Sequencing the Unsequenceable

Formalin-Fixed, Paraffin-Embedded (FFPE) Sample Processing for Successful Target Enrichment

INTRODUCTION

Library construction for Next Generation Sequencing (NGS) using formalin-fixed paraffin-embedded (FFPE) samples offers unique challenges in acquiring high-quality sequencing data due to wide distribution of sample quality. Differences in formalin fixation methods, storage conditions, and age lead to crosslinked and/or degraded nucleic acid and inconsistent extraction yields. Therefore, FFPE extraction and library construction methods must be carefully considered for target enrichment applications. In collaboration, Covaris and Twist Bioscience demonstrate a complete library preparation and target enrichment solution that generates ready-to-sequence multiplexed libraries directly from FFPE tissue of various qualities.

TECHNOLOGY

The workflow leverages the Covaris truXTRAC FFPE total Nucleic Acid Plus Kit and AFA-TUBE TPX shearing with the world-class performance of Twist Bioscience's Library Preparation and Target Enrichment Solutions.

Covaris FFPE Pre-Analytical Products

The truXTRAC FFPE total Nucleic Acid family of kits incorporates the patented Adaptive Focused Acoustics (AFA) technology into the deparaffinization and nucleic acid extraction workflow. Fine-tuned AFA energy settings allow RNA and DNA isolation in parallel from the same sample (no splitting), thereby increasing yields and reducing heterogeneity due to separate sample input. Due to the solvent-free deparaffinization and active extraction process, high quality nucleic acids in sufficient quantity for downstream NGS analysis are obtained. The Covaris truXTRAC FFPE total Nucleic Acid Plus Kit is designed for efficient and sequential extraction of total nucleic acids (RNA and DNA) from FFPE tissue samples using AFA.

Twist Bioscience Target Enrichment Solutions

The Twist Library Preparation Kit for Mechanical Fragmentation enables the construction of adapter ligated libraries ready for downstream targeted enrichment and subsequent sequencing. Compatible with both Twist CDI and UDI adapters, this kit accommodates a wide range of DNA quality and types. When coupled with the Twist Target Enrichment Solution, a full saturation and enrichment of targeted molecules is achieved. This enrichment efficiency, especially with degraded samples, confers the highest level of confidence in variant determination. The uniform capture across the entire target region ensures that extra sequencing is minimized, which lowers sequencing costs, increases sample throughput, and achieves a higher depth of coverage across target regions with uncompromising quality.

METHODS

In this FFPE-specific application, a multistage workflow combining Covaris FFPE extraction and fragmentation with Twist library preparation and target enrichment was carried out under the manufacturer's recommendations. Various FFPE tissue types (N = 6) were extracted a single time and their quantitative quality scores were determined by qPCR using the KAPA hgDNA Quantification and QC Kit (QQC Kit). Extracted samples were then independently fragmented multiple times to a targeted range of 200 bp to 250 bp (N ≥ 3). 100 ng input of fragmented gDNA was then carried through to library preparation. Libraries were then pooled to multiplex (N = 8) samples per capture. Twist Universal Blockers and the Twist Human Core Exome Probes were added to the hybridization reaction for target enrichment. The enriched libraries were sequenced on the NextSeq 500/550 High Output v2 kit (Illumina) to generate 2 x 76 paired-end reads and down-sampled to 150x of targeted bases. Picard HS_metrics tools with a mapping quality of 20 were utilized for sequence analysis.

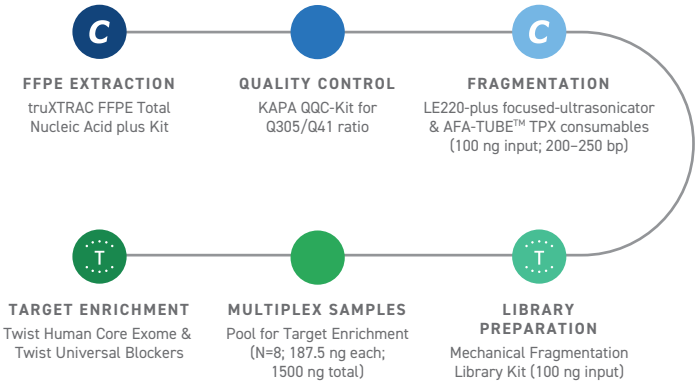
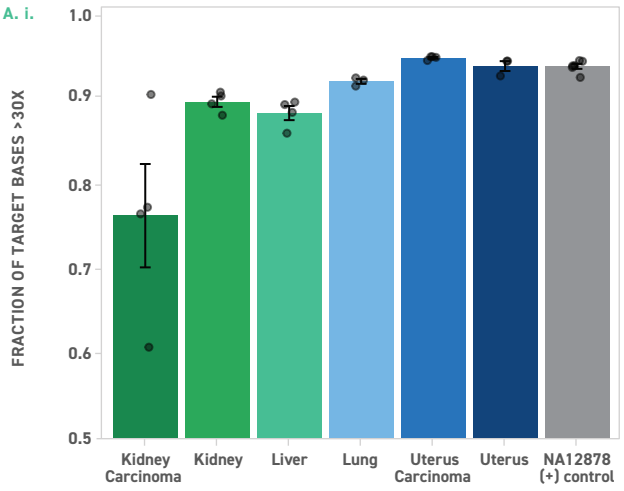


Figure 1: Experimental workflow

RESULTS

A. i.



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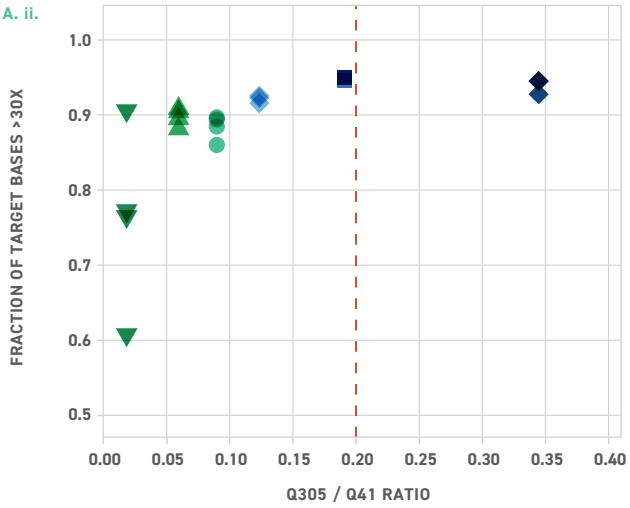

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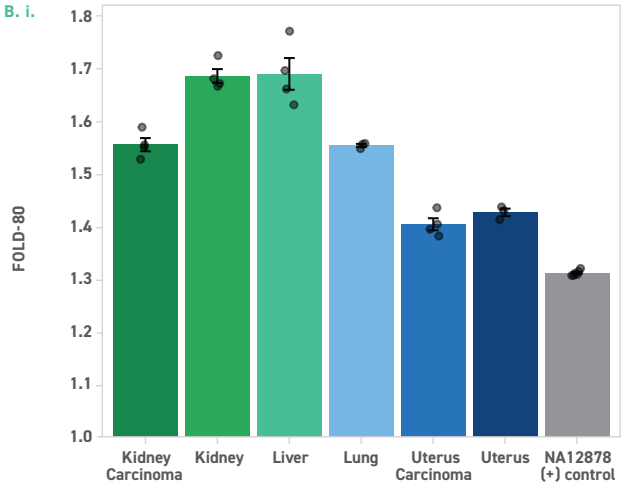

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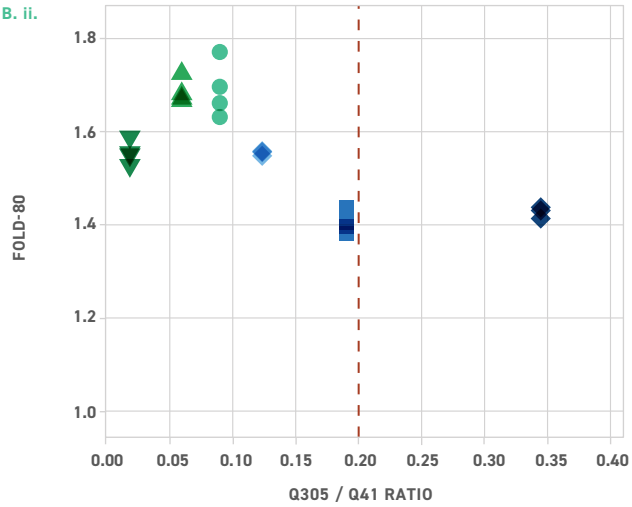
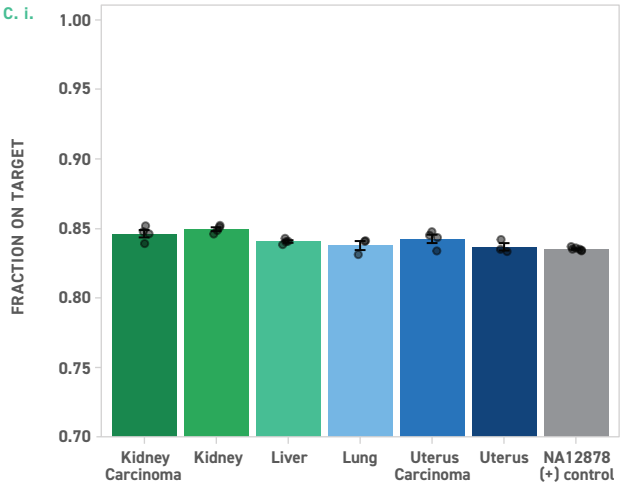


C. i.



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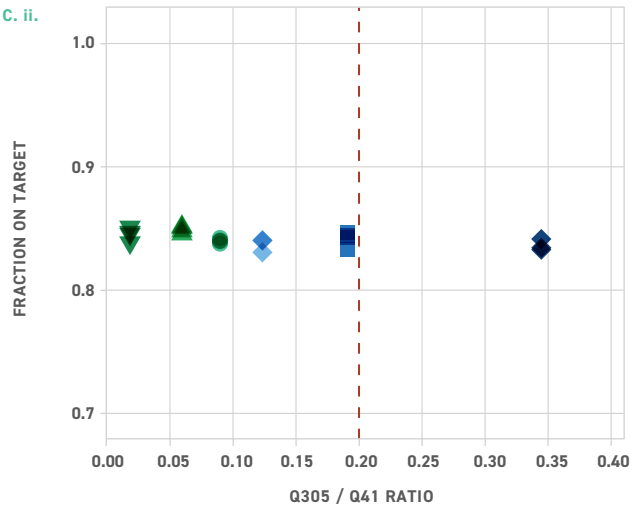

RESULTS

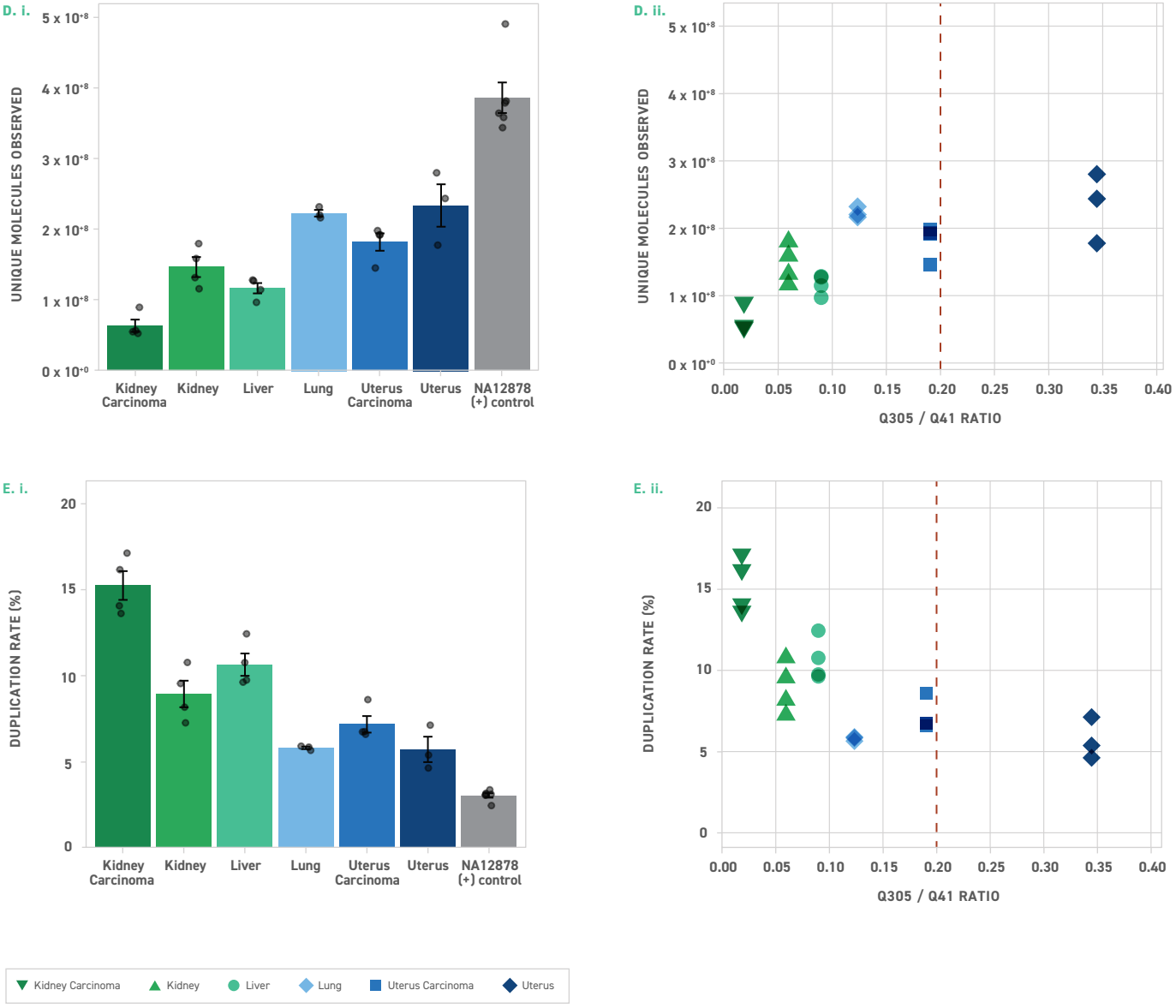

Figure 2. Sequencing Performance of FFPE Extracted Samples. A) to **E)** Summary of sequencing metrics from gDNA libraries prepared using Covaris AFA technology for extraction and fragmentation and Twist Target Enrichment Solution (see **Figure 1**). Experimental workflow was carried out according to manufacturer's recommendations for the respective step (see **Figure 1**). **i)** Bar graphs of sequencing metrics by tissue type and **ii)** scatter plots of quantitative scores of library integrity (Q305/Q41 ratios; KAPA hgDNA Quantification and QC Kit) versus sequencing metrics. Positive control sheared with AFA but not subject to FFPE extraction or Q305/Q41 ratio determination was used. N ≥ 3 for all observations; error bars denote standard deviation.

RESULTS

Using the aforementioned workflow, results from processing numerous FFPE tissue types and qualities with KAPA Q305/Q41 qPCR ratios ranging from 0.34 to 0.02 were presented. With samples presenting Q305/Q41 ratios ≥0.05, sequencing results of 8-plexed libraries demonstrate large improvements in target enrichment efficiency (Picard metrics) that include uniformity (Fold_80 ≤ 1.8), sequencing depth (30x coverage ≥88% with 150x down sampling), and duplication rates (≤11%) when compared to similar published studies.

These results demonstrate a validated solution for library preparation and targeted exome sequencing of FFPE samples that can be integrated into automated workflows. The truXTRAC kit and AFA technology from Covaris generate size specific DNA libraries from FFPE samples that, when paired with Twist's target enrichment solution (Human Core Exome and Universal Blockers), deliver multiplexed libraries for high performance targeted sequencing.

DISCUSSION

Large cohorts of FFPE samples continually provide challenges for obtaining high quality sequencing data. Sample preservation methods, age, sample storage conditions, tissue type, and other factors contribute to a wide distribution of sample quality. When quantifying sample quality, qPCR scores from kits such as the KAPA QQC kit are useful in identifying low quality samples that are unlikely to return adequate sequencing depth. Samples with KAPA QQC Q305/Q41 ratios of <0.2 are typically binned as 'low-quality' and not suitable for sequencing[1].

A workflow for FFPE extraction that combines Covaris truXTRAC products and AFA technology with Twist NGS library products and target enrichment panels was presented. This workflow demonstrated that samples with KAPA QQC Q305/Q41 ratios of ≥0.05 can now be considered for whole exome multiplexed target enrichment and sequencing. As a result, when Covaris and Twist technologies are applied, FFPE samples that would typically be binned as *unsuitable for sequencing* with other workflows can be confidently reclassified as *suitable for sequencing*.

The combination of AFA and Twist Target Enrichment technologies help researchers to unlock more robust data from interesting FFPE samples that have previously been unusable for molecular profiling methods. Gaining the ability to sequence these often rare and challenging samples may give us more insight into the molecular drivers of a variety of unique tumor types and will further extend progress toward personalized and precision medicine.

[1] de Abreu, F, *et al.* (AGBT 2015) *The KAPA Human Genomic DNA Quantification and QC Kit Enables Prediction of Sequencing Performance Through User-Defined Metrics*, Marco Island, FL