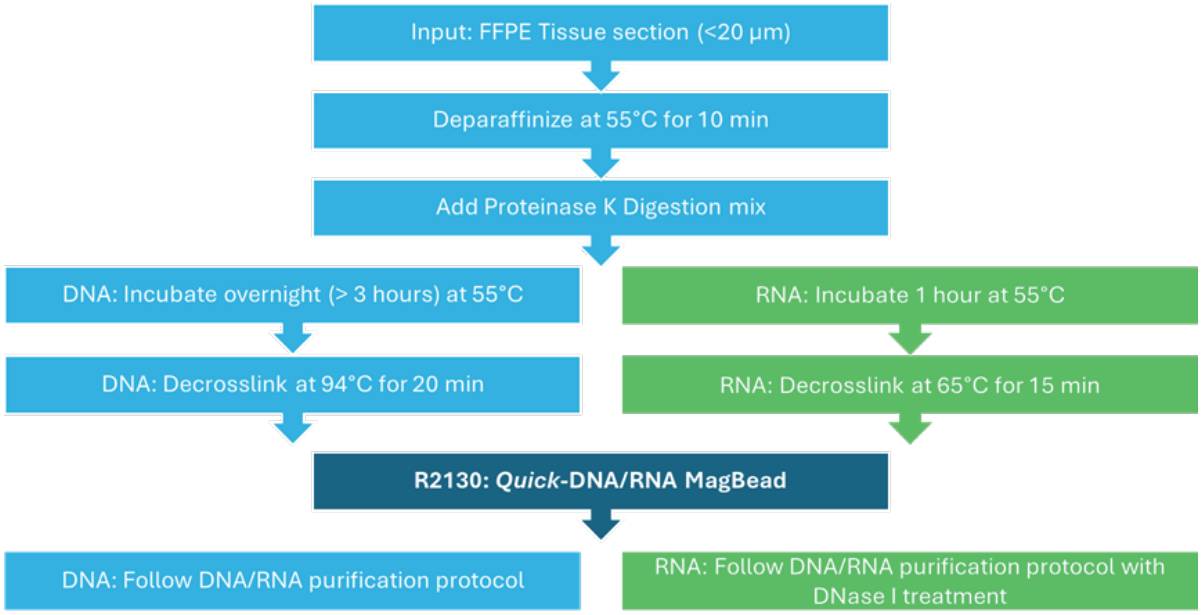

Figure 1: Schematic overview of FFPE tissue DNA/RNA extraction workflow.

NGS Library Preparation and Sequencing

A novel enzymatic method for creating complementary second strands from bisulfite-converted DNA, which is similar to reverse cross-linked and purified FFPE DNA, was selected for whole-genome sequencing library preparation using the Zymo-Seq WGBS (Whole Genome Bisulfite Sequencing) Library Prep Kit (D5465). Purified meningioma DNA samples were normalized to 100 ng, followed by a second strand synthesis reaction and the addition of sequencing adapters. Library amplification was completed using Zymo-Seq™ UDI Index Primers (D3096). Library quality control was performed on the Agilent 4200 TapeStation. RNA was normalized to approximately 125 ng and prepped using the Zymo-Seq RiboFree Total RNA Library Prep Kit (R3000). For the rRNA depletion procedure, samples were incubated for 4 hours. The first Select-a-Size bead cleanup in the protocol was replaced with a cleanup procedure using the RNA Clean & Concentrator™-5 (R1013) due to the abundance of small cDNA fragments. The libraries were amplified for 16 PCR cycles. All libraries were sequenced on the NovaSeq X Plus (Illumina) using the 10B, 300 cycle reagent kit.

Bioinformatics Analysis

Sequence reads from whole-genome sequencing libraries were identified using standard Illumina base calling software. Raw FASTQ files were adapter and quality trimmed, and 15 bases were further trimmed from the 5' end according to the Nextera recommendations using TrimGalore 0.6.4. FastQC 0.11.9 was used to assess the effect of trimming and overall quality distributions of the data. Alignment data were created as BAM files using the hg19 (GATK.GRCh37) genome as reference. Alignment visualization and figures were generated using a combination of R and Python custom modules. The Zymo Research RNA-seq data analysis pipeline was applied to reads generated from libraries prepared with the RiboFree NGS kit. In brief, the Zymo Research RNA-Seq pipeline was originally adapted from

nf-core/rnaseq pipeline v1.4.2 (*https://github.com/nf-core/rnaseq*). The pipeline was built using Nextflow (*https://www.nextflow.io/*). Quality control of raw reads was carried out using FastQC v0.11.9 (*http://www.bioinformatics.babraham.ac.uk/projects/fastqc*). Adapter and low-quality sequences were trimmed from raw reads using Trim Galore! v0.6.6 (*https://www.bioinformatics.babraham.ac.uk/projects/trim_galore*). Trimmed reads were aligned to the reference genome using STAR v2.6.1d (*https://github.com/alexdobin/STAR*). BAM file filtering and indexing was carried out using SAMtools v1.9 (*https://github.com/samtools/samtools*). RNA-seq library quality control was implemented using RSeQC v4.0.0 (*http://rseqc.sourceforge.net/*) and QualiMap v2.2.2-dev (*http://qualimap.conesalab.org/*). Duplicate reads were marked using Picard tools v2.23.9 (*http://broadinstitute.github.io/picard/*). Library complexity was estimated using Preseq v2.0.3 (*https://github.com/smithlabcode/preseq*). Duplication rate quality control was performed using dupRadar v1.18.0 (*https://bioconductor.org/packages/dupRadar/*).

Results

Whole Genome Sequencing (WGS) of DNA

We processed 16 archived brain tumor (meningioma) scroll samples using Zymo Research's high-throughput FFPE tissue DNA/RNA extraction and NGS library preparation workflow to demonstrate its robust performance on challenging, clinically relevant samples. Historically, nucleic acid purification from FFPE samples has been exceptionally time-consuming and labor-intensive, as the fixation and paraffin embedding procedure requires additional sample processing steps — specifically, deparaffinization and reverse cross-linking of the tissue. The need for reagent pipetting and sample transfers between the bench and high-temperature incubation instruments makes large-scale sample processing difficult for a single technician to manage. Therefore, we implemented a 96-well plate format in which tissue scrolls are added to individual wells and then loaded onto the Hamilton Microlab (ML) Prep for high-throughput dispensation of deparaffinization and proteinase K (PK) reaction mixtures to effectively dissociate and separate sample tissue from embedded wax. Entire plates of 96 samples can be alternately loaded then transferred between instruments by a technician, minimizing hands-on time.

Following deparaffinization and PK treatment, the tissue samples are ready for DNA or RNA purification. We applied existing Zymo Research magnetic bead-based purification chemistries, which are compatible with most popular liquid handling robotics platforms frequently found in life sciences research labs. A workflow schematic is summarized in Figure 1. For purification of FFPE tissue scroll samples, we utilized the KingFisher Apex automation platform. We found that its ability to work with smaller volumes (i.e., < 20 µL) was advantageous for FFPE samples, as it enabled smaller reaction and elution volumes, resulting in higher final DNA and RNA concentrations (averaging 26.7 ng/µL for DNA and 41.3 ng/µL for RNA). See Tables 1 and 2 for purification yield and purity assessments.

Table 1: DNA extraction quantification and purity.

Sample #	ng/µL	260/280	260/230
1	15.6	1.90	2.08
2	47.6	1.88	2.21
3	35.6	1.88	2.08
4	25.7	1.85	2.14
5	19.6	1.83	1.81
6	39.0	1.85	1.96
7	34.3	1.87	2.05
8	31.2	1.85	1.98
9	36.4	1.88	2.10
10	18.4	1.87	2.07
11	6.45	1.90	1.96
12	20.8	1.86	1.79
13	34.5	1.89	1.93
14	43.0	1.87	1.94
15	8.3	1.89	1.99
16	10.2	1.88	1.89

Table 2: RNA extraction quantification and purity.

Sample #	ng/µL	260/280	260/230
1	27.9	2.00	0.86
2	43.9	1.97	1.50
3	47.8	1.90	1.27
4	55.0	1.93	1.32
5	76.0	1.95	1.38
6	32.0	1.95	1.23
7	55.0	1.95	1.29
8	40.3	1.95	1.12
9	24.3	1.91	0.93
10	19.3	1.93	0.83
11	39.7	1.96	1.06
12	24.0	1.90	0.83
13	50.0	2.02	1.25
14	71.0	1.97	1.40
15	39.1	1.94	0.98
16	15.7	1.97	0.70

We then proceeded with NGS library prep using the purified DNA material. Sample quality plays a very important role in determining the reliability of the final sequence dataset. Nucleic acids isolated from FFPE tissue are notoriously poor in quality due to the formalin fixation and paraffin embedding process. Using a TapeStation instrument, we assessed DNA and RNA quality and confirmed that it was indeed very low across all samples (mean DIN = 3.2, Figure 2a).

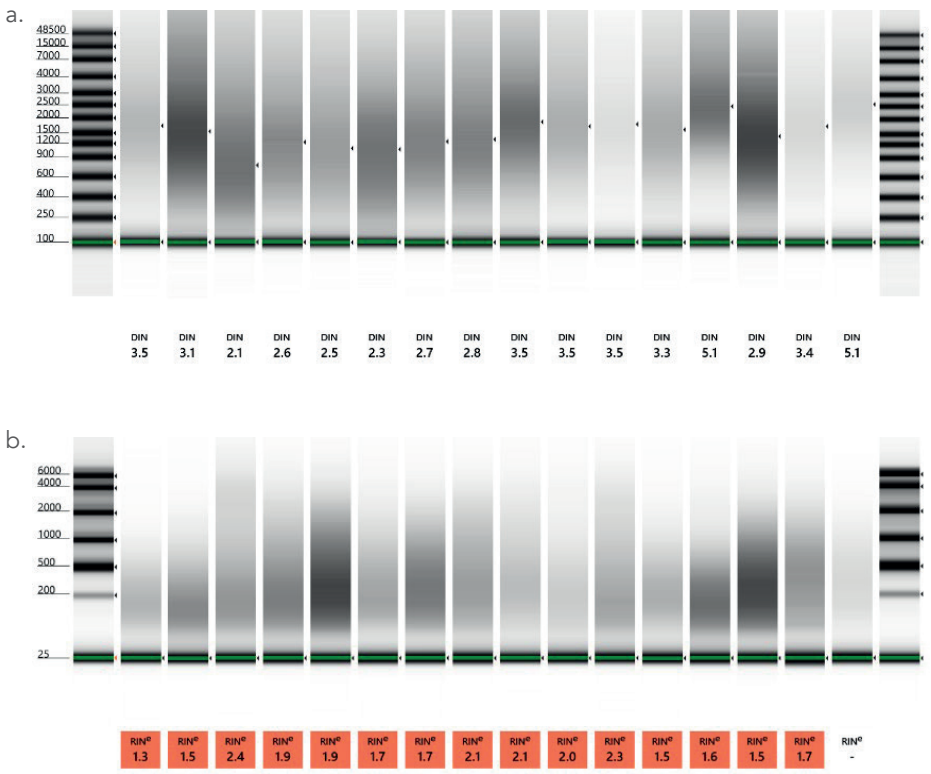

Figure 2: a) Genomic DNA and DIN score and b) RNA and RIN score, extracted from FFPE tissue. Molecular integrity was visualized on an Agilent TapeStation 4200 instrument.

In recent years, several groups observed that ancient biological tissue contains poor-quality DNA, similar to that found in FFPE samples (Stiller et al., 2016; Gansauge et al., 2017). In those studies, the authors demonstrated that a single-stranded (ss)DNA sequencing library preparation approach improved the overall library complexity and resulting sequence data compared to more common double stranded (ds)DNA library prep methods. However, the workflows described in those papers were complicated and required specialized reagents, making them expensive and incompatible with studies involving larger numbers of samples for investigation.

We reasoned that bisulfite conversion, the gold standard used to analyze DNA methylation modifications, produces a very high proportion of ssDNA molecules, closely resembling the DNA observed after the high-temperature treatment needed to reverse cross-links in FFPE tissue. As "The Epigenetics Company", Zymo Research has nearly 25 years of experience in the field of epigenetics research with the highest number of product citations in the field. Amongst the epigenetic products developed by Zymo Research is a method that converts bisulfite-treated ssDNA into dsDNA as part of a robust and efficient whole-genome bisulfite sequencing preparation workflow. Furthermore, the method is compatible with common transposase-based tagmentation and adapterization indexing protocols, enhancing the simplicity and ease of the workflow.

Given the similarities between bisulfite-converted DNA and purified FFPE ssDNA, we applied the Zymo-Seq WGBS Library Prep Kit (D5465) protocol to 100 ng of input DNA to assess its performance in constructing whole-genome sequencing libraries from highly degraded samples. Importantly, the enzymatic second strand synthesis step described in Section 2 of the published protocol successfully generated dsDNA that could be tagmented using Illumina's DNA Prep chemistry (https://files.zymoresearch.com/protocols/d5465-zymo_seq_wgbs_library_kit.pdf). The additional DNA mass generated at that step was not an issue, as the Tagmentation beads from the Illumina DNA Prep Kit normalized the samples during adapterization. After an additional 8 indexing cycles, we only needed a single-sided selection for the resulting library cleanup due to the small DNA fragment insert sizes (Figure 3a).

As a final test, we sequenced all 16 WGS libraries on an Illumina NovaSeq, allocating 462 million 150 bp PE reads per sample on average. Notably, the reported pass filter (>99%) and alignment (≥99.9%) rates were very high whereas the error rates (≤0.56%) were very low. Remarkably, the observed median read depth was nearly 30X across the entire cohort despite the highly fragmented and degraded nature of the starting material (Table 3 and Figure 4). The *Quick*-DNA/RNA purification chemistry employed in our workflow recovers both ssDNA and dsDNA. The WGS method described here can similarly generate NGS libraries from both ssDNA and dsDNA, whereas most NGS library prep protocols are limited to higher molecular weight dsDNA input material. The combined ssDNA and dsDNA purification and fragment capture capacity likely contributed synergistically to produce high average read depth in the data presented here.

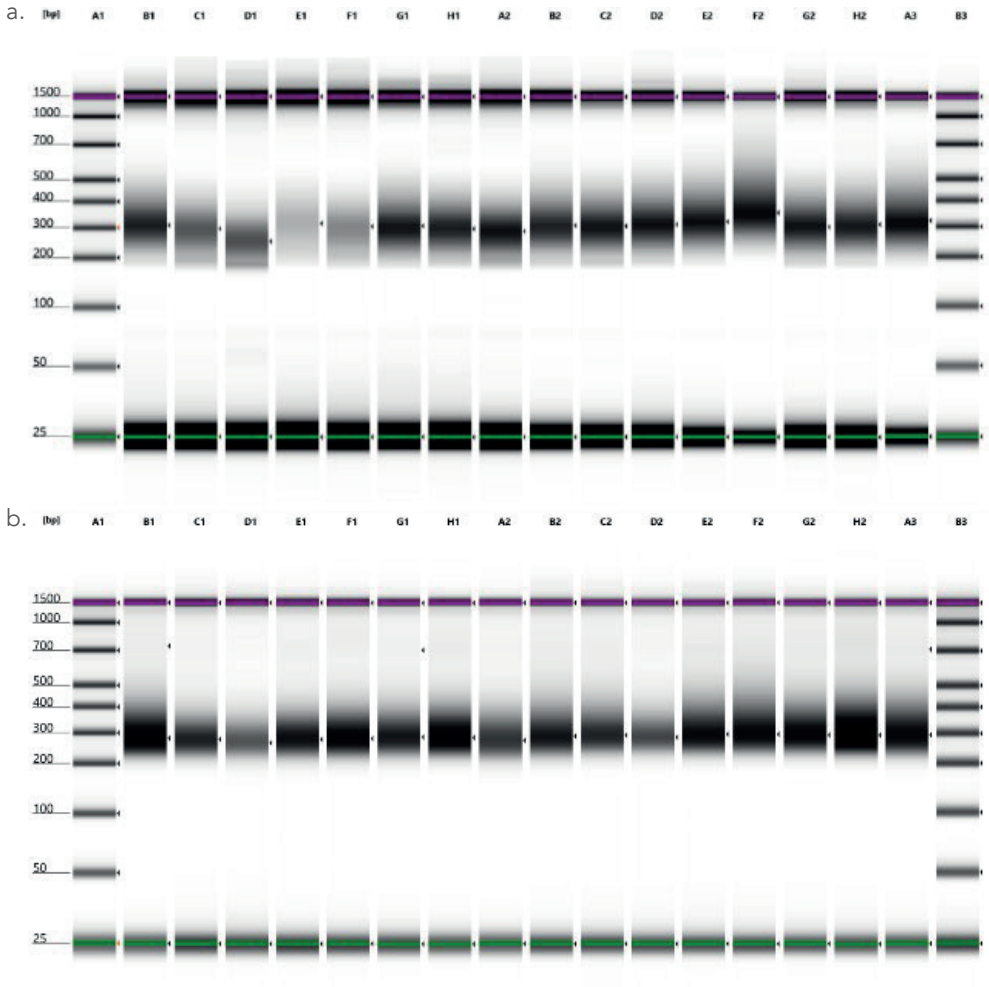

Figure 3: Tapestation QC of a) WGS libraries and b) RNA-seq libraries constructed from FFPE samples 1-16 loaded left to right across the image lanes.

Whole Transcriptome Sequencing (RNA-Seq)

We next evaluated RNA-seq library prep capability to support emerging studies that couple whole transcriptome profiling with genomic sequencing from the same FFPE tissue sample, supporting high-powered multi-omic research. Since RNA purified from FFPE tissue is highly degraded, we reasoned that a ribosomal (r)RNA depletion approach would yield better datasets compared to those designed to enrich the 3' ends of mRNA molecules — for example, polyA enrichment approaches. Our rationale was that enriching around polyA tails would disproportionately incorporate only the very ends of the short, low molecular weight gene transcripts, thus producing read data that makes bioinformatic analysis difficult. Others have drawn similar conclusions, yet most rRNA depletion protocols remain complicated, labor-intensive, or expensive (Liu et al., 2022; Pennock et al., 2019; Jacobsen et al., 2023), making them incompatible with high-throughput workflows. To overcome those challenges, we developed a customized workflow using the Zymo-Seq RiboFree® Total RNA Library Prep Kit (R3000). The RiboFree kit simplifies RNA-seq library preparation by utilizing a novel enzymatic method to deplete abundant ribosomal RNA species. By avoiding probe-based depletion, the workflow is simplified, making it automation-friendly while also reducing hands-on time and reagent costs.

Table 3: General whole genome sequence data summary statistics.

Sample Name	% GC	M Seqs	% PF	Error Rate	M Reads Mapped	% Mapped	Median Depth
brain_1-1	45%	361.0	99.10%	0.52%	715.4	99.90%	23.0X
brain_2-1	46%	459.4	99.20%	0.49%	910.7	99.90%	28.0X
brain_3-1	48%	645.4	99.10%	0.54%	1278.4	99.90%	31.0X
brain_4-1	45%	400.4	99.10%	0.56%	793.2	99.90%	22.0X
brain_5-1	47%	549.7	99.20%	0.50%	1089.7	99.90%	30.0X
brain_6-1	46%	529.0	99.20%	0.54%	1048.7	99.90%	30.0X
brain_7-1	47%	461.6	99.20%	0.51%	914.4	99.90%	27.0X
brain_8-1	47%	616.3	99.20%	0.48%	1221.6	99.90%	35.0X
brain_9-1	45%	188.1	99.10%	0.56%	372.7	99.90%	12.0X
brain_10-1	46%	470.0	99.00%	0.47%	930.3	99.90%	30.0X
brain_11-1	46%	554.4	99.10%	0.50%	1097.6	99.90%	35.0X
brain_12-1	44%	540.3	99.30%	0.51%	1072.7	99.90%	35.0X
brain_13-1	43%	391.7	99.00%	0.53%	775.0	99.90%	27.0X
brain_14-1	45%	459.9	99.10%	0.56%	911.3	99.90%	28.0X
brain_15-1	46%	328.9	99.10%	0.49%	651.5	99.90%	19.0X
brain_16-1	44%	511.6	99.10%	0.46%	1013.2	99.90%	36.0X
Average	**45.63%**	**466.7**	**99.13%**	**0.51%**	**924.8**	**99.90%**	**28.0X**

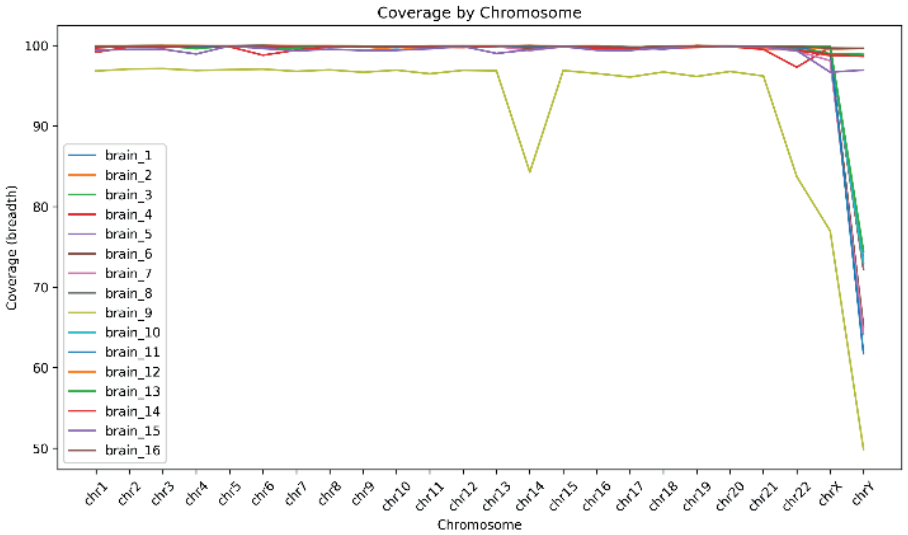

Figure 4: Coverage breadth by chromosome calculated as per-base read depth ≥5X.

We applied our custom workflow to RNA purified from the same meningioma samples used to assess whole genome sequencing and confirmed the expected very low RNA quality (RIN = 1.8, Figure 2b). However, we were able to successfully generate libraries suitable for sequencing (Figure 3b) and subsequently generate an average of 45 million read pairs per library. Basic bioinformatics QC metrics demonstrated a robust and reproducible workflow with high reads passing filter (>97%), alignment (>90%), gene detection (>47,000; featureCounts), and low rRNA contamination (<1%) on average (Table 4). Furthermore, a heatmap based on top gene expression patterns indicated high-quality RNA data, with XIST gene expression reported at far higher levels in female compared to male samples. As expected, USP9Y and other Y-chromosome linked genes showed higher expression in male compared to female samples (Figure 5).

Table 4: Gene expression data statistics.

Sample Name	% GC	M Seqs	% PF	% Aligned	% Dups	% rRNA	% Assigned	M Assigned	Number of Genes Detected
brain_1	44%	41.0	97.60%	93.30 %	21.00%	0.99 %	26.20%	10.2	45,572
brain_2	44%	47.6	96.70%	92.00 %	32.20%	1.35 %	24.40%	10.9	43,143
brain_3	45%	49.9	95.60%	89.90 %	37.50%	1.05 %	14.30%	6.5	50,020
brain_4	43%	49.5	96.40%	91.30 %	27.60%	0.83 %	20.00%	9.2	47,857
brain_5	44%	46.4	97.00%	92.00 %	24.90%	0.55 %	23.00%	10.0	48,965
brain_6	44%	47.9	96.90%	92.00 %	27.30%	1.02 %	20.20%	9.1	49,231
brain_7	43%	42.6	97.30%	93.00%	20.60%	0.47 %	20.60%	8.3	49,003
brain_8	44%	49.2	96.50%	90.30 %	28.40%	1.28 %	21.50%	9.8	48,711
brain_9	43%	44.1	97.00%	91.70 %	26.10%	0.88 %	17.70%	7.3	50,406
brain_10	43%	44.3	97.00%	92.00 %	27.70%	0.86 %	17.60%	7.3	49,975
brain_11	43%	42.5	96.60%	91.80 %	35.70%	0.68 %	27.50%	10.9	43,535
brain_12	43%	44.8	97.50%	92.90 %	25.70%	0.82 %	25.60%	10.9	47,028
brain_13	44%	44.3	97.80%	93.70 %	26.40%	0.55 %	28.00%	11.8	46,842
brain_14	44%	44.4	97.40%	93.00 %	25.10%	0.51 %	24.80%	10.4	47,345
brain_15	43%	41.6	97.70%	94.10 %	20.10%	0.49 %	27.00%	10.7	44,300
brain_16	43%	41.4	97.50%	92.80 %	27.50%	1.15 %	27.40%	10.8	46,449
Average	**43.56%**	**45.1**	**97.03%**	**92.24%**	**27.11%**	**0.84%**	**22.86%**	**9.6**	**47,399**

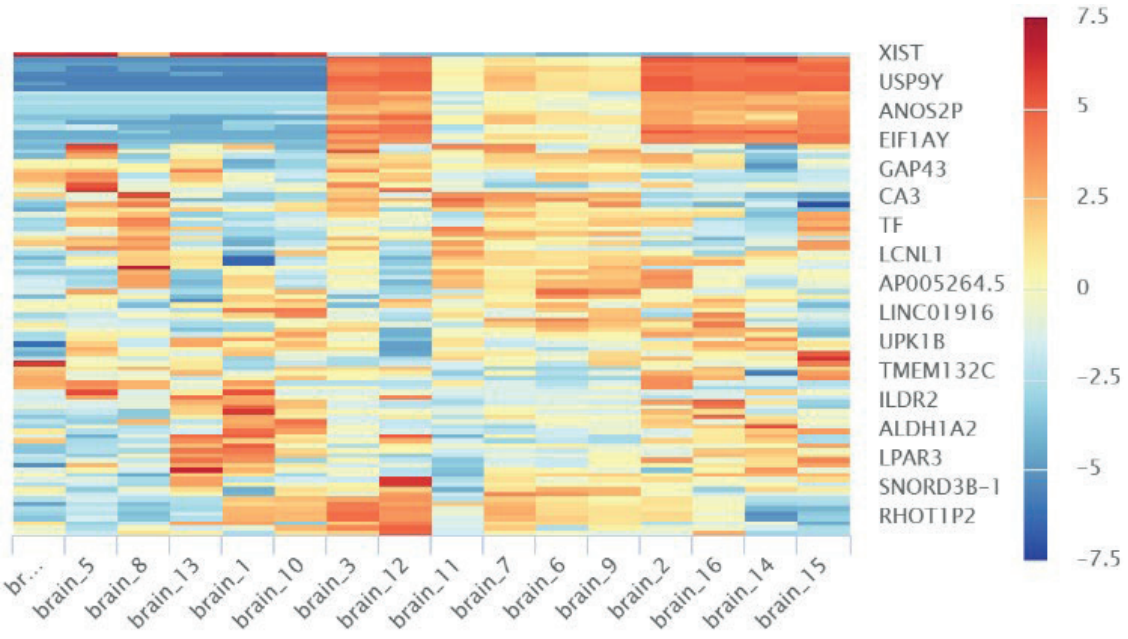

Figure 5: Top gene expression patterns heatmap from all 16 meningioma purified RNA samples.

Discussion

There has been a lot of very recent interest amongst scientific research as well as investor communities as to how data science will revolutionize a variety of industries, especially biomedical sciences. With the successful rollout of Artificial Intelligence (AI) platforms such as ChatGPT, Claude, Llama, DeepSeek, and others, the attention has switched from AI engines in and of themselves to their practical applications (RESI Conference Boston, 2024). With the advent of ultra-high throughput sequencing platforms and the near realization of the $100 genome, genome data that can fuel AI-driven biomedical research is now accessible in a way that was previously not possible. However, as Nicolo Fusi, Senior Principal Research Manager at Microsoft Research in Cambridge, Massachusetts, recently put it when discussing AI's potential for precision medicine, "We don't [necessarily] need more data, we need better data" (PMWC Silicon Valley, 2025).

Utilization of FFPE material in biomedical research is one such source of rich biomedical data. The potential exploitation of FFPE sample data generation using NGS technology has been limited by the relative difficulty and poor quality of the DNA/RNA inherent with the sample collection process. The methods and workflows described here demonstrate that high-quality sequence data can be generated from FFPE samples. Using specialized chemistry and dedicated protocols we described how the sample prep (purification and NGS library prep) steps can be automated to increase throughput, reduce hands-on time, and improve reproducibility and robustness of downstream sequence data. Furthermore, the resulting sequence data proved to be of high complexity, alignment, and overall coverage, making them suitable for in-depth bioinformatic analysis. Finally, the methods described here are translatable and scalable to any test or research lab processing samples and generating data for molecular biology applications.

At Zymo Research we are proud to be part of the current renaissance in genomics-led biomedical research. We provide state-of-the-art products and services that streamline and standardize every stage of an NGS-driven study. Our innovative chemistries and proven automated workflows, along with the expertise of our data scientists, ensure that even challenging samples, whether from environmental sources, clinical biopsies, or those with poor quality (FFPE) or even ultra-low picogram quantity inputs, can be quickly and reliably prepared for generating NGS data and ready for downstream applications. We remain committed to pioneering new research tools and next-generation sequencing analysis services techniques to meet any ongoing and future challenges that arise. While Zymo Research provides some of the most technologically advanced products in the industry, everything we do is driven by the fundamental belief that, "The beauty of science is to make things simple".

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