FFPE sample preparation simplified with BeatBox: A xylene-free, high-throughput workflow for in-depth tissue proteome analysis

Authors: Katharina Limm, Silvia Würtenberger, Marcello Stein, Nils A. Kulak and Katrin Hartinger
PreOmics GmbH, Planegg/Martinsried, Germany

Introduction

Formalin-fixed, paraffin-embedded (FFPE) tissue is an invaluable resource for retrospective studies to investigate molecular mechanisms or to discover novel biomarkers.[1] Since clinical FFPE specimens are collected and stored routinely by hospital pathology departments, large libraries that are linked to clinical metadata are readily available.[2,3]

FFPE tissue sample preparation for proteomic analyses is extremely challenging as formalin-fixation makes proteins difficult to extract. Harsh conditions must be applied to reverse protein cross-linking.[2] In addition, paraffin interferes in the downstream liquid chromatography-mass spectrometry (LC-MS) analysis which is why most protocols include an initial xylene-based deparaffinization step that is time-consuming, poses a health hazard due to high levels of toxicity, and results in low reproducibility with the risk of sample loss.[4,5]

We present an optimized solution using the BeatBox tissue homogenizer and iST proteomic sample preparation technology that simplifies, speeds-up and standardizes FFPE sample preparation (Fig. 1). The workflow eliminates the need for xylene-based deparaffinization, and enables fast and robust processing of up to 96 samples in parallel from starting material to clean peptides within one working day. We benchmarked the BeatBox FFPE workflow against a traditional sonication approach and evaluated its effectiveness for deep proteomic analysis of FFPE tissue in comparison with fresh frozen tissue.

Keywords

Proteomics, FFPE, BeatBox, high-throughput sample preparation, iST technology, HR-mass spectrometry, LC-MS

Key takeaways

• Streamlined FFPE sample preparation workflow combining BeatBox and iST technology

• High-throughput processing of up to 96 samples/day to ready-to-measure peptides

• Optimized xylene-free approach without deparaffinization for LC-MS-based proteomics

• Fast, easy-to-use, and standardized protocol for high levels of reproducibility and reduced hands-on time

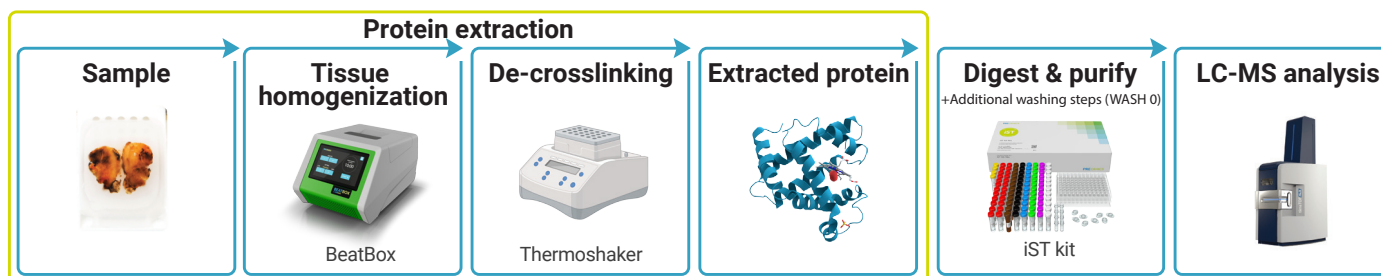• BeatBox workflow performance with FFPE samples is equivalent to working with fresh frozen tissue

Figure 1 | Overview of the BeatBox FFPE workflow. High-throughput LC-MS-based proteomics workflow for FFPE tissue without xylene-based deparaffinization using the BeatBox platform in combination with optimized iST sample preparation including additional washing steps.

PreOmics Application Note – FFPE sample preparation simplified with BeatBox: A xylene-free, high-throughput workflow for in-depth tissue proteome analysis

Version 1.0 - For research use only **Page | 1 of 5**

PREOMICS

Methods *for protocol details please see reference 6

Tissue samples

Studies were performed using FFPE as well as fresh frozen mouse cardiac muscle, kidney and liver tissue provided by the Research Institute of Molecular Pathology in Vienna and the Histology Facility at Vienna BioCenter Core Facilities (Austria).

For the experiments, 10 µm FFPE tissue "full" curls containing paraffin (approximately 37, 78, and 143 mm² tissue area for cardiac muscle, kidney, and liver, respectively), deparaffinized FFPE tissue from "full" curls of the same size, and 1-2 mg of fresh frozen tissue were compared. A xylene-based protocol was employed for deparaffinization.

Sample preparation

Table 1 provides an overview of all sample preparation workflows. FFPE samples ("full" curls or tissue deparaffinized with xylene) were homogenized either with BeatBox (10 min, HIGH setting, with BeatBox Tissue Kit 96x) or with bead-based sonication (10 cycles, 30 sec on, 30 sec off, 1.5 mL tubes).[7] Fresh frozen tissue was only homogenized using the BeatBox (10 min, STANDARD setting, with BeatBox Tissue Kit 96x).

After homogenization, FFPE samples were incubated at 95°C to de-crosslink and solubilize protein. Subsequently, all samples were digested and purified according to the FFPE protocol with the iST 96x Kit (PreOmics GmbH).[6] PreOmics' iST workflow saves time and streamlines the proteomic sample preparation by employing an easy-to-use, three-step, all-in-one kit. For samples from FFPE "full" curls without the xylene-based deparaffinization step, the peptide purification on the iST cartridge was optimized by adding WASH 0 buffer to ensure complete removal of paraffin.

LC-MS/MS analysis and data analysis

Peptides were resuspended in LC-LOAD (iST Kit, PreOmics GmbH), and a 300 ng sample analyzed on an EASY-nLC™ 1200 system (Thermo Fisher Scientific) coupled to a timsTOF-HT mass spectrometer (Bruker Daltonics) in DIA-PASEF mode using a 30-minute gradient.

Raw files were analyzed using DIA-NN V1.8[8] in library-free mode and searched against the UniProt FASTA database of *Mus musculus* (Swiss-Prot entries; downloaded 2022-02-14). The false discovery rate was set to 1% on the precursor level and evaluated against decoy precursors. Enzyme specificity was set as C-terminal to arginine and lysine, using trypsin as protease, and a maximum of one missed cleavage was allowed in the database search. Statistical analysis was performed using Perseus (V 1.6.15.0).

Table 1 | Overview of compared sample preparation workflows for FFPE tissue (deparaffinized with xylene or "full" curls) and fresh frozen tissue for LC-MS analysis.

	FFPE tissue				Fresh frozen tissue
Xylene-based deparaffinization	Yes		No ("full" curls)		-
Tissue homogenization	Sonication	BeatBox	Sonication	BeatBox	BeatBox
Digestion	iST workflow				iST workflow
Purification	iST workflow (standard)		iST workflow (optimized with WASH 0)		iST workflow (standard)

Results and Discussion

The new BeatBox FFPE workflow is faster and more efficient than traditional sonication workflows

Two major factors influence protocol speed and efficiency: the deparaffinization method and the parallelization capability of subsequent sample preparation. Traditional xylene-based deparaffinization can mean 1.5 hours of additional time with many manual steps. Furthermore, sample homogenization may be low throughput and requires repeated time-consuming cycles if the device is incompatible with multiwell plates.

The BeatBox workflow allows FFPE sample preparation without xylene-based deparaffinization and can process up to 96 samples in a plate in parallel. In comparison with the traditional sonication workflow with xylene-based deparaffinization, this approach saves up to 4 hours of time and thereby enables high-throughput processing of 96 FFPE samples within one working day (Fig. 2).
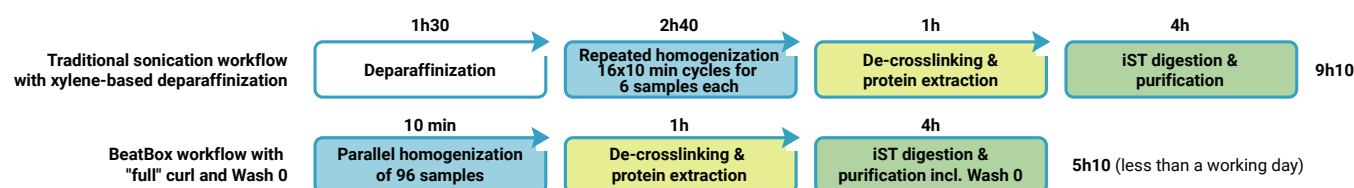

Figure 2 | Comparison of protocol times between the BeatBox FFPE workflow starting from "full" curl with optimized iST purification using WASH 0 for paraffin removal and the traditional sonication workflow with initial xylene-based deparaffinization for 96 samples (see also Table 1).

PreOmics Application Note – FFPE sample preparation simplified with BeatBox: A xylene-free, high-throughput workflow for in-depth tissue proteome analysis

Version 1.0 - For research use only **Page | 2 of 5**

The BeatBox FFPE workflow permits in-depth proteomic analyses without prior xylene-based deparaffinization

The BeatBox workflow and the sonication-based workflow were compared using both "full" curls and deparaffinized FFPE tissue. Samples were prepared and measured as described in the materials and methods section (see Table 1 for overview).

For the three different mouse tissue types – cardiac muscle, kidney, and liver – BeatBox homogenization increased the proteomic depth on average by 14-43%, depending on tissue type (Fig. 3). This observation held true for both FFPE tissue types, deparaffinized using xylene (Fig. 3A) as well as "full" curls prepared without the xylene-based deparaffinization step, and only treated with an optimized peptide purification on the iST cartridge by applying WASH 0 buffer (Fig. 3B). WASH 0 buffer ensures total removal of the paraffin to prevent impurities clogging the cartridge or the analytical column.

Using the BeatBox FFPE workflow, both deparaffinized tissue and "full" curl samples overlapped >91% regarding identified proteins for all analyzed tissue types (data not shown) and the number of identified proteins showing valid values differed by only 1-2% between deparaffinized and "full" curl FFPE tissue (Figure 3).

The results yield two remarkable findings: First, the resulting protein IDs reveal a better performance of the BeatBox compared with the sonication device. Second, the optimized peptide purification using WASH 0 renders a separate xylene-based deparaffinization step unnecessary, for both the BeatBox and the sonication-based workflow. Thus, combining protein extraction on BeatBox with optimized iST-based digest and peptide purification to process FFPE tissue offers considerable time savings already at the sample preparation step and allows for deeper proteomic analyses.
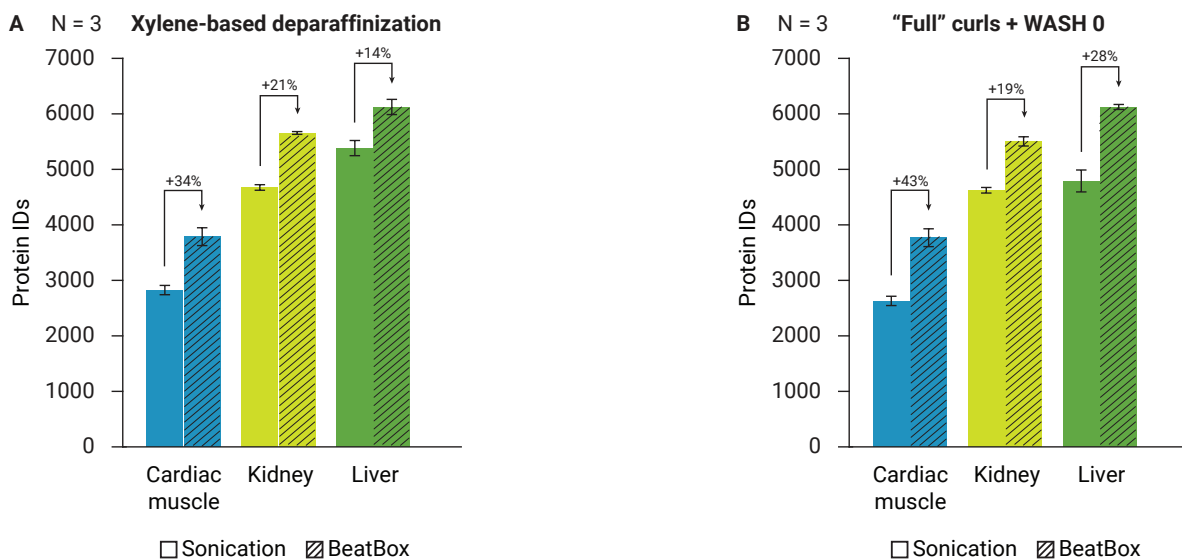

Figure 3 | Comparison of protein identifications after BeatBox and sonication-based FFPE workflows. FFPE tissue samples from mouse cardiac muscle, kidney, and liver were homogenized in triplicate either using the BeatBox or a standard sonication device. Samples were prepared using the iST technology and then analyzed via LC-MS. FFPE samples were either deparaffinized using xylene **(A)** or used as "full" curls treated with an optimized iST purification using WASH 0 buffer **(B)**. Error bars represent the standard deviation.

The BeatBox FFPE workflow exhibits remarkable repeatability

Figure 4 illustrates the technical variability of the BeatBox FFPE workflow. Coefficients of variation (CV) within quadruplicates from deparaffinized FFPE samples and "full" curl FFPE samples were below 10% for all assessed tissue types. This emphasizes the high repeatability of the homogenization process with BeatBox and makes the workflow ideal for the proteomic analysis of large sample cohorts.
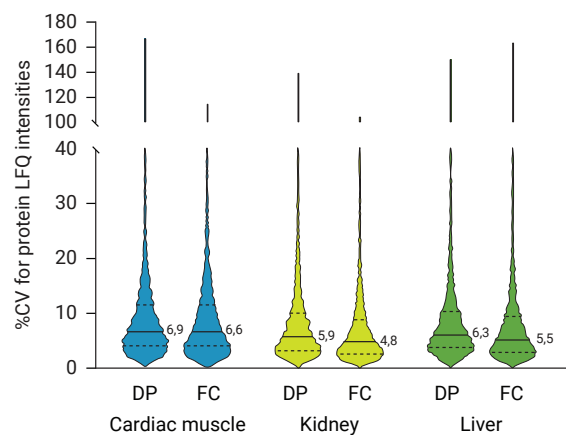

Figure 4 | Assessment of technical variability of the BeatBox FFPE workflow. Coefficient of variation (CV) in % for protein LFQ intensities within replicates (n=4, serial sections) from FFPE samples processed with the BeatBox workflow and analyzed via LC-MS. FFPE samples were either deparaffinized using xylene (**DP**) or used as "full" curls treated with an optimized iST purification with WASH 0 buffer (**FC**). The median CVs are shown as numbers next to the violin plots.

PreOmics Application Note – FFPE sample preparation simplified with BeatBox: A xylene-free, high-throughput workflow for in-depth tissue proteome analysis

Version 1.0 - For research use only   **Page | 3 of 5**

BeatBox workflow processes FFPE and fresh frozen tissue with similar efficiency

FFPE "full" curls and fresh frozen tissue samples were prepared with the BeatBox workflow and measured with LC-MS as described In the materials and methods section (see Tab. 1 for overview). The resulting data showed that the number of proteins identified using "full" curls and fresh frozen samples was comparably high (Fig. 5A), exhibiting an overlap of 79-87% of shared proteins depending on tissue type (data not shown). For fresh frozen tissue, roughly 4300, 5800, and 6300 proteins were identified for mouse cardiac muscle, kidney, and liver tissue, respectively. "Full" curl FFPE

tissue resulted in only 8-14% lower numbers of proteins (around 3700, 5200, and 5800, respectively). This shows that the BeatBox workflow achieves in-depth proteome coverage, even with FFPE tissue (Fig. 5A).

The dynamic range of protein abundance determined from LC-MS data of either FFPE "full" curls, or fresh frozen tissue demonstrated comparable protein depth. This further confirmed the efficiency of protein extraction from FFPE tissue when the BeatBox is used (Fig. 5B). This paves the way to analyze FFPE tissue samples without compromising on the quality of the results.
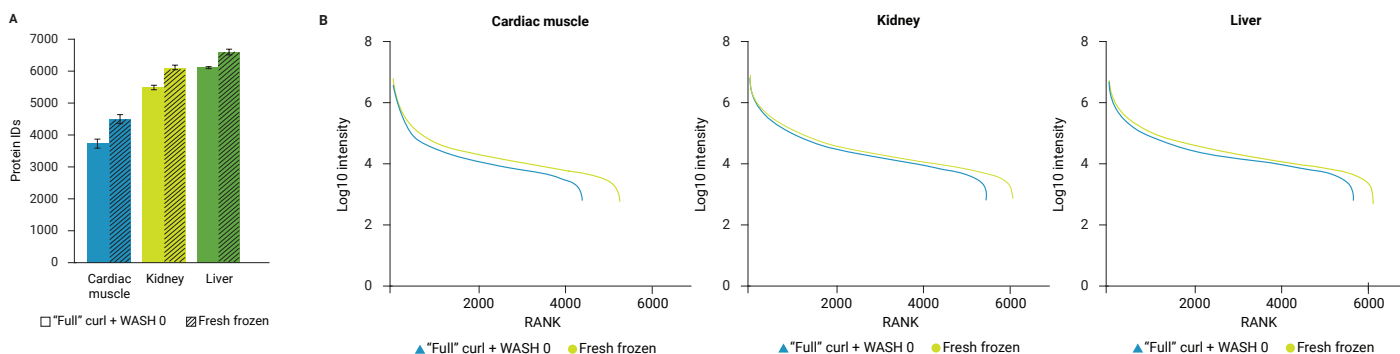

Figure 5 | Comparison of identified proteins and dynamic range obtained from FFPE tissue and fresh frozen tissue homogenized on BeatBox. FFPE "full" curl samples (**FC**) and fresh frozen samples (**FF**) from mouse cardiac muscle, kidney and liver were homogenized in quadruplicate on the BeatBox, then prepared using iST technology and analyzed via LC-MS. FC samples were treated with an optimized iST purification using WASH 0 buffer (see Tab. 1 for overview). Shown are proteins identified (**A**), as well as the dynamic range and protein abundance as S-curves (**B**).

Conclusions

Despite being readily available, scientists often refrain from using FFPE tissue in mass spectrometry (MS)-based proteomic studies as sample preparation is technically demanding and often time-consuming. The presented solution combining the BeatBox and iST technology provides a simple, fast, and robust way to process FFPE tissue for LC-MS-based proteomics. The approach saves up to 4 hours of time compared with traditional workflows and allows the preparation of 96 samples in a single working day.

Analyses of FFPE tissue from mouse cardiac muscle, kidney, and liver showed that the BeatBox workflow yields greater proteome coverage than a traditional sonication approach. The results further indicated that optimized washing during

the iST peptide clean-up effectively removed paraffin from FFPE samples, taking away the need for a separate xylene-based deparaffinization procedure. This workflow is less toxic, has fewer manual steps, and is highly reproducible, making it ideal for the analysis of large sample cohorts. Finally, benchmarking FFPE tissue samples against fresh frozen tissue showed a similar deep proteome coverage and dynamic range of protein abundances.

In summary, using the BeatBox workflow to process stored FFPE tissue from biobanks reduces reliance on fresh frozen samples and will advance proteomic analyses in a variety of application areas ranging from basic research to clinically relevant studies.

PreOmics Application Note – FFPE sample preparation simplified with BeatBox: A xylene-free, high-throughput workflow for in-depth tissue proteome analysis

Version 1.0 - For research use only   **Page | 4 of 5**

PREOMICS

Products

Product	Manufacturer	Product Code
BeatBox Instrument	PreOmics GmbH	P.O.00144
BeatBox Tissue Kit 96x	PreOmics GmbH	P.O.00121
iST Kit 96x	PreOmics GmbH	P.O.00050
WASH 0 (10 mL)	PreOmics GmbH	P.O.00095

Ordering information:

http://www.preomics.com/quote

order@preomics.com

Acknowledgements

References

1. Coscia, F. et al. A Streamlined Mass Spectrometry–Based Proteomics Workflow for Large-scale FFPE Tissue Analysis. The Journal of Pathology (2020). DOI: 10.1002/path.5420.

2. Obi, NE. et al. Biomarker Analysis of Formalin-Fixed Paraffin-Embedded Clinical Tissues Using Proteomics. Biomolecules (2023). DOI: 10.3390/biom13010096

3. Piehowski DP. et al. Residual Tissue Repositories as a Resource for Population-Based Cancer Proteomic Studies. Clinical Proteomics (2018). DOI: 10.1186/s12014-018-9202-4.

4. Kalantari N., Bayani M., and Ghaffari T. Deparaffinization of Formalin-Fixed Paraffin-Embedded Tissue Blocks Using Hot Water Instead of Xylene. Analytical Biochemistry (2016). DOI: 10.1016/j.ab.2016.05.015

5. Mitsa G. et al. A Non-Hazardous Deparaffinization Protocol Enables Quantitative Proteomics of Core Needle Biopsy-Sized Formalin-Fixed and Paraffin-Embedded (FFPE) Tissue Specimens. International Journal of Molecular Sciences (2022). DOI: 10.3390/ijms23084443

6. PreOmics protocol: BeatBox Tissue Kit 96x coupled to iST 96x | Formalin-fixed, paraffin-embedded (FFPE) tissue; https://www.preomics.com/resources

7. PreOmics protocol: iST Kit for FFPE Tissues 8x; https://www.preomics.com/resources

8. Demichev, V., Messner, C.B., Vernardis, S.I. et al. DIA-NN: neural networks and interference correction enable deep proteome coverage in high throughput (2020). https://doi.org/10.1038/s41592-019-0638-x

PreOmics GmbH
Martinsried | Germany

PreOmics Inc.
Billerica, MA | USA

Version 1.0 - For research use only **Page | 5 of 5**
www.preomics.com