From Tissues to LC-MS-ready Protein Digests: Automated Sample Preparation Enabled by Adaptive Focused Acoustics® (AFA®) Technology

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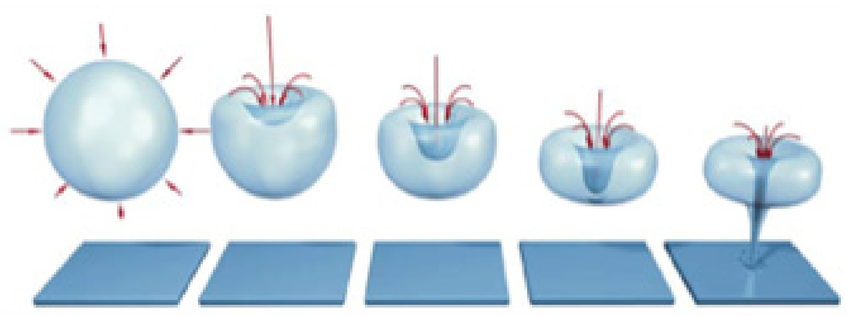
Background and Introduction

LC-MS based analysis of peptide hydrolysates obtained from digested proteins is important for pre-clinical evaluation of compound libraries, as well as for discovery and analysis of biochemical pathways in translational medicine. While the last couple of decades witnessed tremendous growth in analytical technology such as high-resolution accurate mass spectrometry, liquid chromatography, and informatics (enabling workflows from data analysis to data management), lagging progress in sample extraction from biological matrices and purification of analytes often results in inefficient and poor-quality data, regardless of the end-detection analytical tools. Here, we introduce a comprehensive and fully automatable sample preparation workflow that incorporates Adaptive Focused Acoustics (AFA)-enabled efficient cell lysis, protein extraction, protein/bead binding, and on-bead trypsin digestion. This workflow requires only three liquid transfer steps, resulting in a robust process and guaranteeing high quality data.

Adaptive Focused Acoustics (AFA) Technology



- Ultra-high frequency electronics and transducers produce and focus acoustic waves.
- Fluctuations cause dissolved gasses to form microscopic bubbles that grow, oscillate, and collapse.
- Acoustic energy can be adapted and shear forces can be low to gently mix samples or keep magnetic beads in suspension, but also high enough to disrupt biomolecule complexes or even fragment macro molecules.

Method

Suspension and adherent cells were grown in standard 96-well polystyrene culture plates (Corning) following ATCC culturing recommendations.

The following workflow is fully automatable: Suspension and adhesive cultures were washed with 1X PBS. To detach adherent cells, 40 µL of PBS per well was added and plates were subjected to AFA in the R230 Focused-ultrasonicator. AFA energy settings have been optimized for detachment (75 joules/10 scans) and subsequent homogenization (60 joules/10 scans) of the cell suspension directly in polystyrene culture plates (***Figure 1***).

Homogenized cell suspensions were transferred to a Covaris 96 AFA-TUBE TPX 150 Plate, following addition of SDS (final concentration 1%). AFA treatment (13200 joules) in a row-by-row treatment mode results in one step cell lysis, DNA shearing (reduction of viscosity), and protein extraction. Extracted proteins are reduced and alkylated in the same plate, and then bound to magnetic beads following Protein Aggregation Capture (PAC) conditions. During bead binding AFA (255 joules/10 scans) was applied. The bead-bound proteins were washed and resuspended in ammonium bicarbonate (ABC) buffer/trypsin solution. For hydrolysis, samples were incubated with intermittent AFA-mixing for 1 hour (90 joules/126 scans) at 34 °C. Released peptides were collected by harvesting the supernatant, which was finally transferred to a new 96-well plate, dried using a speed vac, and reconstituted in buffer for LC-MS analysis (***Figure 2***).

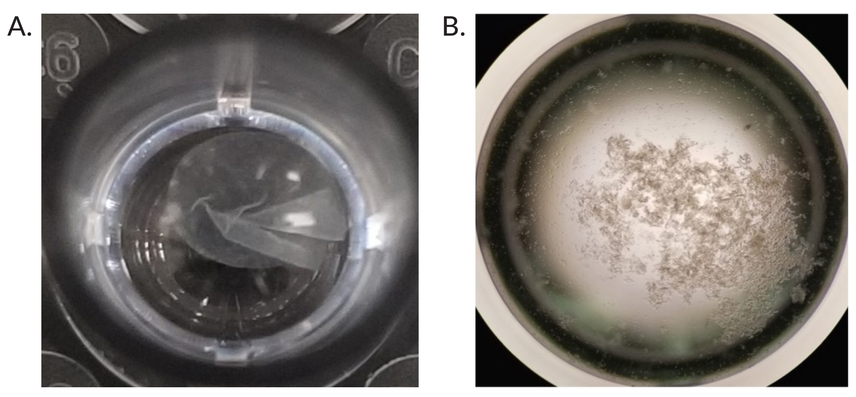


Figure 1. Displacement of Adherent Cells with AFA. Polystyrene cell culture plates with HCT 116 cells (80% confluent) are treated with low energy AFA settings to effect detachment of adherent cells in a single confluent cell-layer (***A***). A second AFA treatment optimized for increased turbulence breaks apart the cell sheet into a cell suspension to facilitate transfer by pipetting into the 96 AFA-TUBE TPX 150 Plate (***B***).

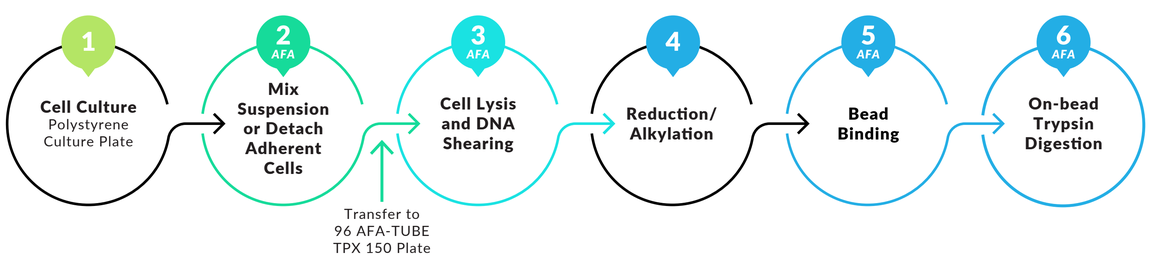


Figure 2. AFA-enabled Workflow. AFA conditions for cell detachment, cell lysis, DNA shearing, and protein extraction were developed with HCT 116 cells (***Figure 1***). AFA conditions for bead-binding and on-bead trypsin digest were developed using K562 protein extract (***Figure 3***).

Results

The AFA-enhanced PAC workflow was optimized using commercial K562 protein extract. After proteins were reduced and alkylated, AFA-inferred mixing was used to enhance bead-binding. Non-selective (total protein) binding with AFA was similar to that observed with thermomixer, conferring a small advantage to binding efficiency (~2 - 8 % improvement; data not shown).

On-bead digestion experiments were performed with 1:10 or 1:50 trypsin ratios. On-bead trypsin digestion was done by using either a thermomixer for 1 and 16 hours or AFA for 1 hour. AFA-enhanced mixing resulted in increased protein and peptide IDs as compared to both 1 and 16 hour heat block controls (***Figure 3***). The analysis of the digests was performed on a Fusion Lumos Tribrid Mass Spectrometer coupled to an Easy nLC 1200 HPLC system. The data was analyzed using Proteome Discoverer.

Adherent cell detachment workflows were compared using either trypsinization or AFA to recover HCT 116 cells from polystyrene culture plates. The AFA method is faster, fully automatable, and significantly more user friendly than the trypsin method (***Table 1***).

The full workflow, starting from adherent cell culture was done with HCT 116 cells. Following detachment by either AFA or trypsinization, cells were transferred into a Covaris 96 AFA-TUBE TPX 150 Plate, subjected to AFA-based lysis and DNA shearing, and prepared for AFA-enhanced trypsin digestion for 1 hour (***Figure 4***). Cells displaced with AFA resulted in a similar number of protein and peptide IDs as compared to standard trypsinization.

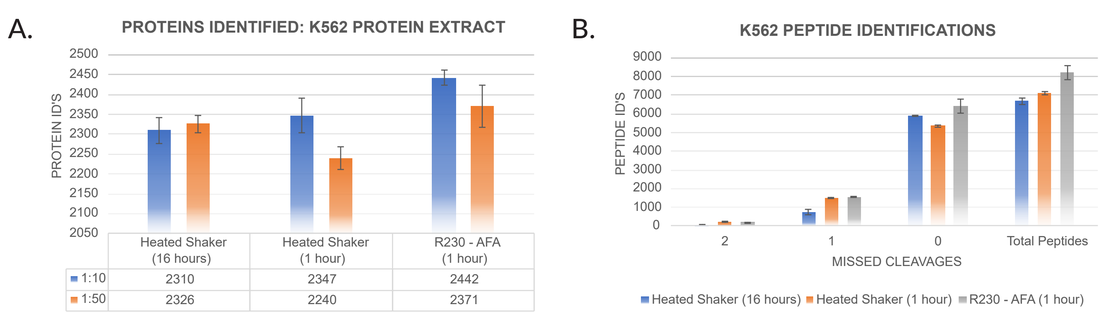
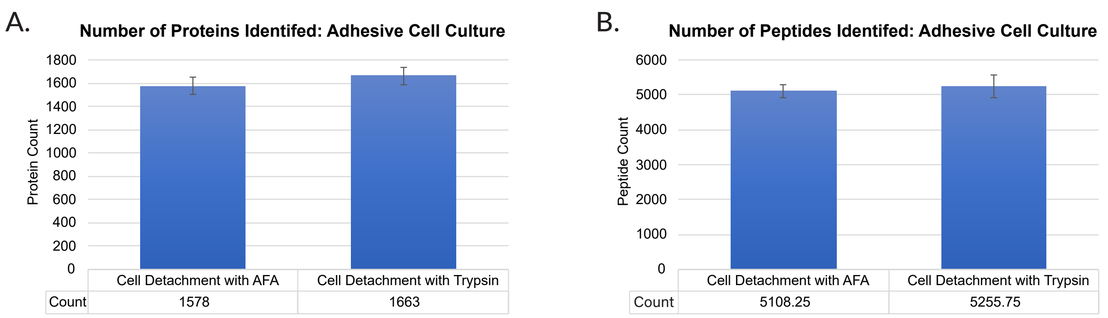


Figure 3. LC-MS Analysis of Tryptic Digestion of K562 Cell Protein Extract. K562 protein extract was bound to magnetic beads using AFA-enhanced mixing and then digested with trypsin (using trypsin to protein ratios of 1:10 or 1:50) by either incubating in a heated shaker (1 hour or ON at 37 °C) or by applying AFA in an R230. High confidence protein identifications are shown (***A***) as well as a comparison of missed cleavages and peptide identifications between digestion methods (***B***).

Table 1. Adherent Cell Detachment Workflow Comparison. A comparison of AFA and trypsinization techniques for the displacement of adherent cells from 96-well polystyrene cell culture plates shows the advantage of AFA.

	R230 w/ AFA	Trypsinization
Wash Steps	2	3
Pipetting Steps	4	8
Centrifugation	N/A	2x
Time (min)	10	20 - 30
Automatable	Yes	No



C.

Category Name (accession #)	Percent of protein category hits against total number	
	R230 - AFA (%)	Trypsinization (%)
extracellular matrix protein (PC00102)	0.3	0.6
cytoskeletal protein (PC00085)	7.4	7.0
transporter (PC00227)	5.3	5.3
scaffold/adaptor protein (PC00226)	3.4	3.5
DNA metabolism protein (PC00009)	1.6	1.8
cell adhesion molecule (PC00069)	0.7	0.8
intercellular signal molecule (PC00207)	0.5	0.5
protein-binding activity modulator (PC00095)	4.4	4.7
viral or transposable element protein (PC00237)	0.3	0.2
RNA metabolism protein (PC00031)	10.3	9.5
calcium-binding protein (PC00060)	1.2	1.3
gene-specific transcriptional regulator (PC00264)	2.4	2.4
defense/immunity protein (PC00090)	0.9	0.8
translational protein (PC00263)	7.5	7.2
metabolite interconversion enzyme (PC00262)	13.9	13.5
protein modifying enzyme (PC00260)	8.0	8.7
chromatin/chromatin-binding, or -regulatory protein (PC00077)	3.2	3.3
transfer/carrier protein (PC00219)	0.7	1.0
membrane traffic protein (PC00150)	3.2	2.9
chaperone (PC00072)	3.1	3.1
cell junction protein (PC00070)	0.0	0.1
structural protein (PC00211)	0.2	0.2
storage protein (PC00210)	0.1	0.0
transmembrane signal receptor (PC00197)	1.1	1.4
Total Gene Hits in %	79.7	79.8

Figure 4. LC-MS Analysis of Tryptic Digestion of Protein Extract from HCT 116 Cells. A comparison of AFA and trypsin detachment methods. After detachment of cells with either AFA (R230) or trypsinization, cells are transferred into a 96 AFA-TUBE TPX 150 and lysed by applying AFA. The HCT 116 protein extract is then bound to magnetic beads using AFA-enhanced mixing, followed by AFA-enhanced trypsin digestion. High confidence protein (***A***) and peptide (***B***) identifications are shown. PANTHER analysis of protein categories classified by gene hits and LC-MS analysis of tryptic digestion of protein extract from HCT 116 cells (***C***) show similar protein ID coverage.

Discussion

- Covaris AFA enables an automatable workflow starting from cell culture (adherent or suspension) to LC-MS ready protein hydrolysates.
- AFA conditions (energy, plate scanning or row-by-row treatment) are highly tunable and are employed throughout the workflow to accomplish four different functions.
- AFA simplifies adherent cell detachment by eliminating the need for cumbersome enzymatic incubation steps and improving user convenience.
- Following displacement of adherent cells, the entire workflow takes place in a Covaris 96 AFA-TUBE TPX 150 Plate, limiting transfer steps to a total of 3, and enabling the AFA treatments necessary for subsequent steps.
- Cell lysis and DNA shearing result from a single AFA treatment, extracting proteins and eliminating high viscosity due to high molecular weight nucleic acids.
- PAC workflow facilitates sample preparation by enabling compatibility with a wide range of buffer systems and simplifying cleanup.
- Immobilized protein (on-bead) trypsin digestion with AFA for 1 hour improves protein and peptide identifications over the standard overnight heat block incubation.

For any questions regarding this poster or its contents, please contact Ulrich Thomann (uthomann@covaris.com) or Patrick McCarthy (pmccarthy@covaris.com).