High Performance Multiplexed Whole Exome Sequencing from Problematic FFPE Tissues

Mark Consugar[1], Leonardo Arbiza[1], Kristin Butcher[1], Siyuan Chen[1], Hutson Chilton[1], Richard Gantt[1], Yehudit Hasin-Brumshtein[1], Jim Laugharn[2], Jayne Simon[2], Ulrich Thomann[2], Christina Thompson[1], Martina Werner[2], Ramsey Zeitoun[1]

[1]Twist Bioscience; [2]Covaris, Inc.

1. Abstract

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.

This workflow leverages the Covaris truXTRAC® FFPE total Nucleic Acid Plus Kit and oneTUBE-10 shearing with the world-class performance of Twist Bioscience's Target Enrichment Solutions. Covaris, the Gold Standard for mechanical DNA shearing in NGS applications, offers pre-analytical products that leverage Adaptive Focused Acoustics® (AFA®) technology. In this FFPE-specific application, the Covaris truXTRAC FFPE total Nucleic Acid Plus Kit and oneTUBE-10 shearing on the LE220-plus Focused-ultrasonicator enables full emulsification of paraffin and disaggregation of tissue for highly efficient nucleic acid extraction and generation of size-specific DNA libraries. With the Twist Bioscience Human Core Exome kit, the resulting libraries are indexed, pooled, and target enriched with uniquely optimized DNA probes to generate ready-to-sequence high quality multiplexed libraries.

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 are presented. With samples presenting Q305/Q41 ratios ≥0.05, sequencing results of 8-plexed libraries demonstrate large improvements in general 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 Bioscience's superior target enrichment workflow, deliver multiplexed libraries for high performance targeted sequencing.

2. Covaris AFA Technology

Mutation detection-based sequencing is becoming increasingly important in both research and the clinic. Sample preparation is recognized as the limiting factor for sensitivity and specificity of biomarker detection. Adaptive Focused Acoustics (AFA) is an advanced acoustic technology enabling the mechanical processing of samples by Focused-ultrasonicators. AFA employs highly controlled bursts of focused high-frequency acoustic energy to efficiently and reproducibly process samples in a temperature-controlled and non-contact environment. This focused and efficient delivery requires a minimal amount of energy input avoiding the adverse effects of excess energy such as damaging heat, experimental variability, and sample over-processing typical of ordinary sonicators.

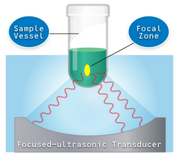
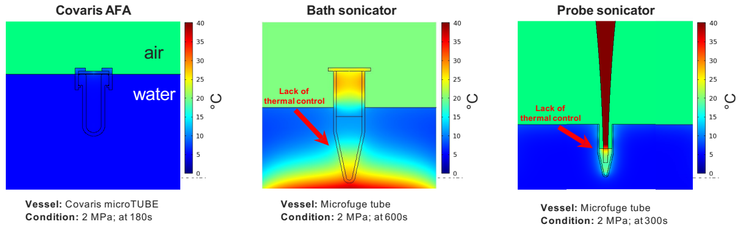


Figure 2.1. Illustrative representation of AFA technology.



Vessel: Covaris microTUBE
Condition: 2 MPa; at 180s

Vessel: Microfuge tube
Condition: 2 MPa; at 600s

Vessel: Microfuge tube
Condition: 2 MPa; at 300s

Figure 2.2 Thermal profile comparison of AFA with probe and bath sonicators. Note the superior thermal profile around the sample with application of AFA.

3. Covaris FFPE Pre-Analytical Products

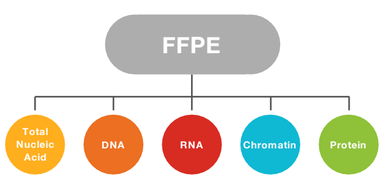


Figure 3.1 Sample types that can be extracted from FFPE samples with Covaris truXTRAC FFPE Family of pre-analytical products and AFA technology.

The truXTRAC FFPE total Nucleic Acid family of kits incorporates the patented AFA technology into the deparaffinization and nucleic acid extraction workflow (Figure 3.1). 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 truXTRAC FFPE total NA Plus Kit is designed for efficient and sequential extraction of total nucleic acids (RNA and DNA) from Formalin-Fixed, Paraffin-Embedded (FFPE) tissue samples using AFA (Figure 3.2).

Benefits for DNA Extraction:
- Highly amplifiable DNA due to increased fragment length and quality
- Drastically reduced **Q**uantity **N**ot **S**ufficient rates
- More DNA from less tissue - preservation of valuable sample
- Homogeneous extraction guarantees coverage of entire tissue examined

Benefits for RNA Extraction:
- High DV_{200} scores of the extracted & purified RNA
- Drastically reduced **Q**uantity **N**ot **S**ufficient rates
- Exon-shuffling and gene-fusion detection is optimized due to increased length of extracted transcript

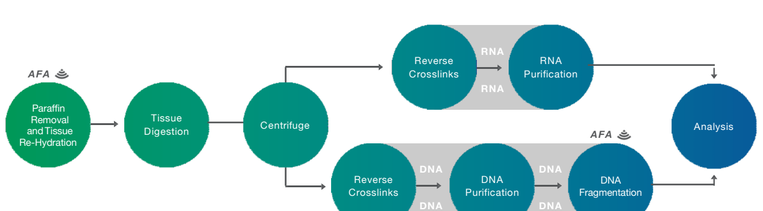


Figure 3.2. truXTRAC FFPE total Nucleic Acid Kit workflow.

4. Twist Target Enrichment Panels

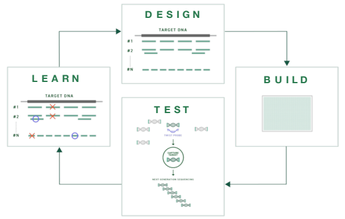


Figure 4.1 Design-Build-Test-Learn: Workflow of design-build-test-learn strategy that was used to generate process for designing a target enrichment system.

A Design-Build-Test-Learn (**DBTL**) strategy was implemented towards developing a framework for generating reproducibly high-performing panels for target enrichment and sequencing (Figure 4.1).

This iterative learning approach requires each step to be performed with reproducible results towards building on results of previous iterations. The reproducibility and expected performance of both the **build** and **test** steps of the DBTL system is presented. The reproducibility data is shown for a representative 800 kb panel consisting of roughly 7,400 probes. Replicates were synthesized 1 month apart.

Build: An NGS quality control step is performed on every custom panel generated where probe representation is measured post-production. This ensures the process completed as expected and the probe content and representation reflects the intended design. Reproducibility between two panels based on NGS probe counting is high and supports DBTL (Figure 4.2).

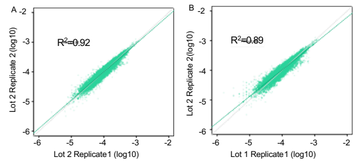


Figure 4.2 Lot to Lot Variability From Build: Each synthesis involves amplification step. A panel containing roughly 7,400 probes (800 kb) was re-synthesized ~1 month apart (Lot1 and Lot2), with two amplification replicates in each Lot (Replicate 1 and 2) **A**) Reproducibility of probe representation within same synthesis, different amplifications. **B**) Reproducibility of probe representation between syntheses.

Test: An NGS target enrichment probe to probe performance was done to ensure reproducible capture and testing of the built panel (Figure 4.3). The overall sequencing HS metrics also showed high concordance between lots.

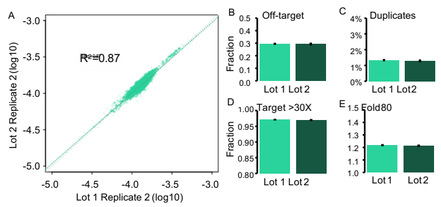


Figure 4.3 Lot to Lot Variability From Test: Data was down-sampled to 150x of target size and analyzed using Picard Metrics with a mapping quality of 20; N = 2. **A**) Lot to lot reproducibility capture per probe. **B-E**) Reproducibility of probe target enrichment performance between syntheses.

Following the optimization of each portion of the cycle the results were used to design high-performance panels in a first attempt. Six panels ranging from 0.02 Mb to 13 Mb were synthesized and shown to have high coverage metrics (30x coverage) which was made possible by a multivariate optimization of key metrics (Figure 4.4).

A

Panel Name	Size (Mb)	Probes	Genes
Mitochondrial DNA	0.02	139	37
Cancer Hotspot	0.04	384	50
Neurodegenerative	0.6	6,024	118
Cancer + Hotspot	0.8	7,446	127
Actionable Cancer	1.7	19,661	522
Pan-Cancer	3.2	31,002	578
Exploratory Cancer	13.3	135,937	5,442

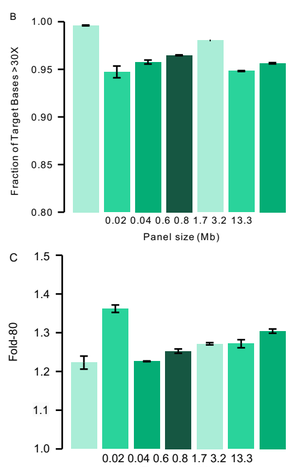


Figure 4.4 First Pass Capture Performance*: Information of capture performance across 6 different panels. **A**) Description of panels and size. **B**) Uniformity (Fold 80) **C**) 30x Coverage performance of each panel as defined by Picard HS metrics.

*Hybrid capture was performed using several target enrichment panels (Twist Bioscience) using 500 ng of gDNA (NA12878; Coriell) per single-plex pool following manufacturer's recommendations. Sequencing was performed with a NextSeq® 500/550 High Output v2 kit (Illumina®) to generate 2x76 paired end reads. Data was downsampled to 150x of target size and analyzed using Picard Metrics with a mapping quality of 20; N = 2.

5. Barcode Independent Universal Blockers

One metric that limits performance of multiplex hybrid capture is the percentage of bases on-target. To address this, commercial hybrid capture systems typically employ two types of blocker systems to improve this metric for human samples: **(1)** Cot DNA, a product enriched for repetitive human gDNA and **(2)** adapter blockers, designed to limit adapter cross-hybridization. While sequence specific adapter blockers provide acceptable performance for small barcode sets, they hinder adoption of expanded index sets, restrict changing barcode length between experiments, and prohibit use of unique dual indices (UDI) or unique molecular identifiers (UMI).

A universal blocker system was developed to address these bottlenecks by increasing capture of on-target reads independent of barcode sequence length or design for all TruSeq® compatible adapters (**Figure 5.1**). Functionality is also retained regardless of target panel size (**Figure 5.2**) and across singleplex and multiplex target enrichment workflows (see **Section 6**). As a result, this universal adapter blocker technology drastically increases on- and near-bait capture, allows for index flexibility between experiments, simplifies design and execution of multiplex experiments, and directly reduces sequencing costs.

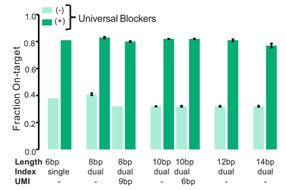


Figure 5.1: Performance is independent of barcode design. On-target performance of universal blockers (Twist Bioscience) across a variety of single and dual index TruSeq-compatible adapters. Individual libraries were generated from a single genomic source (NA12878; Coriell) and TruSeq-compatible adapters with barcode lengths ranging from 6 to 14 bp (UMI length not included). Hybrid capture was performed either in the absence or presence of universal blockers using an exome target enrichment panel (33.1 Mb; Twist Bioscience) using 500 ng of gDNA per individual library following the manufacturer's recommendations for 16-hour hybridization reactions. Cot DNA was present in all samples. Fraction of bases on-target is defined by the equation 1 - PCT_OFF_BAIT. Error bars denote one standard deviation or range of observations; N ≥ 2.

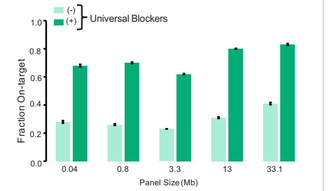


Figure 5.2: Performance is independent of panel size. On-target performance of Universal Blockers (Twist Bioscience) across a variety of panel sizes. Individual libraries were generated from a single genomic source (NA12878; Coriell) and 8 bp dual-indexed TruSeq-compatible adapters. Hybrid capture was performed either in the absence or presence of universal blockers with target enrichment panels of various sizes (0.04Mb to 33.1Mb; Twist Bioscience) and 500 ng of gDNA per individual library following the manufacturer's recommendations for 16-hour hybridization reactions. Cot DNA was present in all samples. Fraction of bases on-target is defined by the equation 1 - PCT_OFF_BAIT. Error bars denote range of observations; N = 2.

6. Multiplex Hybridization

Target enrichment probes should also be designed match the desired throughput of the system. Probes were designed to maximize the capture of unique molecules and minimize sequencing duplicates to delivery high multiplex performance. Twist Target Enrichment workflows were designed to maintain high-performance with up to a 16-plex capture. This is demonstrated on three panels of 800 kb, 3.3 Mb and a fixed Exome of 33.1 Mb. Consistent capture coverage at 30x is observed across all samples and multiplexing conditions (**Figure 6.1**). While an increase in duplicate rate is expected as the number of samples in a multiplex increases, the magnitude of this increase is minimal. For an 800kb panel duplication rate increases from 1.8% to 2.7% between 1-plex and 16-plex captures, respectively, and similar observations were made with larger panels. The minimal impact to performance is confirmed with consistent 30x coverage.

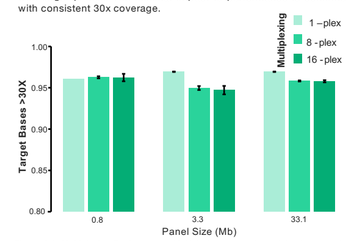


Figure 6.1: Multiplexing Performance. Multiplexing was performed for three panels at three degrees of multiplexing. Hybrid capture was performed using target enrichment panels of various sizes (800kb, 3.3Mb, or 33.1 Mb; Twist Bioscience) using a total of 1500 ng library (NA12878; Coriell) per multiplexed pool following manufacturer's recommendations. **a)** 800 kb cancer panel, **b)** 3.3 Mb cancer panel, and **c)** Exome panel. N = 2.

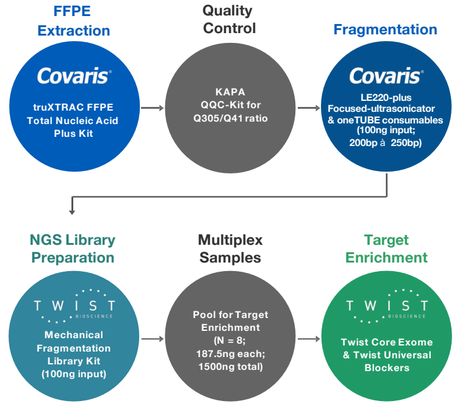
7. Targeted Enrichment of FFPE Samples

Working with large cohorts of FFPE samples presents many challenges including poor yields during extraction, wide distribution of sample quality due to formalin fixation methods, archival storage conditions, and chemical modifications that limit downstream conversion into libraries suitable for NGS. These factors directly impact sequencing quality with lower library diversity, poor uniformity, higher duplication rates, and lower sequence coverage which constrains the number of samples per sequencing run. Additionally, FFPE derived NGS libraries are typically target enriched as single-plex samples due to these factors, limiting high throughput applications.

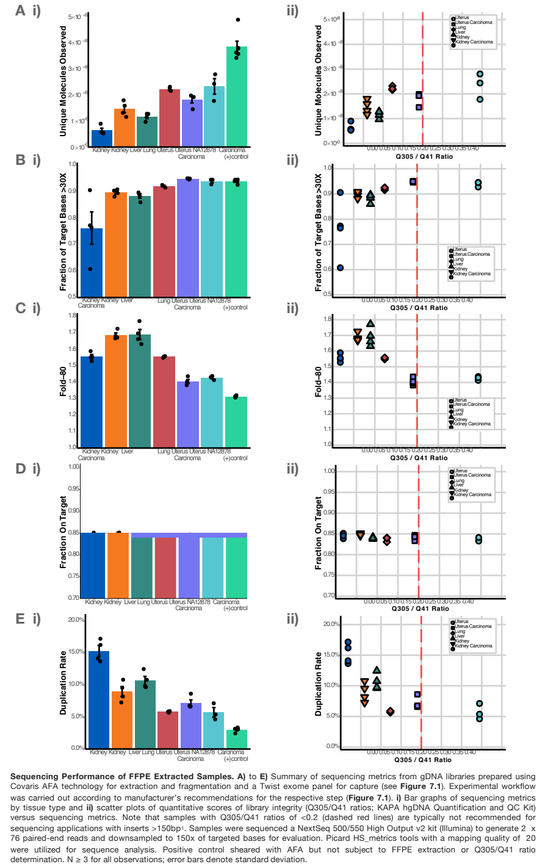
Quantitative scores utilizing qPCR can be utilized to identify low quality FFPE extracted DNA samples and eliminate them from evaluation to optimize sequencing resources. One such kit is the KAPA® hgDNA Quantification and QC Kit (QQC Kit). An independent evaluation of this kit suggests that FFPE extracted DNA with Q305/Q41 ratios <0.2 should not be carried forward for sequencing applications with insert sizes >150bp[1].

Covaris truXTRAC FFPE total Nucleic Acid Plus Kit and oneTUBE-10 shearing on a LE220-plus Focused-ultrasonicator was combined with Twist Bioscience's Target Enrichment Solutions to address these current limitations around extraction, target capture, and sequencing performance. With only 100 ng of gDNA input required for NGS library creation, this workflow alleviates concerns around FFPE extraction efficiency and variability of library fragment size (**Figure 7.1**). The demonstrated automation friendly workflow enables multiplex target enrichment with high quality sequencing performance on an exome panel (33.1Mb) across a variety of tissue samples with Q305/Q41 ratios well below current recommendations (**Figure 7.2**).

[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



Experimental Workflow. Multistage workflow combining Covaris AFA Technology for FFPE extraction and fragmentation with Twist Library Creation and Target Enrichment. Various FFPE samples (N = 6) were extracted a single time and their Q305/Q41 ratios determined. Extracted samples were then independently fragmented multiple times to a targeted range of 200bp to 250bp (N ≥ 3) and carried through the remaining steps of the workflow.



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 a Twist exome panel for capture (see **Figure 7.1**). Experimental workflow was carried out according to manufacturer's recommendations for the respective step (**Figure 7.1**). **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. Note that samples with Q305/Q41 ratios of <0.2 (dashed red lines) are typically not recommended for sequencing applications with inserts >150bp[1]. Samples were sequenced a NextSeq 500/550 High Output v2 kit (Illumina) to generate 2 x 76 paired-end reads and downsampled to 150x of targeted bases for evaluation. Picard HS_metrics tools with a mapping quality of 20 were utilized for sequence analysis. Positive control sheared with AFA but not subject to FFPE extraction or Q305/Q41 ratio determination. N ≥ 3 for all observations; error bars denote standard deviation.

8. Summary

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.

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'.

- Covaris truXTRAC products and AFA technology enable high quality extraction and shearing of DNA from FFPE samples.
- Twist target enrichment workflows and panels provide reliable multiplex performance across a wide range of panel sizes.
- Combining Covaris AFA FFPE extraction products and AFA fragmentation with Twist library creation and multiplex target enrichment allows for sequencing of challenging FFPE samples (Q305/Q41 ratios ≥0.05) with uniformity and 30X depth of coverage values that are currently unmatched in the literature for exome-sized target enrichment panels.
- Improved uniformity for sequencing of FFPE samples translates directly to more samples per lane and reduced sequencing costs for a desired depth of coverage.