

Automated Protein Extraction from Clinical Samples with the Covaris ML230 Focused-ultrasonicator

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Abstract

Many research projects in translational or clinical laboratories require automated, hands-off solutions for protein sample preparation which enable better reproducibility, increased efficiency, higher quality results, and faster turnaround time. This application note presents protocols for simultaneous multi sample processing from diverse inputs in a convenient 8-strip format. Solutions are described for protein extraction from formalin-fixed and paraffin-embedded (FFPE) tissue, fresh frozen tissue, and cultured cells for mass spectrometry-based (MS) proteomics using short gradient runs. These protocols have been developed on the recently launched Covaris ML230 Focused-ultrasonicator and optimized for a Single Pot Solid Phase Sample Preparation (SP3) workflow. The same extraction workflows can be adapted applying other clean-up methodologies as well as different downstream analyses such as western blotting or ELISA. The workflow presented here using the Covaris ML230 Ultrasonicator provides an ideal solution for mid-throughput protein extraction with batch-sizes around 40 protein extractions per day.

Introduction

Sample preparation significantly impacts the data quality derived from the sample of interest including model systems as well as primary clinical samples. This is especially relevant and important in the field of proteomics research, as proteins are an extremely diverse community of macromolecules, present in an extraordinarily wide range of concentrations, and they cannot be amplified. Many methods and protocols to isolate proteins are available, of which Covaris Adaptive Focused Acoustics® (AFA®) has unique advantages towards:

- Covering the diversity of samples (FFPE, LCM, fresh tissue, cells, bacteria, yeast...)
- Reducing human error
- Decreasing hands-on time
- Increasing speed and throughput
- Significantly improving reproducibility and reliability

The efficiency of AFA for pre-analytical proteomics has been demonstrated on numerous instruments, in various conditions (volume, buffer, sample type) and throughputs (single tubes, 8-strips, 96 and 384-well plates). Covaris Focused-ultrasonicators are proven to be an extremely robust and flexible solution for mid- to high-throughput protein processing, regardless of buffers and clean-up methods: it is fully efficient with approaches like SP3 [1,2] or S-Trap [3]. Given the relevance of AFA in proteomics sample preparation workflows, we adapted

those protocols to our most recently released instrument, the Covaris ML230 Focused-ultrasonicator: this compact device is able to process single tubes as well as strips in a simultaneous manner, offering the perfect solution for processing up to 40 samples per day.

Materials

Required Equipment

Covaris

- Covaris ML230 Focused-ultrasonicator ([PN 500656](#))
- AFA-TUBE TPX 8-strips ([PN 520292](#)) and caps ([PN 500639](#))
- PS ML230 Rack 8 AFA-TUBE TPX Strip ([PN 500699](#))
- truXTRAC tissue lysis buffer (TLB) ([PN 520284](#))

Other Suppliers

- Thermocycler
- Magnetic Beads
- Magnetic Rack

Samples

For autopsy informed, consent was given by the next of kin, and autopsies were performed on the legal basis of §1 SRegG BE of the autopsy act of Berlin. Animal material was obtained from an approved study by the local animal ethics committee (LAGeSo Berlin; T 0096/02) and carried out in accordance with EU Directive 10/63/EU as well as in line with the ARRIVE guidelines.

Methods

Protocols were adapted from previous collaborations on higher throughput instruments (LE220-plus and LE220R-plus) for fresh tissues and cells [2], and non-toxic FFPE [4] protein extraction. Those methods generally use SDS based buffers and SP3 as the clean-up and digestion method. Other buffers [5] and other clean-up methods [3,6] can be used. You can contact applicationsupport@covaris.com or download our Proteomics Quick Guide for up-to-date and optimized protocols.

Protein Extraction from FFPE Samples

Scrolls from human liver samples (1 to 2 mg FFPE tissue) were prepared and trimmed to remove excess paraffin. Sixteen samples were processed in single wells of two 8 AFA-TUBE TPX Strips (**Figure 1**) filled with 75 μL (Strip 1) or 150 μL (Strip 2) of Covaris Tissue Lysis Buffer (TLB), an SDS based buffer. A combination of heating (95 °C) and AFA (settings in **Table 1**) was used to reverse crosslinks and remove paraffin from the tissue samples. This unique and patented ultrasound-focused process allows paraffin to emulsify without any organic solvent, resulting in a milky sample solution which can be processed further without intermediate filtration steps.

Volume (μL)	PIP (W)	% DF	CPB	Repeats	Pulse or Time Continuous (s)	Delay Duration(s)	Temp (°C)	Dither
75	375	25	50	20	10	10	18	3 mm Y dither @ 20 mm/s
150	350	25	200	N/A	300	N/A	18	3 mm Y dither @ 20 mm/s

Table 1. AFA treatment settings for FFPE tissue deparaffinization and homogenization.

Different approaches exist for protein clean up. Amongst them, the SP3 protein aggregation [1] method allows for direct removal of all contaminants and protein digestion and peptide release in the same vessel.

To remove paraffin and SDS and for subsequent protein purification, proteins are captured by magnetic beads followed by thorough alcohol washes. Peptides are released in the supernatant through proteolytic on-bead digestion, thus avoiding additional transfer steps and keeping the process 'single-pot' as described in other publications [2,4-6]. The standard protocol is depicted in **Figure 1**.

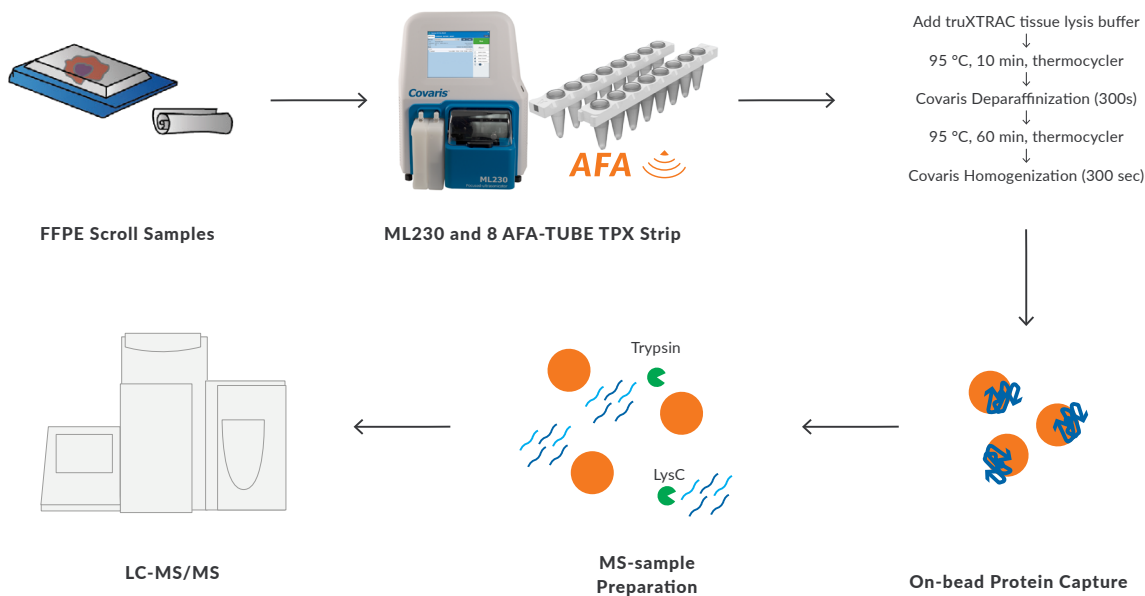
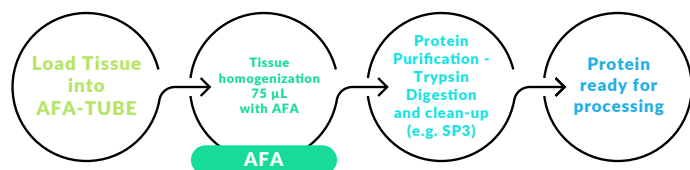


Figure 1. Workflow of protein extraction from FFPE in a 8-strip format. FFPE scrolls are dispensed in the 8-strip. TLB buffer is added, and samples are heated to 95 °C in a thermocycler. They undergo a first AFA treatment to emulsify the paraffin. A one-hour treatment at 95 °C allows to reverse crosslink the tissue. A second AFA treatment is made to homogenize the tissue and release the proteins. Beads are added for protein capture and after several washes the proteins are digested with trypsin. Adapted from Schweizer et al., Covaris application note [M020141](#).

Alkylation and reduction (TCEP/CAA) are combined in one step at 95 °C. On beads capture is performed under a 10:1 bead to protein ratio (1:1 hydrophilic/hydrophobic), with 50% ACN. Different washes with ethanol are made, before a final acetonitrile wash. Proteins are then reconstituted in ABC with 1:100 trypsin and digested over night at 37 °C.

Protein Extraction from Fresh Tissue

Twenty-four samples from the same rat liver (2 to 8 mg) were collected in AFA-TUBE TPX 8-strips filled with 75 µL of lysis buffer (1% SDS in 0.1 M ammonium bicarbonate (ABC) + 1.25x protease inhibitor cocktail). Samples are processed directly for protein extraction with one or two rounds of AFA treatment, depending on the input. The same purification approach (SP3) described for FFPE samples (without deparaffinisation and de-crosslinking) is used for cleaning and digesting the proteins.



Volume (µL)	PIP (W)	DF (%)	CPB	Repeats	Pulse or Time Continuous (s)	Delay Duration(s)	Temp (°C)	Dither
75	375	25	50	20	Pulsing 30*10sec	10	12	3 mm Y dither @ 20 mm/s

Table 2. AFA treatment settings for fresh tissue homogenization.

Protein Extraction from Cultured Cells

Sixteen replicates of 400,000 HeLa cells were dispensed in 2 AFA-TUBE TPX 8-strips filled with 75 µL of lysis buffer (1% SDS in 0.1 M ABC + 1.25x protease inhibitor cocktail). Samples are processed directly for protein extraction with one round of AFA treatment. The same purification approach (SP3) described for fresh tissue samples is used for cleaning and digesting the proteins.

Volume (µL)	PIP (W)	DF (%)	CPB	Repeats	Pulse or Time Continuous (s)	Delay Duration(s)	Temp (°C)	Dither
75	375	25	50	50	Pulsing 30*10sec	10	18	3 mm Y dither @ 20 mm/s

Table 3. AFA treatment settings for cultured cells homogenization.

Results

Protein extraction from FFPE samples

Peptides were quantified after digestion using Thermo Fluorometric Kit resulting on average in 25 to 50 µg total quantity. Five micrograms of peptides were run on a 5 min gradient using scanning Swath (Agilent Infinity II - Sciex 6600 Q-TOF). For data analysis, DIA-NN was used with an in-house project specific spectral library, generated with gas-phase fractionation on Thermo Q-Exactive Plus [7-8].

Results were analyzed at 1%FDR. Around 15,000 peptides (Figure 2) and 2,500 proteins groups were found for samples processed in 75 µL (Figure 3 right panel), and about 4% more for both for samples extracted in 150 µL (Figure 2 & 3, left panel), suggesting that 75 µL was not enough to fully cover the scroll and emulsify all the paraffin, resulting in some protein loss. Median CV was found at 14.2% for 150 µL and 13.7% for 75 µL, for the overall workflows from protein extraction to raw data analysis, in consistency with previous studies using AFA. Approximately 7% of variance can be attributed to sample digestion and LC_MS analysis. Variance distribution across protein groups is very conserved between the two sets of tested volumes.

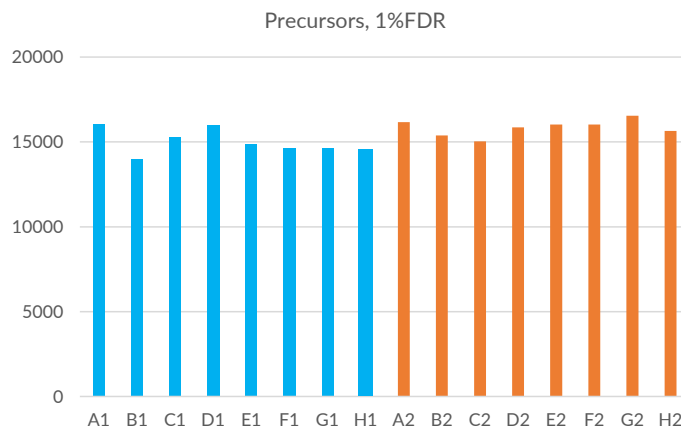


Figure 2. FFPE samples: precursor count at 1%FDR. Letters indicates the different wells in strips 1 (75 µL) and 2 (150 µL).

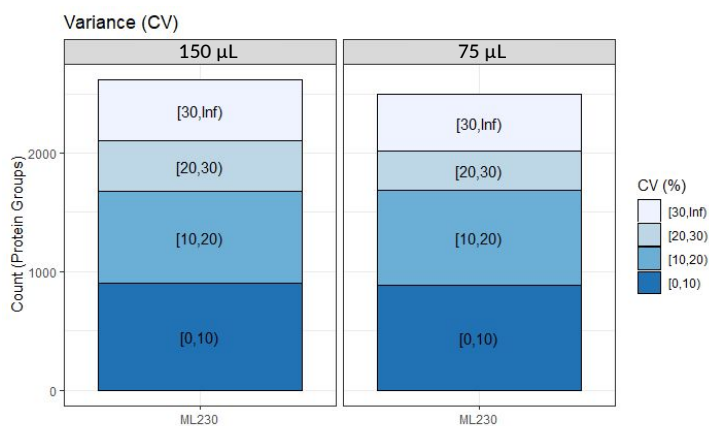


Figure 3. Variance distribution across protein groups (FFPE). More than 2,000 proteins are quantified below 30% and almost 1,000 below 10% variance.

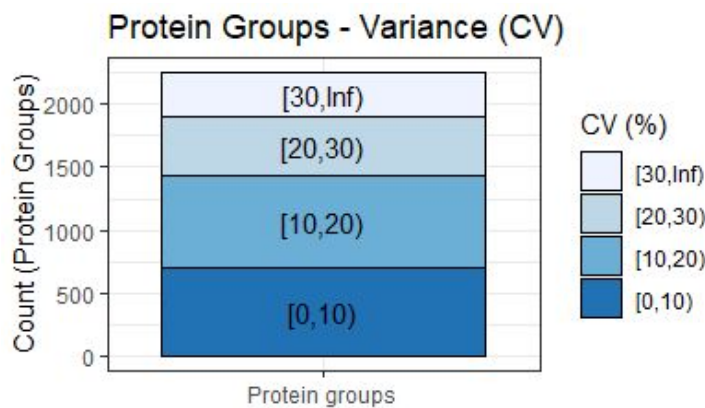


Figure 5. Variance distribution across protein groups (Fresh frozen).

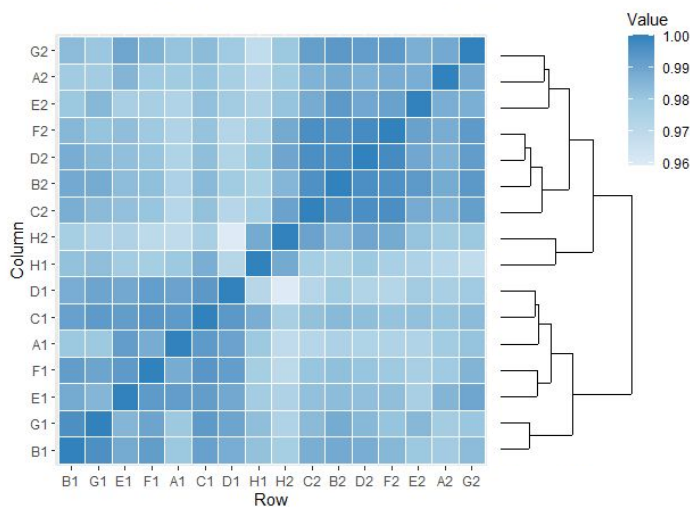


Figure 4. Pearson correlation coefficients across and between strips (FFPE). Letters indicates the different wells in strips 1 (75 µL) and 2 (150 µL).

Pearson correlations across all proteome measurements were high with values above 0.96, showing high workflow reproducibility (**Figure 4**). This correlation happens to be very high even between the two different volumes. Protein extraction from fresh tissue Peptides were quantified after digestion using Thermo Fluorometric Kit resulting on average in 30 to 100 µg total quantity. Five micrograms of peptides were run on 5 min gradient using scanning Swath (Agilent Infinity II - Sciex 6600 Q-TOF). For data analysis, DIA-NN was used with an in-house project specific spectral library, generated with gas-phase fractionation on Thermo Q-Exactive Plus.

Results were analyzed at 1%FDR. Around 17,500 peptides (not shown) and 2,250 proteins groups were identified (**Figure 5**), fully in line with other similar studies [9].

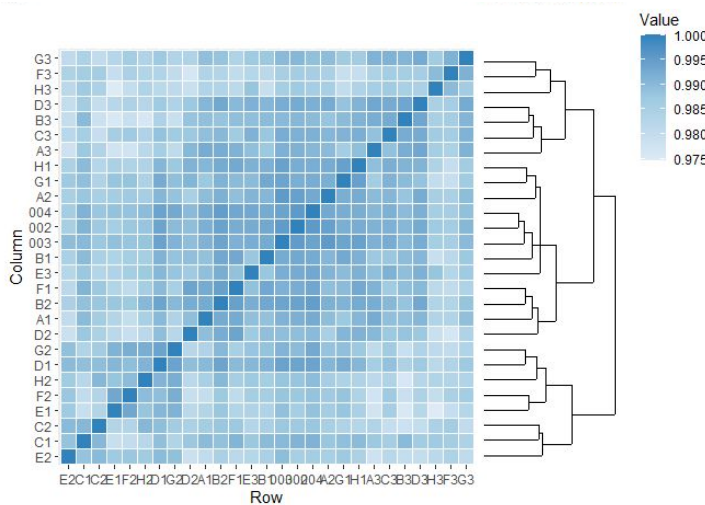


Figure 6. Correlation analysis (Pearson) shows high reproducibility of the workflow. Hierarchical clustering separates strip 3 but fails to distinguish Covaris strip 1 and 2. Technical replicates for sample digestion and LC-MS cluster together (002,003,004).

Reproducibility was very good with median Calculated median CV found at 15%. Like in the FFPE experiments, the consistency of identification between replicates was very high with all Pearson correlation values above 0.97 (**Figure 6**).

Protein Extraction from Cultured Cells

Peptides were quantified after digestion using Thermo Fluorometric Kit resulting in up to 30 µg total quantity. Five micrograms of peptides were run on 5 min gradient using scanning Swath (Agilent Infinity II - Sciex 6600 Q-TOF). For data analysis, DIA-NN was used with an in-house HeLa spectral library, generated with gas-phase fractionation on Sciex 6600 Q-TOF.

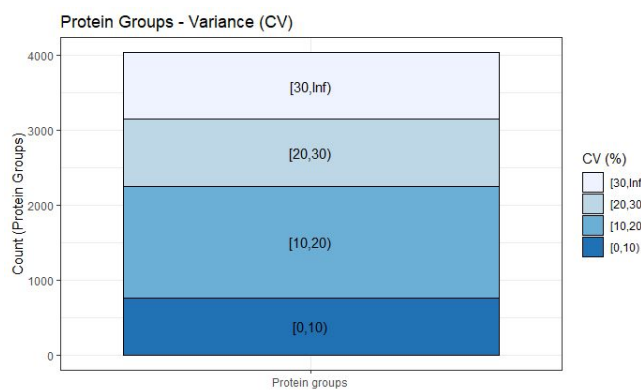


Figure 7. Variance distribution across protein groups (HeLa cells).

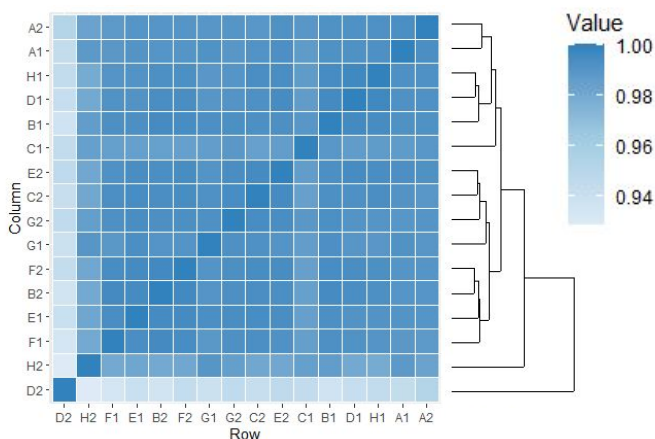


Figure 8. Pearson correlation coefficients (HeLa cells).

Results were analyzed at 1%FDR. Around 30,000 peptides (not shown) and 4,000 proteins groups were identified (Figure 7), which was very satisfactory with regards to the gradient length, and compatible with other studies [2]. Of interest, the calculated median CV was a slightly higher (18.1%) when compared to the other samples run on the ML230 (Figure 6). An explanation came from the Pearson correlation analysis, with one sample (D2, Figure 8) being clearly outside of the range of correlation found for all the other replicates. Without this sample, CV improves significantly to reach 13.9%.

Conclusions

The employed protein extraction workflow is easy to adapt to the ML230 Covaris Focused-ultrasonicator and displays highly consistent and reproducible results for the various sample inputs tested (cultured cells, fresh tissue and FFPE samples). Peptide and protein identifications are very good for short gradients and

average compared to conventional 2h gradient analysis. Longer gradients have been used with successful results on mouse liver [10]. The extraction process is fully compatible with a single pot approach, e.g. using SP3 to clean up and digest the proteins [2,10]. Results are highly comparable to those found on similar samples processed on different Covaris instruments [2, 4, 11-12].

Here we show a fast, efficient, and reproducible mid-throughput protein extraction workflow that is ideal for sample series of 10 to 40 samples. Consumables and workflows are highly reproducible. Therefore, no or minimal batch effects are expected. This allows preparation of hundreds of samples within a week and enables mid-sized studies in pre-clinical and clinical research with, e.g.:

- Samples from the clinic, such as fresh frozen tissue material
- Samples from pathology or biobanks such as PFA, FFPE or DBS samples
- Targeted assays for marker protein identification

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