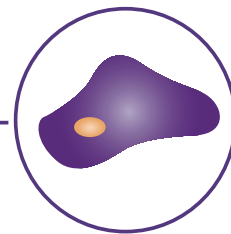
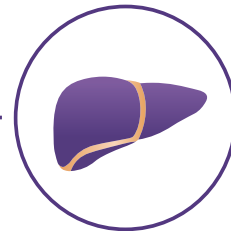


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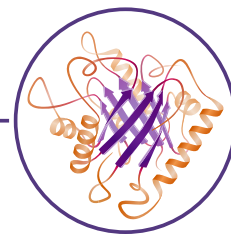
Sample Preparation for Proteomics Applications with PIXUL™



Cells



Tissue



Proteins

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Introduction

Sample preparation in Proteomics studies is challenging due to the complexity of the proteome. The first and often most critical step in the sample preparation protocol is cell lysis which is carried out using sonication. Due to its accuracy, sensitivity, and flexibility Mass Spectrometry has become the gold standard for analyzing the proteome. In recent years it has also found its way into the clinical setting.

In this scenario reproducibility and the ability for high-throughput processing is crucial. With Active Motif's [PIXUL™ Multi-Sample Sonicator](#) it is possible to shear 96 samples with 12 different settings in a high-throughput fashion, which can be applied to many different sample types. With this TechNote, we want to demonstrate this flexibility by comparing the lysis of HEK293T cells, and mouse liver tissue samples with the [PIXUL Multi-Sample Sonicator](#) to a standard probe sonicator.

After cell lysis, samples were subjected to LC-MS/MS analysis on an LTQ Orbitrap Elite instrument. Comparison of both sonication methods was performed by evaluating common QC parameters, including the protein extraction efficiency, the number of identified proteins, and the presence of unwanted protein modifications.

HEK293T Cells

Sample Preparation

Sample preparation for MS-Analysis was performed using the Protifi S-Trap™ sample processing technology. In brief: A cell pellet containing 5.4M HEK293T cells was resuspended in a buffer containing 5 % SDS in 50 mM TEAB pH 8.5. Cell lysis was enhanced via Sonication. The standard approach is lysis using a probe sonicator (VCX130 with a 3 mm probe, Sonics and Materials, Newtown, USA) with 3x15 s pulses, 15 W output, and 30 s on ice between individual bursts.

This approach was compared to the PIXUL Multi-Sample Sonicator with the following settings: 5 min of shearing, 50 pulses of 2 MHz with a frequency of 1 kHz, 20 Hz burst rate, and 2 wells per sample which were pooled later in the process.

After sonication, DTT and IAA were added to reduce and alkylate the proteins and proteins were digested with 1 µg Trypsin at 37 °C o/n. 1.5 µg of the resulting peptide mixture was then analyzed by nanoLC-MS/MS on an LTQ Orbitrap Elite instrument using standard 2 hour runs and CID fragmentation.

Protein Concentration

The first QC measure taken in the protocol was to determine whether the protein yield was comparable between the standard probe sonicator and the PIXUL. To evaluate protein concentration, samples were subjected to BCA analysis and analyzed in triplicate.

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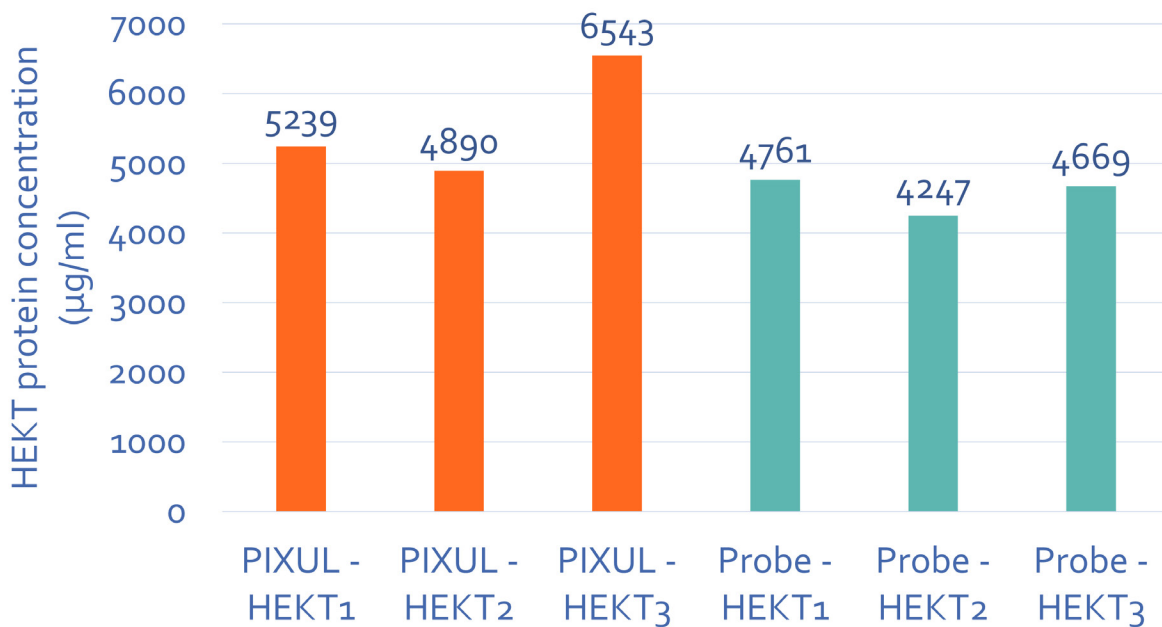


Figure 1: Protein Concentration Measurement with BCA

Three samples processed with the standard probe sonicator or the PIXUL were subjected to BCA analysis and the protein concentration is shown in µg/ml. Orange: Samples processed with PIXUL. Green: samples processed with the probe sonicator.

Figure 1 shows a comparison between three samples processed with the standard probe sonicator and three separate samples processed with PIXUL.

Protein concentration of samples processed with PIXUL are in the same range (4890 - 6543 µg/ml) as samples processed in the standard way (4247 - 4761 µg/ml), indicating a similar, if not slightly better protein yield when using PIXUL compared to the standard method.

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Sample name	# of identified peptides	# of identified protein groups
HEKTPIXUL_rep1	9497	2404
HEKTPIXUL_rep2	10020	2431
HEKTPIXUL_rep3	9080	2268
HEKTProbe_rep1	9619	2458
HEKTProbe_rep2	9577	2480
HEKTProbe_rep3	9559	2381

Table 1: Comparison of the Number of identified Peptides and Proteins.

Number of Identified Peptides

Despite small differences in the extracted protein concentration, for each sample the same peptide amount was injected for LC-MS/MS analysis. LC-MS/MS runs of all 6 samples were searched separately using the MaxQuant algorithm (version 1.6.17.0) with mainly default settings, including a false discovery rate set at 1% on both peptide and protein level.

Spectra were searched against the human Swiss-Prot Reference proteome database (www.uniprot.org) (database release version of 2021_01, containing 20,621 protein sequences).

The top three rows represent PIXUL data and the lower three rows the standard probe sonicator.

The number of peptides and proteins that were identified was comparable between both groups (PIXUL: 9080-10020, Standard: 9559-9619), indicating a similar ability to identify peptides and proteins in HEK293T cell lysates. The variability between replicates, however, is slightly higher with PIXUL.

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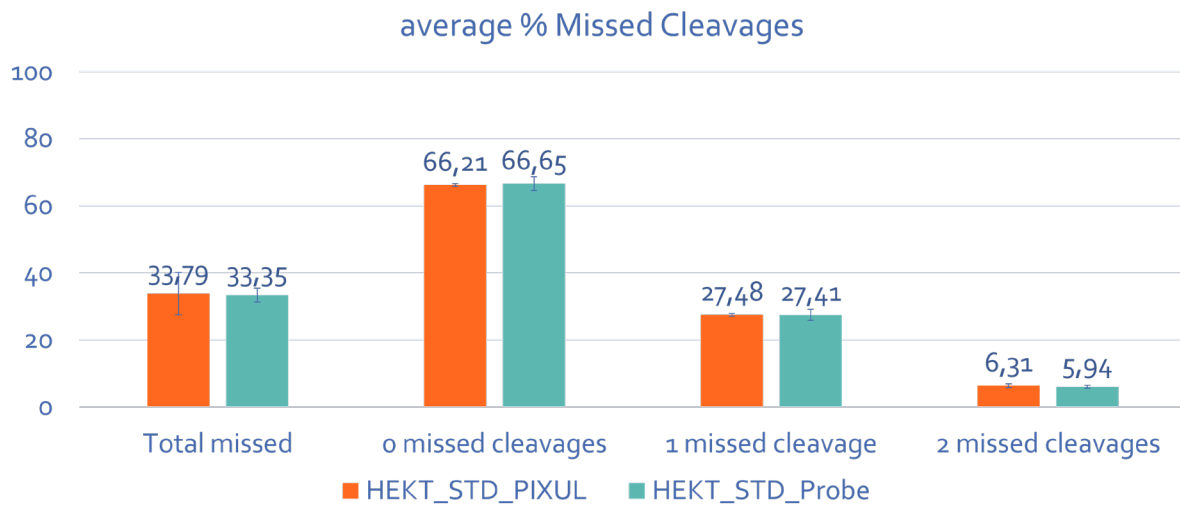


Figure 2: Average percentage of missed cleavages.

Missed Cleavages

Next, the number of missed trypsin cleavages was evaluated. Missed cleavages represent an instance where Trypsin did not cleave the peptide as expected after each Lysine or Arginine residue. During database searching, up to 2 missed cleavages were allowed as a standard setting, hence the number of peptides with 0, 1, or 2 missed cleavage events could be assessed.

The average percentage of missed cleavages is shown for PIXUL (orange) and standard Probe Sonicator (green). The total percentage of missed cleavages is comparable between PIXUL and the standard probe sonicator (33,79 vs 33,35). Furthermore, the fraction of 0, 1 or 2 missed cleavage events is comparable, suggesting a similar degree of missed cleavages in samples prepared with PIXUL compared to the standard probe sonicator.

Overall, the number of missed cleavages is acceptable given the fact that no (e.g., endoLys) pre-digestion was performed.

Modifications

Last, the average percentages of Acetylated Protein N-termini and Oxidized Methionine residues (methionine sulfoxide) were assessed as measures for the ability to detect protein modifications and as general quality parameters for sample preparation including sonication.

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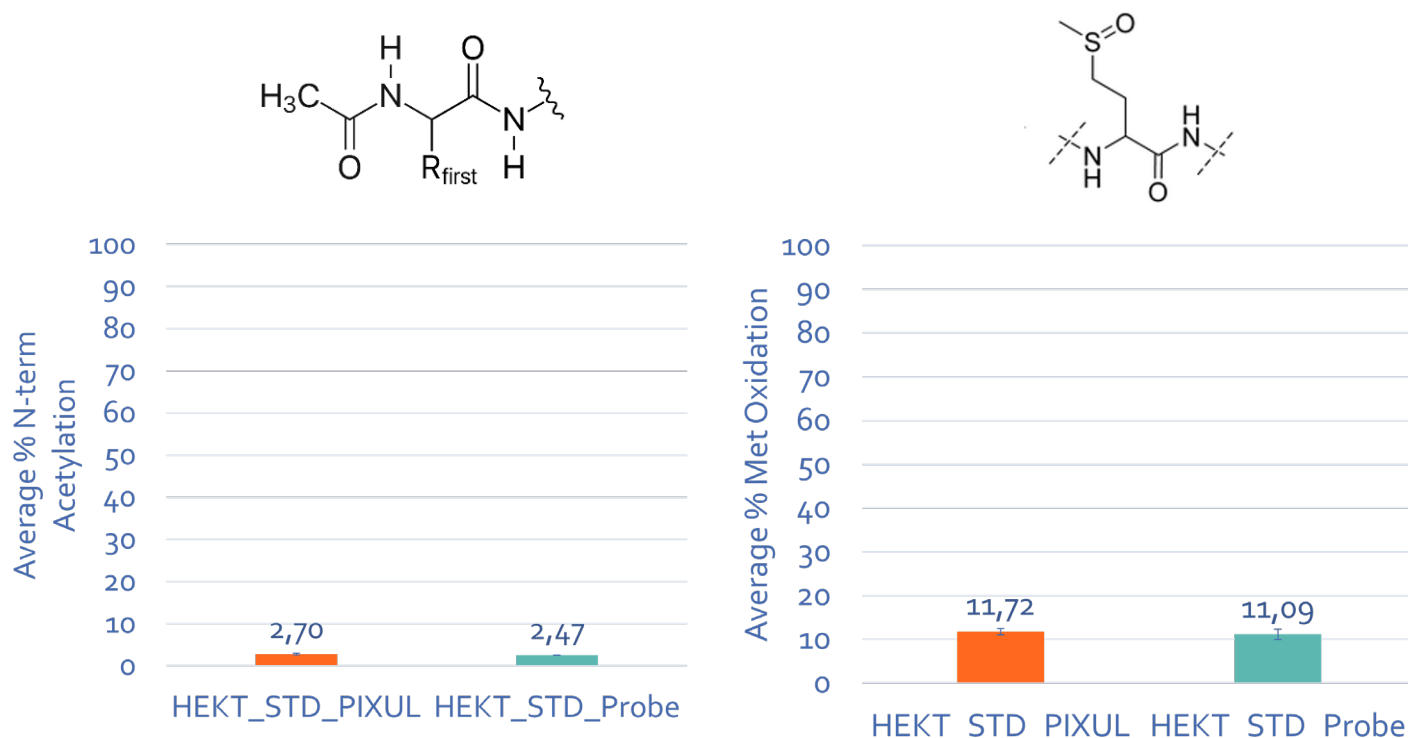


Figure 3: Average percentage of protein N-terminal acetylation and oxidized methionine.

LEFT: The average percentage of protein N-terminal acetylation is shown for PIXUL (orange) and standard probe sonicator (green).

RIGHT: The average percentage of oxidized methionine is shown for PIXUL (orange) and standard Probe Sonicator (green).

The percentage of acetylated protein N-termini in samples processed with PIXUL (2.7 %) as well as the percentage of oxidized methionines (11.72 %) was in the same range as the percentage observed with the standard probe sonicator (2.47 %, 11.09 %).

Altogether, these results showed that for Proteomics sample preparation of standard culture cells, the PIXUL gives comparable results to standard Probe sample preparation protocol with the benefit of higher throughput.

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Mouse Liver Tissue

Sample Preparation

Sample preparation for MS-Analysis was performed using the Profiti S-Trap™ sample processing technology. In brief: 100 mg of fresh mouse liver tissue was resuspended in a buffer containing 5 % SDS in 50 mM TEAB pH 8.5. Tissue disruption was performed using a MACS tissue dissociator (Miltenyi Biotech).

The standard approach then includes sonication of the sample using a probe sonicator (VCX130 with a 3 mm probe, Sonics and Materials, Newtown, USA) with 3x15 s pulses, 15 W output and 30 s on ice between individual bursts.

This approach was compared to the [PIXUL Multi-Sample Sonicator](#) with the following settings: 5, 15, 30 or 60 min of shearing, 50 N pulse with 1 kHz, 20 Hz burst rate and 8 wells per sample which were pooled later in the process.

After sonication DTT and IAA were added to reduce and alkylate the proteins and proteins were digested with 1 µg Trypsin at 37°C o/n. 1.5 µg of the resulting peptide mixture was then analyzed by nanoLC-MS/MS on an LTQ Orbitrap Elite instrument using standard 2 hour runs and CID fragmentation.

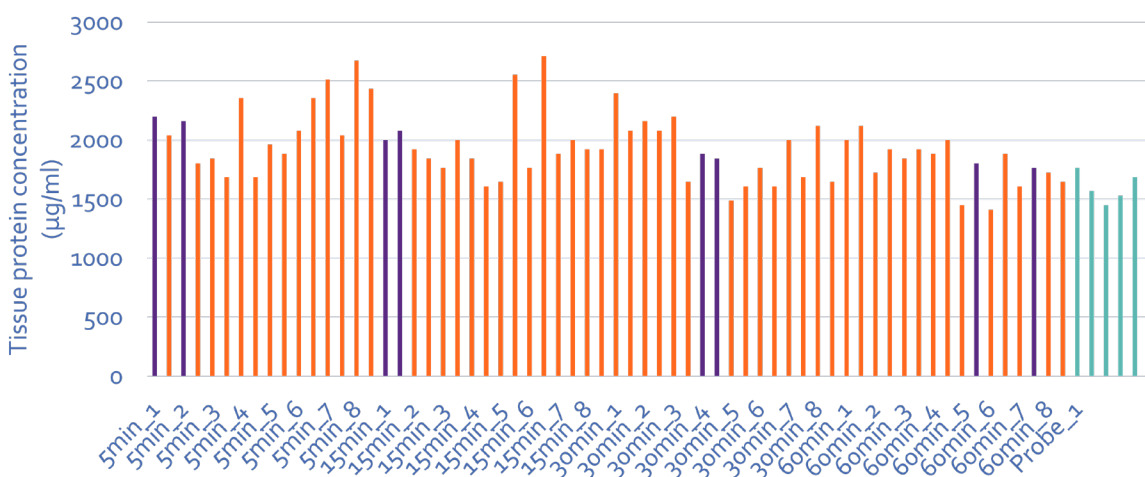


Figure 4: The samples processed with the standard probe sonicator or the PIXUL were subjected to BCA analysis and the protein concentration is shown in µg/ml.

Orange: Samples processed with PIXUL, green: samples processed with the probe sonicator, blue: samples selected for further analysis.

$P\text{-value}_{5min} = 0.00048$

$P\text{-value}_{15min} = 0.00552$

$P\text{-value}_{30min} = 0.014$

$P\text{-value}_{60min} = 0.022$

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Protein Concentration

The first QC measure taken in the protocol was, whether the Protein yield was comparable between the standard Probe Sonicator and the different durations used on the PIXUL. To evaluate Protein concentration, samples were subjected to BCA analysis and analyzed in triplicate.

Figure 4 shows a comparison between six samples processed with the standard probe sonicator and 8 samples processed in duplicates for 4 different time points (5 min, 15 min, 30 min and 60 min) with PIXUL.

Protein concentration of samples processed with PIXUL are in the same range (1409 - 2710 µg/ml) as samples processed in the standard way (1449 - 1764 µg/ml), indicating a similar, if not slightly better protein yield when using PIXUL compared to the standard method. In addition, there was no obvious difference between the samples sheared for varying amounts of time on the PIXUL, indicating that 5 min of shearing is already sufficient to yield protein concentrations sufficient for downstream analysis.

Sample name	# of identified peptides	# of identified protein groups
TissuePIXUL5min_rep1	7319	1441
TissuePIXUL5min_rep2	7265	1434
TissuePIXUL15min_rep1	7280	1359
TissuePIXUL15min_rep2	7359	1375
TissuePIXUL30min_rep1	7716	1457
TissuePIXUL30min_rep2	8380	1689
TissuePIXUL60min_rep1	7345	1493
TissuePIXUL60min_rep2	7721	1493
TissuePIXUL60min_rep1	7345	1493
TissuePIXUL60min_rep2	7721	1493

Table 2: Comparison of the Number of identified Peptides and Proteins.

Top eight rows represent data of two replicates of each of the four durations run on PIXUL and lower two rows represent the standard probe sonicator.

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Number of Identified Peptides

The first QC measure taken in the protocol was, whether the Protein yield was comparable between the standard Probe Sonicator and the different durations used on the PIXUL. To evaluate Protein concentration, samples were subjected to BCA analysis and analyzed in triplicate.

Despite small differences in the extracted protein concentration, for each sample the same peptide amount was injected for LC-MS/MS analysis.

LC-MS/MS runs of selected samples were searched separately using the MaxQuant algorithm (version 1.6.17.0) with mainly default settings, including a false discovery rate set at 1% on both peptide, and protein level. Spectra were searched against the human Swiss-Prot Reference proteome database (www.uniprot.org) (database release version of 2021_01, containing 20,621 protein sequences).

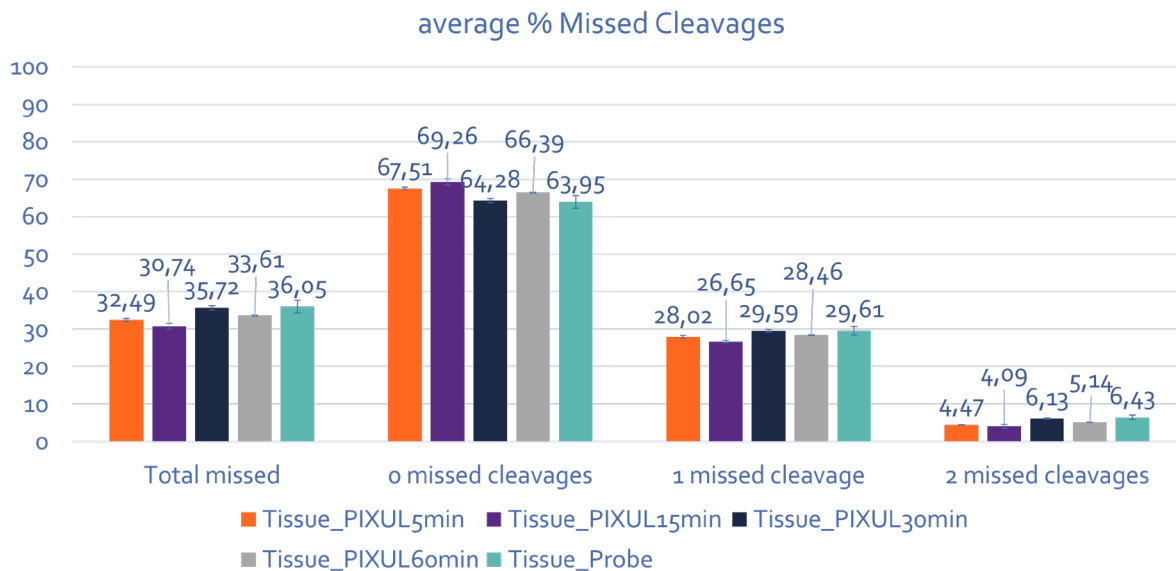


Figure 5: Average percentage of missed cleavages.

The average percentage of missed cleavages is shown for PIXUL (orange, purple, black, grey) and Probe sonicator (green).

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The number of peptides and proteins that were identified were comparable between both groups (PIXUL: 7265-8380, Standard: 7803-8124) indicating a similar ability to identify peptides and proteins in mouse liver tissue. Furthermore, the ability to identify peptides did not vary substantially between samples using different processing times on the PIXUL, meaning that 5 minutes of sonication is sufficient to completely and efficiently lyse liver tissue.

Missed Cleavages

Next, the number of missed trypsin cleavages was evaluated. Missed cleavages represent instances where trypsin did not cleave the peptide as expected after each Lysine or Arginine residue. During database searching up to 2 missed cleavages were allowed as a standard setting, hence the number of peptides with 0, 1 or 2 missed cleavage events could be assessed.

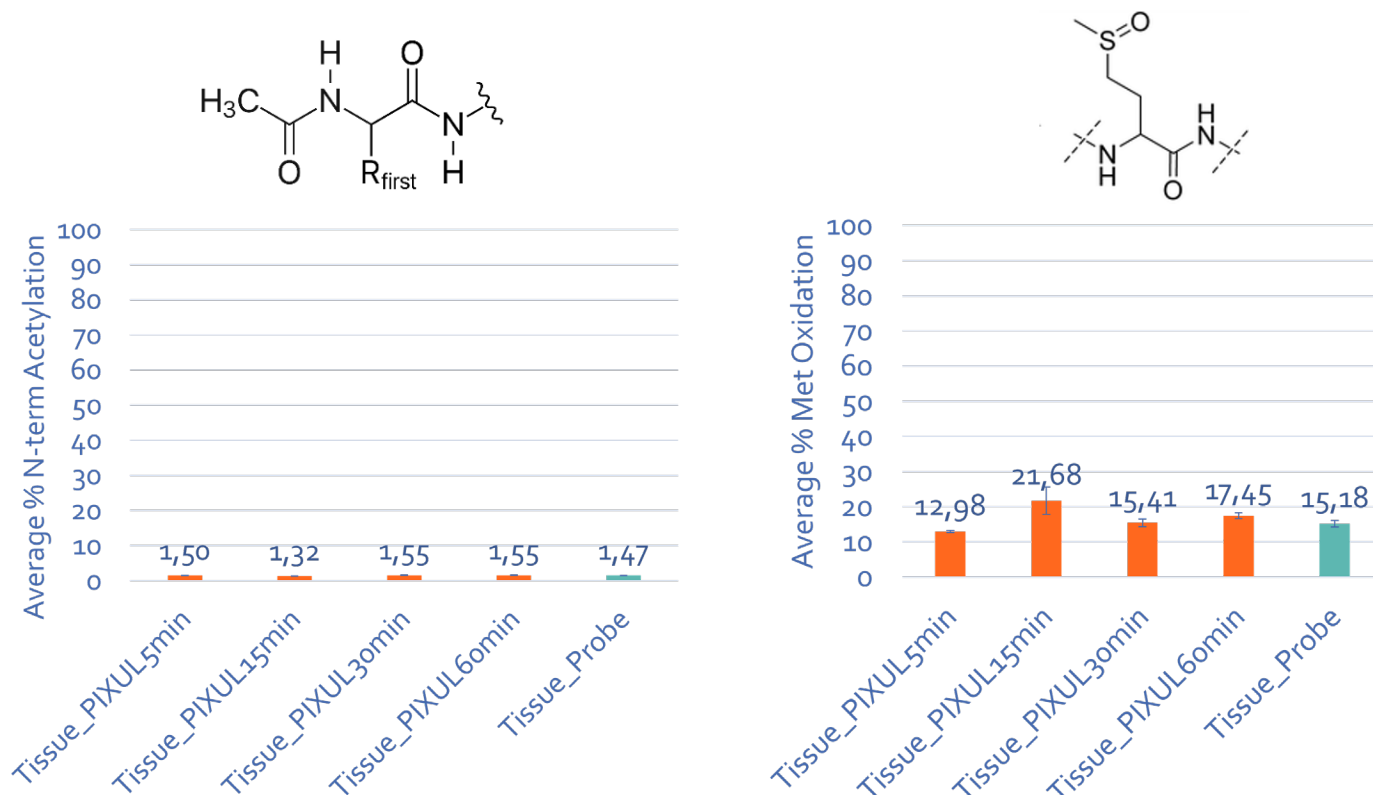


Figure 6: Average percentage of Protein N-terminal acetylation and oxidized methionine.

LEFT: The average percentage of Protein N-terminal acetylation is shown for PIXUL (orange) and standard Probe Sonicator (green). **RIGHT:** The average percentage of oxidized methionine is shown for PIXUL (orange) and standard Probe Sonicator (green).

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The total percentage of missed cleavages is comparable between PIXUL and the standard probe sonicator (30,74-35,72 vs 36,05). Furthermore, the fraction of 0, 1 or 2 missed cleavage events is comparable, suggesting a similar degree of missed cleavages in samples prepared with PIXUL compared to the standard probe sonicator. Moreover, the fraction of 0, 1 or 2 missed cleavages among the varying processing time on PIXUL is comparable. Overall, the number of missed cleavages is acceptable given the fact that no (e.g., endoLys) pre-digestion was performed.

Modifications

Last, the average percentages of acetylated protein N-termini and oxidized Methionine residues (methionine sulfoxide) were assessed as measures for the ability to detect protein modifications and as general quality parameters for sample preparation including sonication.

The percentage of acetylated protein N-termini as well as the percentage of oxidized methionine in samples processed with PIXUL was in the same range as samples processed with the standard probe sonicator.

Altogether, these results showed that for Proteomics sample preparation of mouse liver tissue as a representation for animal tissue samples, the [PIXUL Multi-Sample Sonicator](#) gives comparable results to the probe sample preparation protocol with the added benefit of higher throughput.

Summary

With this short TechNote, we demonstrate the suitability of PIXUL Multi-Sample Sonicator for Proteomics preparation of HEK293T cells and mouse liver tissue samples using the S-Trap System. Results were compared to the standard way of processing those samples using a probe tip sonicator.

To evaluate the suitability of the samples for LC-MS/MS analysis, the extracted protein concentration, the number of identified peptides and proteins, the percentage of missed trypsin cleavages and the presence of common protein modifications was measured. For all evaluated sample types and quality parameters, the PIXUL performed equally well or better as the standard probe tip sonicator, with the benefit of being able to process 96 samples in a short period of time. The reduced hands-on time further enables the development of semi-automated workflows for Proteomics sample preparation.

Data courtesy of the VIB Proteomics Core.





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