Proteomics from FFPE Samples – Adaptive Focused Acoustics® (AFA®) Enabled Robust Sample Preparation Leads to Confident Data

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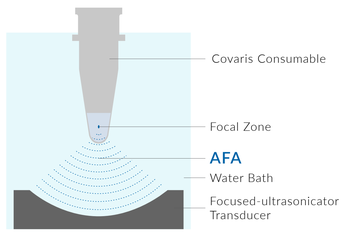
Introduction and Background

Formalin-fixed paraffin-embedded (FFPE) tissue samples are a readily available resource for studying the molecular mechanisms of disease. Although FFPE tissue samples have been used for genomics studies for years, they only recently gained recognition in proteomics. This is due to the inherent difficulty in extracting proteins from these highly crosslinked, dehydrated and paraffin-embedded samples. Even with the gain of sensitivity in LC-MS analysis, quantitation and identification of proteins from such samples needs to be improved, mostly starting with sample preparation.

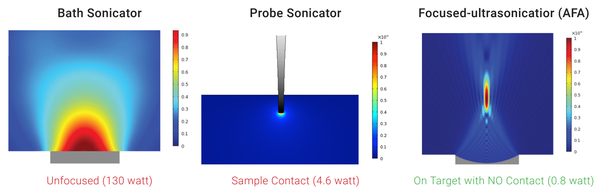
Conventional protein sample preparation workflows starting with FFPE samples often result in highly degraded, incompletely decrosslinked proteins associated with low yields. Adaptive Focused Acoustics (AFA) Technology can be deployed to dramatically increase the efficiency of protein extraction and downstream purification. More importantly, it also improves the digestion of proteins, resulting in significantly reduced turnaround times and higher peptide complexity.

In this study, we report development of a comprehensive and robust workflow that, in a modular format, allows deparaffinization, protein extraction & decrosslinking, protein purification, and trypsin digestion, with minimal sample transfer steps.

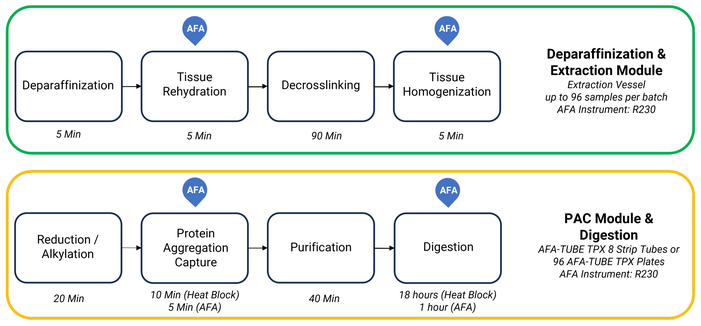


Covaris Technology:
- Less Energy
- More Control
- Maintain Isothermal Process

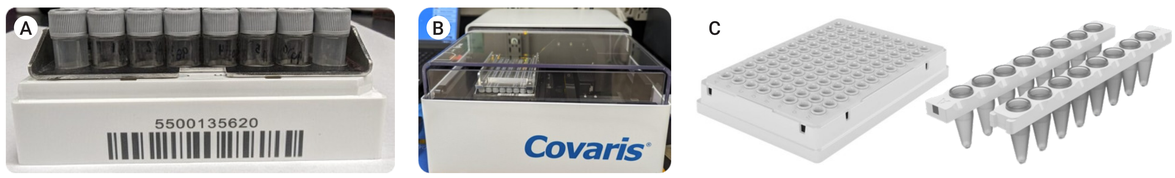
Characteristics of Adaptive Focused Acoustics (AFA) Technology. High-frequency acoustic energy is precisely focused onto the sample, minimizing energy waste that would otherwise cause sample heating, and thereby maximizing its impact on the sample. Thanks to sophisticated technology, energy can be controlled, enabling the user to tune AFA conditions for their specific needs, e.g., cell disruption (high energy) or gentle mixing (low energy).



AFA-enhanced Protein Extraction and PAC Workflow



The workflow is broken down into two modules. Deparaffinization, tissue rehydration, & decrosslinking is performed in AFA-compatible extraction vessels in the R230 Focused-ultrasonicator (batches of 8–96 are possible). The resulting lysate is transferred to an AFA-TUBE TPX (8 Strip or 96-well Plate) and reduced/alkylated prior to Protein Aggregation Capture (PAC) enhanced with AFA. Following wash steps, on-bead trypsin digestion is performed in ammonium bicarbonate buffer using either standard overnight or AFA-accelerated treatment (shown to the left).



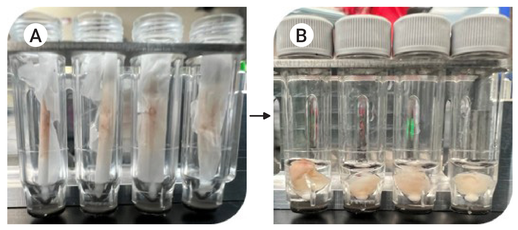
R230 Focused-ultrasonicator and extraction tubes. FFPE samples are loaded into individual extraction tubes (**A**). After deparaffinization, the tubes are placed into an R230 Focused-ultrasonicator for acoustic treatment (**B**). The resulting homogenate is transferred into a 96 AFA-TUBE TPX Plate or an 8 AFA-TUBE TPX Strip for further processing (**C**).

Methods

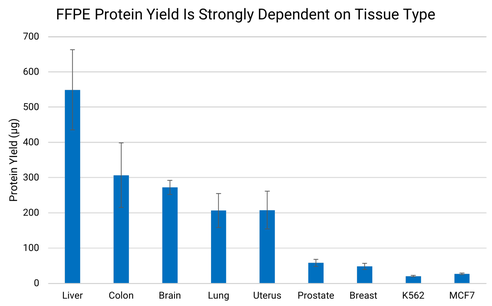
FFPE samples from different normal and cancerous tissues and two cell pellet FFPE control blocks (K562, AMSBIO 3070-0110; MCF7, AMSBIO 3010-0210) were used for this evaluation.

One 10 µm FFPE scroll was added to the extraction vessel. Rehydration buffer (50 µL) and Deparaffinization Solution (450 µL) were added, and the samples were incubated at 56 °C for 5 minutes. Separation of the aqueous phase containing the tissue and the organic phases was achieved by centrifugation, and the organic phase was discarded. Tissue Lysis Buffer (100 µL) was added, and the tissues were rehydrated and partially homogenized by applying AFA in a Covaris R230 Focused-ultrasonicator. Proteins were decrosslinked by incubation at 90 °C for 90 minutes, following another AFA-enhanced homogenization step.

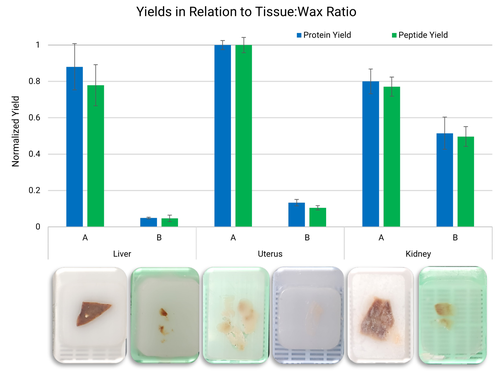
The resulting homogenate was transferred to an AFA-TUBE TPX (8 Strip or 96-well Plate) and reduced and alkylated. Proteins were captured with AFA-based mixing on carboxylated magnetic beads adapting the PAC protocol (Hughes, C.S., Moggridge, S., Müller, T. et al. Single-pot, solid-phase-enhanced sample preparation for proteomics experiments. Nat Protoc 14, 68–85 (2019)). After wash steps, the protein-loaded magnetic beads were suspended in Trypsin buffer (50 mM ammonium bicarbonate, pH 7.0-8.5). Hydrolysis was performed either with 1 hour of AFA treatment on an R230 or in a heat block for 2–18 hours. The resulting released peptides can be directly used for LC-MS analysis after desalting (for example, on a C18 matrix).



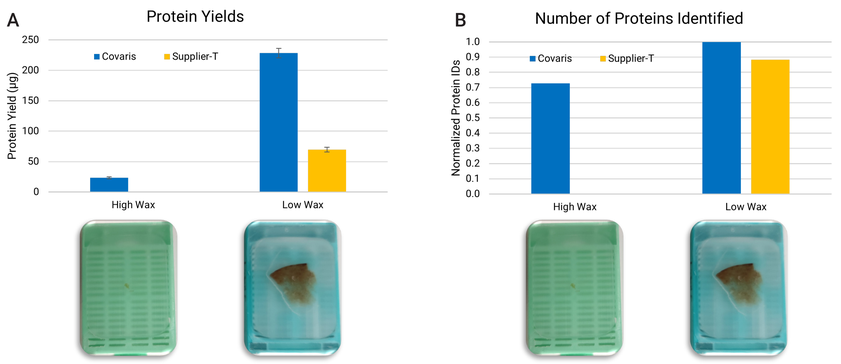
Shown are extraction tubes that contain one 10 µm FFPE scroll each (**A**). After adding Rehydration Buffer and Deparaffinization Solution, the vessel is incubated at 56 °C for 5 minutes. Following centrifugation, the aqueous phase containing the tissue and the organic phase containing the majority of the paraffin are separated, and the organic phase is discarded (**B**). Tissue Lysis Buffer containing SDS is added, then the tissue is rehydrated and scrubbed clean from any residual paraffin by treatment with AFA in the R230 instrument. The resulting homogenate is then decrosslinked and subjected to another AFA treatment to homogenize the tissue. It is then transferred to the PAC workflow module vessel for protein purification and trypsinization.



Protein yields were measured from different tissue types (10 µm scrolls each) that were subjected to the described workflow. Protein was measured colorimetrically (BCA Protein Assay, ThermoFisher 23227) in the lysate supernatant derived after AFA-based homogenization. Extractions were performed with 10 µm sections. Note the wide range of protein yields that can be accommodated in the downstream PAC workflow with only one sample transfer. K562 and MCF7 are FFPE blocks that contain cell culture pellets and act as a control.



Protein yields measured by the BCA assay and fluorometrically-measured peptide yields are presented for three tissue types in FFPE blocks with high and low wax to tissue ratios, respectively. Samples with large and small tissue areas were selected to demonstrate the efficiency of the presented modular workflow. Extractions were performed with 10 µm sections in quadruplicate and overnight (non-AFA-enhanced) trypsin digestion.



The herein described modular workflow ("Covaris") was compared to a commercially available kit ("Supplier T"). Two liver tissue blocks, one with high wax and one with low wax-to-tissue ratio were used (10 µm scroll each). The differences in yield are significant (**A**) and demonstrate the superior extraction efficiency that can be achieved by applying AFA to rehydration and homogenization.

Proteins were then subjected to the modular PAC protein purification and trypsin digestion workflow. Resulting released peptides were desalted on a C18 HPLC column prior to separation on a Thermo Fisher Scientific PepMap™ RSLC C18 EASY-Spray™ Column. An Orbitrap Fusion™ Lumos™ mass spectrometer (Thermo Fisher, San Jose, CA, USA) coupled online to an EASY-nLC 1200 (Thermo Fisher, San Jose, CA, USA) was used to perform the RP-nLC/ESI-MS analysis. The proteins extracted following the Covaris workflow resulted in 12% more protein identifications than the alternative product (**B**).

Discussion and Conclusions

- Covaris presents a highly efficient deparaffinization method using a non-toxic chemistry combined with AFA-enhanced scrubbing of the tissue. This results in:
  - Efficient decrosslinking
  - Significantly higher protein yields as compared to traditional methods
- Due to its energy tunability, AFA was optimized to improve steps throughout the modular workflow, enhancing:
  - Deparaffinization, tissue rehydration and homogenization
  - Non-biased protein binding to magnetic beads
  - Protein digestion
- Covaris FFPE protein extraction is compatible with a wide range of tissue types, regardless of tissue-to-wax ratio, and produces significantly higher yields than other commercial kits/products. Protein and peptide yields are highly reproducible.
- Covaris FFPE workflows are highly versatile and can be adapted to accommodate a variety of customer needs.

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