

Comprehensive Sample Prep Workflow for Deparaffinization, Extraction, Purification, & Accelerated Digestion of Proteins from Formalin-Fixed Paraffin-Embedded Tissue

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Introduction and Background

Formalin-fixed paraffin-embedded (FFPE) tissue samples are a valuable resource for studying the molecular mechanisms of disease. FFPE tissue samples are widely used in genomics and have been gaining popularity in proteomics. Despite several technical advancements in LC-MS, identification or quantitation of proteins in tissues embedded in paraffin can be complicated owing to protein cross-linking. Most sample preparation workflows for FFPE samples often result in highly degraded proteins with low yields. Adaptive Focused Acoustics® (AFA®) Technology is extensively used for efficient extraction, purification, and expedited digestion of proteins from complex biological matrices, such as FFPE. In this study, we report development of comprehensive and robust workflows with Covaris technology for deparaffinization, extraction, purification, and digestion of proteins starting with just one 10 µm FFPE scroll.

Workflow

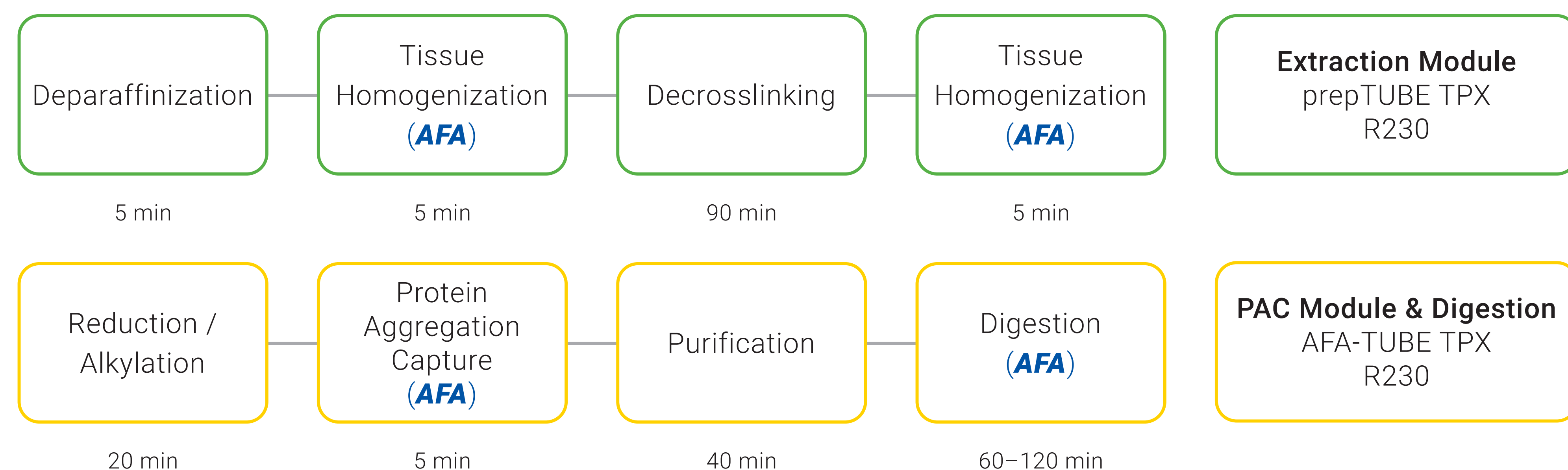


Figure 1: Schematic Covaris workflow diagram. The workflow is separated into two modules. Deparaffinization, tissue homogenization, and decrosslinking is performed in batches of 8 or 16 samples with the prepTUBE TPX in the R230. The resulting lysate is transferred to an AFA-TUBE TPX and reduced/alkylated prior to Protein Aggregation Capture (PAC) which is enhanced with AFA. Following purification, accelerated trypsin digestion is performed in ABC buffer under 1-2 hour AFA-treatment.



Figure 2: prepTUBE TPX and AFA-TUBE TPX. Several prepTUBE TPX loaded with a 10 µm FFPE kidney scroll and a single AFA Bead (A), FFPE kidney tissues after deparaffinization (B), and AFA-TUBE TPX Strips (C). Paraffin is removed from FFPE samples with a non-toxic Deparaffinization Solution. Following decrosslinking and tissue homogenization, the resulting lysate is transferred into the AFA-TUBE TPX for the remainder of the workflow.

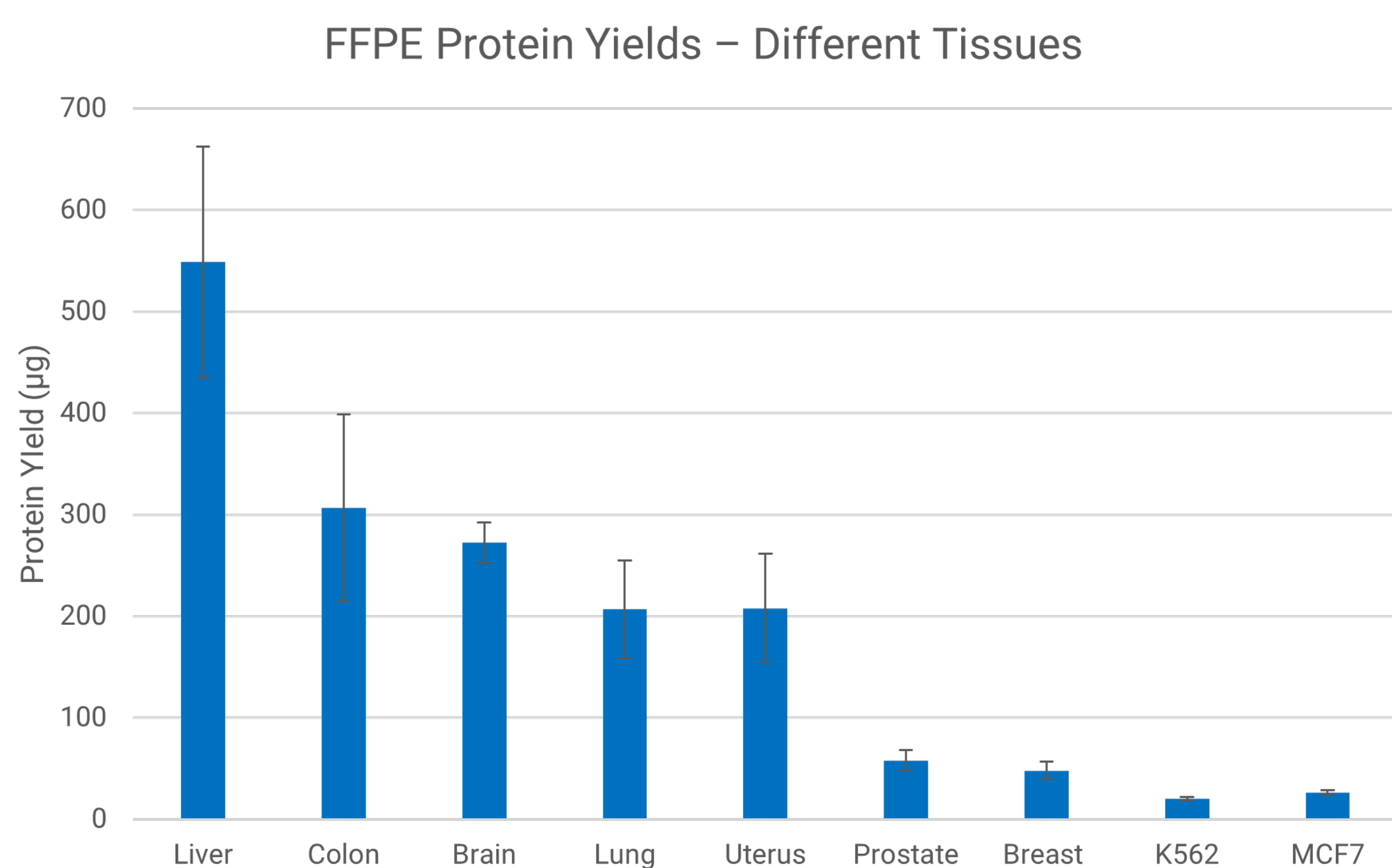


Figure 3: Protein Yield from FFPE Tissues. Colorimetrically-derived yields of protein extracted from 7 FFPE tissues and 2 FFPE cell pellet blocks. Extractions were performed with 10 µm sections. Protein yields from FFPE samples are highly variable due to factors such as fixation time, ratio of tissue to paraffin, type of tissue (density), age and storage conditions of FFPE blocks.

Methods

Covaris

- Non-toxic deparaffinization
- AFA-enhanced tissue homogenization (33,600 Joules | 2X)
- Decrosslinking (90 °C | 90 min)
- Centrifugation (5,000 x g | 15 min)
- Transfer lysate into AFA-TUBE TPX
- Reduction/Alkylation
- AFA-enhanced protein aggregation capture (255 Joules | 10X)
- Magnetic bead purification
- AFA-enhanced trypsin digestion (107 Joules | 126X)

Other/Traditional

- Xylene deparaffinization
- Ethanol washes (100%, 95%, 80%, 50%)
- Micropestle grinding
- Probe sonication (70 Amps | 10X)
- Decrosslinking (95 °C | 120 min)
- Centrifugation (14,000 x g | 10 min)
- Transfer lysate into 1.5 mL tubes
- Reduction / Alkylation
- Trypsin digestion
- Magnetic bead purification (KingFisher)

LC-MS

- Desalting (Acclaim PepMap 100 C18 HPLC column)
- Separation (PepMap RSLC C18 EASY-Spray Column)
- Mass Spectrometry (Orbitrap Fusion Lumos coupled to an EASY-nLC 1200)
- Raw data analysis with Proteome Discoverer 2.4.1.15
- Parameters: 90-minute gradient, 50 ng peptide input, 300 nL/min flow rate, 1% FDR

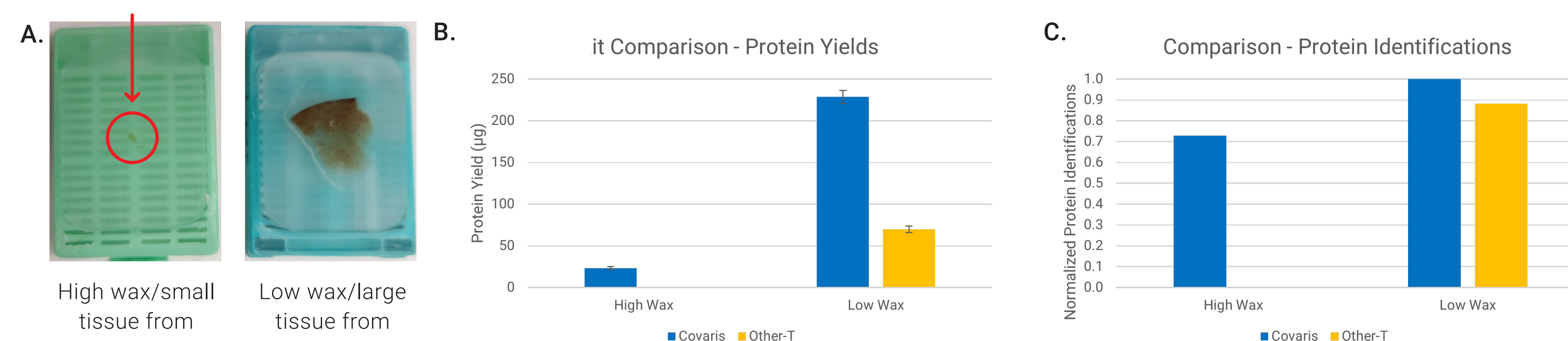


Figure 4: Method Comparison. A comparison between the Covaris method and a commercially available product with high and low wax FFPE liver samples (A) following manufacturer recommended protocols. Covaris resulted in higher protein yields than the competition for low wax samples and was the only kit to successfully extract a measurable amount of protein from high wax samples (B). LC-MS data from peptides generated with the Covaris method resulted in 12% more protein identifications than the alternative product (C). Classification of identified proteins using PANTHER version 18 (Thomas et al. Protein Society 2022, 31(1)) indicated that sample preparation with Covaris resulted in twice as many DNA metabolism proteins, 70% more membrane traffic proteins, 60% more transcriptional regulators, and 45% more transporter proteins as compared to the commercial product.

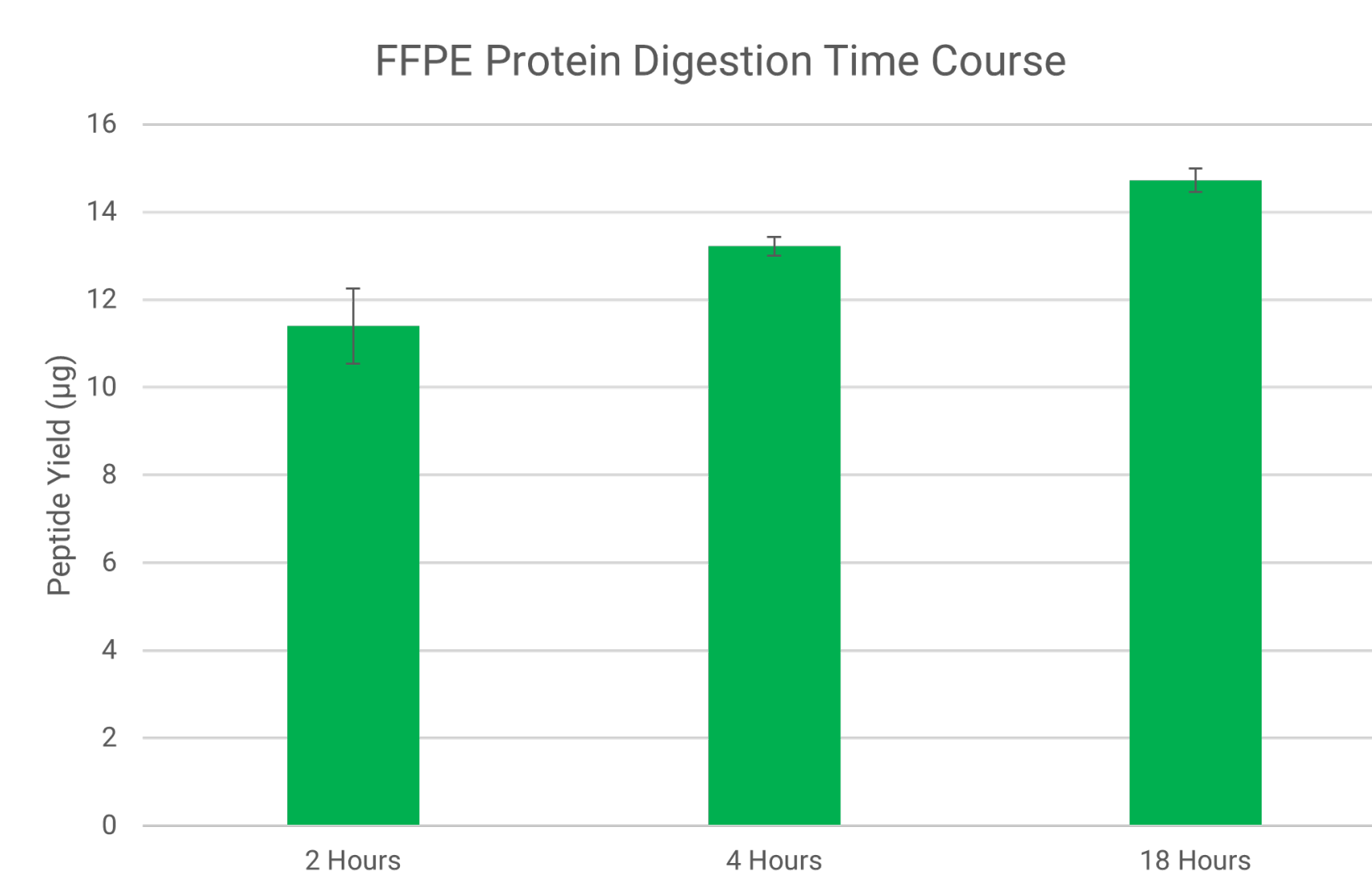


Figure 5: Digestion without AFA. Covaris also offers a flexible, modular version of our FFPE protein sample preparation workflow that does not require AFA for protein aggregation capture or enzymatic digestion. Utilizing a standard 1.5 mL microcentrifuge tube and thermoshaker, a trypsin digestion time course of proteins extracted from an FFPE kidney section produces sufficient peptides after 4 hours. This versatile protocol allows maximization of yields if desired by extending digesting time to overnight.

Flexible Workflow

- Non-toxic deparaffinization
- AFA-enhanced tissue homogenization (33,600 Joules | 2X)
- Decrosslinking (90 °C | 90 min)
- Centrifugation (5,000 x g | 15 min)
- Transfer lysate into 1.5 mL tube
- Reduction / Alkylation
- Magnetic bead purification
- Trypsin digestion

Discussion and Conclusion

- Covaris offers a non-toxic alternative to deparaffinization of FFPE samples.
- The AFA-enhanced deparaffinization results in significantly higher protein yields as compared to traditional methods (xylene / horn sonicator / pestle), allowing for dramatic reduction of input (Figure 4).
- AFA conditions (energy, scanning or single row treatment) are highly tunable and are employed throughout the workflow to accomplish up to three different functions.
- Tissue homogenization and DNA shearing result from the same AFA treatment, extracting proteins and eliminating viscosity due to high molecular weight nucleic acids.
- The Covaris method is compatible with a wide range of tissue types and sizes, and results in higher yields than other commercial products.
- Covaris FFPE workflows are highly versatile and can be adapted to accommodate a variety of customer needs.