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Application Note

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Complete Workflow for In-Depth Proteome Analysis of Multiple FFPE Formats

This application note showcases an end-to-end workflow combining *BeatBox* homogenization, *iST* sample preparation, and *PepSep Advanced* columns for in-depth proteome analysis across multiple FFPE sample formats.

Key Takeaways

- **Streamlined FFPE processing:** *BeatBox* and *iST* provide a simple, reproducible workflow for proteomics sample preparation.
- **Flexible sampling:** FFPE curls and punches can be processed using the same workflow.
- **Robust performance:** *PepSep Advanced* maintained consistent depth across FFPE formats, with improved quantitative precision.
- **Tissue-specific effects:** Sampling format influenced heart proteomes more than liver proteomes.
- **Comparable Peptide Coverage:** *PepSep Advanced* achieved largely overlapping peptide coverage with the benchmark column.

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Complete Workflow for In-Depth Proteome Analysis of Multiple FFPE Formats

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Keywords

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iST

Introduction

Formalin-fixed, paraffin-embedded (FFPE) tissues are valuable resources for retrospective biomarker and disease studies due to their broad availability in biobanks and pathology archives. However, formalin-induced crosslinking, paraffin, variable sample amounts, and different sampling formats complicate standardized proteomic analysis.[1-3]

The *PreOmics BeatBox–iST* FFPE workflow provides a xylene-free approach that combines tissue homogenization, decrosslinking, digestion, and peptide cleanup for up to 96 samples in a single working day. The *BeatBox Tissue Kit 96x* enables high-throughput processing of up to two FFPE curls, while the *BeatBox Tissue Kit 24x* provides greater flexibility in terms of sample quantity, allowing for the processing of up to five FFPE curls.[4-6]

In this study, FFPE mouse heart and liver samples were processed as curls or punches using the *BeatBox Tissue Kit 24x* workflow. Sample preparation was combined with *PepSep Advanced*-coupled LC-MS analysis and *Spectronaut* data processing (Figure 1). The workflow was evaluated for scalability, proteome depth, quantitative reproducibility, differences between curl and punch preparations, and complementary peptide detection across LC configurations.



Figure 1. End-to-end FFPE proteomics workflow. FFPE curls or punches were processed using a *BeatBox-iST* sample preparation workflow, analyzed by *PepSep Advanced*-coupled LC-MS (nanoElute2-timsTOF HT system, Bruker), and processed in *Spectronaut 20*.

Materials and Methods

Experimental design and sample preparation

FFPE mouse heart and liver tissues were sectioned into 10-µm full curls or sampled as approximately 1 × 1 mm tissue punches. Each condition was processed in quadruplicate as independent sample-preparation replicates. Curl and punch samples originated from the same FFPE block.

Workflow scalability was evaluated by comparing two mouse liver curls processed using the *BeatBox Tissue Kit 96x* (plate format) with two to five curls processed using the *BeatBox Tissue Kit 24x* (tube format). Subsequent experiments in the 24x format used either two curls or a single tissue punch.

For this study, modified *BeatBox–iST* workflows optimized for FFPE curls and punches were used. FFPE curls were placed in LYSE buffer, heated at 95 °C for 10 min in a thermoshaker, homogenized using *BeatBox* for 10 min, and then incubated at 95 °C for 60 min in a thermoshaker for heat-induced decrosslinking. FFPE punches were first homogenized using *BeatBox* for 10 min without LYSE buffer. LYSE buffer was then added, and the samples were heated at 95 °C for 10 min in a thermoshaker, homogenized again using *BeatBox* for 10 min, and incubated at 95 °C for 60 min in a thermoshaker for heat-induced decrosslinking. Total extracted protein was quantified using the Pierce BCA Protein Assay Kit – Reducing Agent Compatible (ThermoFisher Scientific), and 100 µg of protein from each preparation was subjected to Trypsin/LysC digestion using *iST* DIGEST at 37 °C for 3 h. Paraffin was removed by an optimized *iST* peptide purification procedure on *iST* cartridges using WASH 0 as an additional washing step. Dedicated protocols for FFPE sample preparation using *BeatBox* and *iST*, including workflows for both FFPE curls and FFPE punches, are available online and were followed where applicable.[6,7]

LC-MS/MS

Purified peptides (300 ng) were analyzed using a nanoElute 2 HPLC equipped with either a *PepSep Advanced* column (Bruker; 25 cm × 75 µm i.d., 1.5 µm particles) coupled to a *CaptiveSpray 2* emitter (Bruker; 10 µm i.d.) or an integrated-emitter C18 comparator column (25 cm × 75 µm), operated with a 24-SPD gradient and coupled to a timsTOF HT mass spectrometer in dia-PASEF mode.

Data analysis

Raw data were processed in *Spectronaut 20* using directDIA+ against the reviewed *Mus musculus* UniProt proteome (UP000000589; January 2024), with precursor and protein-group identifications controlled at 1% FDR.

Quantitative analyses were based on log2-transformed, MaxMedian-normalized *Spectronaut* quantities. Protein-group and peptide coefficients of variation (CVs) were calculated for features quantified in at least two of four sample-preparation replicates.

Protein-level principal component analysis (PCA) was performed using complete protein profiles only, without missing-value imputation. Differential protein abundance was analyzed using limma with robust empirical Bayes moderation. Limma-derived p-values were corrected for multiple testing using the Benjamini–Hochberg procedure. Differential proteins were identified using a two-sided S0-dependent significance envelope ($S_0 = 0.20$, $\alpha = 0.05$, $d_f = 5$).[8]

STRING-DB Cellular Component enrichment was performed using preparation-associated proteins, with all quantified proteins serving as the background set.[9] Proteins exclusive to curls or punches were required to be detected in at least two of four replicates of the respective condition and absent from the other condition. Column-exclusive peptides were likewise required to be detected in at least two of four replicates with one LC configuration and absent from the other.

Results and Discussion

Scalable processing maintains proteome depth

The *BeatBox Tissue Kit 24x* (tube workflow) was benchmarked against the established *BeatBox-iST* FFPE workflow in 96-well plate format using FFPE liver curls (Figure 2). Across equivalent tissue input, both workflows delivered comparable protein-group identifications and protein extraction yields, demonstrating consistent performance between the plate- and tube-based formats (Figures 2A,B). Two liver curls produced similar proteome depth in both workflows and were therefore used as the reference condition for subsequent comparisons.

To evaluate the scalability of the tube-based workflow, tissue input was increased from two to five liver curls. Total protein extraction yield increased with tissue input,

reaching approximately 2 mg from five liver curls (Figure 2B). Despite the substantially higher protein yield obtained at increased tissue input, a fixed 100 µg aliquot from each preparation was used for digestion and subsequent LC-MS analysis. Under these conditions, protein-group identifications remained stable across all input amounts, indicating that increased tissue loading does not compromise proteome depth (Figure 2A).

Together, these results demonstrate that the 24x format expands FFPE input flexibility while maintaining proteome depth and was therefore selected for subsequent curl and punch experiments.

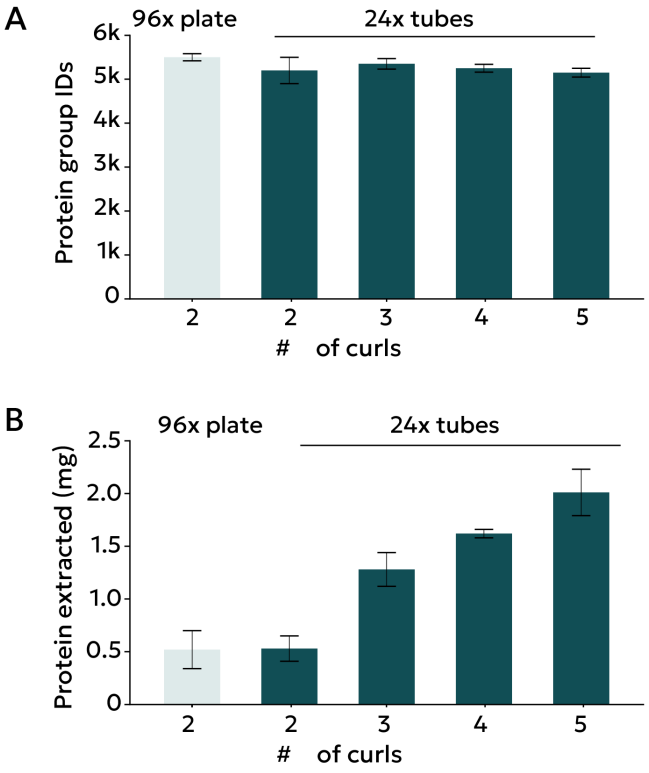


Figure 2. Scalable processing of FFPE liver curls. (A) Protein-group identifications across the 96x plate and 24x tube workflows. (B) Total protein extraction yield with increasing curl input.

Robust FFPE proteomics across curls and punches

Mouse heart and liver samples were processed as either two curls or one punch using the *BeatBox-iST 24x* workflow and analyzed with either a *PepSep Advanced* column or an integrated-emitter C18 comparator column. Within each tissue type, comparable numbers of peptides and protein groups were identified (Figure 3A,B). Heart preparations yielded approximately 45,000–50,000 peptides and 5,000–5,400 protein groups, while liver preparations yielded approximately 70,000–75,000 peptides and 6,500–7,000 protein groups. In addition, low peptide- and protein-group CVs across all sampling formats demonstrated consistent quantitative performance regardless of whether curls or punches were used (Figure 3C,D). These findings show that the *BeatBox-iST* workflow enables robust and reproducible FFPE proteomics across both sampling formats.

Tissue-dependent proteomic differences between curl and punch preparations

To evaluate the influence of FFPE sampling format, curl and punch preparations from the same tissue block were compared at the protein-group level. Protein-group-level PCA was dominated by tissue type, with PC1 explaining 63% of the total variance. PC2 (not shown) explained approximately 10% and primarily separated the LC columns, whereas PC3 explained approximately 8% and separated heart curl and punch preparations. In contrast, liver curl and punch preparations remained closely grouped (Figure 4A).

In heart, curls and punches shared 4,844 proteins, representing 84% of the combined detected proteome. Additionally, 613 proteins were detected exclusively in punch preparations and 323 exclusively in curl preparations (Figure 4B).

Differential-abundance analysis further revealed distinct protein profiles between heart curl and punch preparations (Figure 4C). Punch-associated proteins were enriched for nuclear and chromosome-related cellular components, whereas curl-associated proteins were enriched for plasma-membrane and cell-periphery compartments (Figure 4D).

The pronounced differences observed in heart, together with the close similarity between liver curl and punch preparations, suggest that the influence of FFPE sampling format may be tissue dependent. Spatial proteomics has demonstrated substantial regional proteomic heterogeneity within cardiac tissue, including distinct proteomic signatures between millimeter-scale anatomical regions.[10] Liver is also spatially heterogeneous, but its organization follows repeating lobular units with well-defined porto-central molecular and proteomic zonation.[11] These differences in tissue architecture may influence how strongly a localized punch differs from a whole-section curl at the sampling scale used here. Previous FFPE proteomics studies comparing tissue punches and thin sections similarly reported high overall proteomic similarity while retaining a degree of biological variation between sampling formats.[12] Block-specific tissue composition may additionally contribute to the differences observed in the present study.

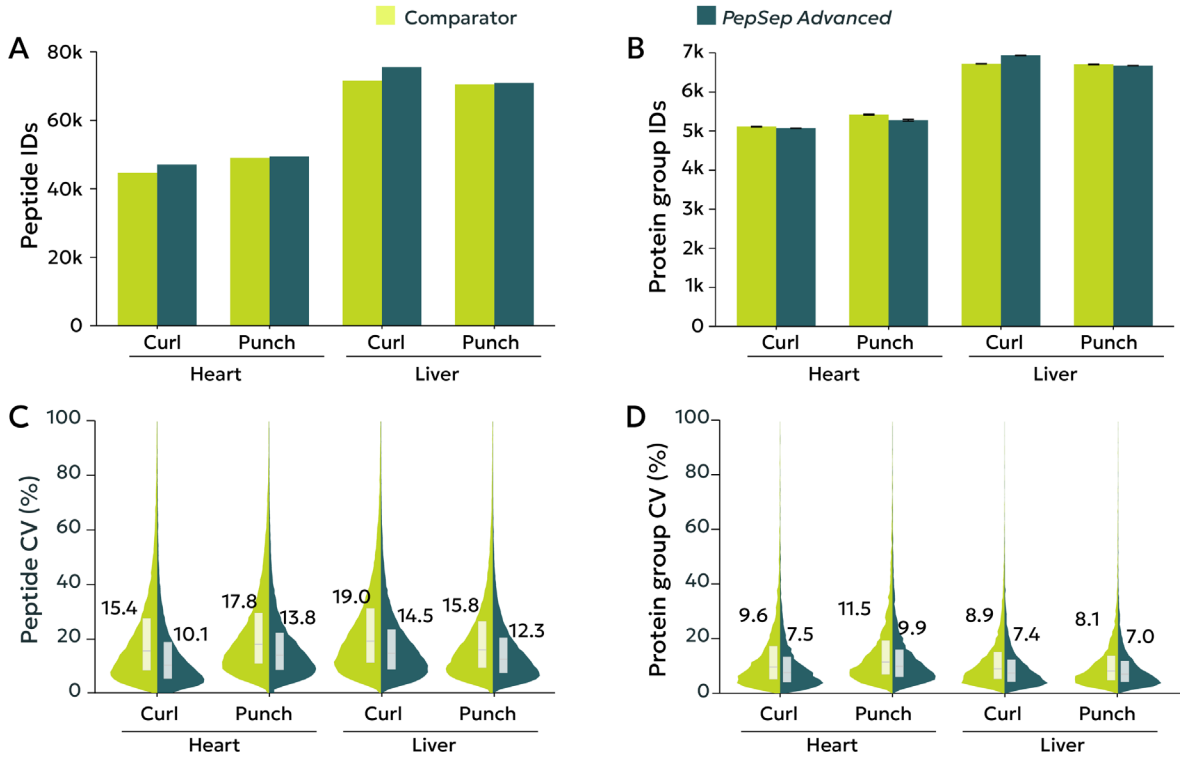


Figure 3. Workflow performance across FFPE sampling formats and column formats. (A) Peptides and (B) protein groups identified in heart and liver FFPE curl and punch preparations, and the corresponding (C) peptide and (D) protein group coefficient of variation (CV) distributions. Colors indicate the LC column used: *PepSep Advanced* (dark green) and comparator (light green).

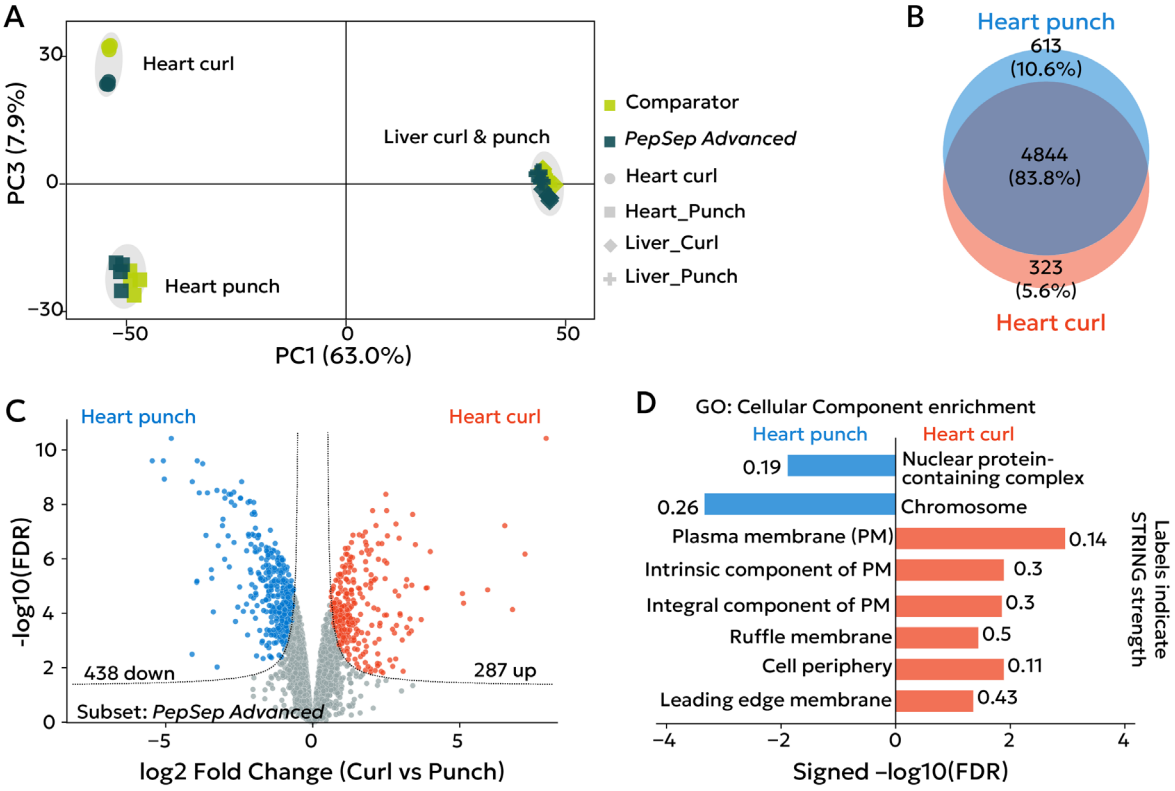


Figure 4. Sampling-format-dependent differences in FFPE heart tissue. (A) Protein-level principal component analysis (PCA) based on quantified protein abundances from FFPE heart and liver samples prepared as curls or punches and analyzed using two LC column types. Colors indicate the LC column used: *PepSep Advanced* (dark green) and comparator (light green). (B) Protein overlap between heart curl and punch preparations. (C) Differential abundance analysis comparing proteins quantified in heart punch and curl samples. (D) STRING-DB Cellular Component enrichment analysis of preparation-associated proteins. Panels B–D are based on the *PepSep Advanced* dataset.

PepSep Advanced provides comparable depth and improved quantitative reproducibility

Across all tissues and sampling formats, *PepSep Advanced* achieved peptide and protein group identification numbers comparable to those obtained with the comparator LC column, with only minor differences in peptide identifications (Figure 3A,B). Notably, *PepSep Advanced* consistently yielded lower median peptide and protein-group CVs. At the peptide level, median CVs ranged from 10.1–14.5% with *PepSep Advanced* compared with 15.4–19.0% for the comparator (Figure 3C). These results demonstrate that *PepSep Advanced* provides comparable proteome depth while delivering improved quantitative reproducibility.

Column-dependent differences at the peptide level

To assess column-dependent differences, peptide profiles obtained with *PepSep Advanced* and the comparator LC column were compared. Peptide-level PCA showed clear separation by tissue type as the primary source of variance, followed by consistent secondary separation between the *PepSep Advanced* and comparator LC columns within each tissue group (Figure 5A). To characterize differences in peptide coverage in greater detail, liver punch samples were selected for overlap and hydrophobicity analyses.

Of the combined peptide set, 70,017 peptides were shared between the two LC columns, representing 72% of detected peptides. *PepSep Advanced* contributed 15,320 exclusively detected peptides (16%), while 11,186 peptides (12%) were exclusively detected with the comparator (Figure 5B).

Column-exclusive peptide populations showed distinct Kyte-Doolittle hydrophobicity distributions (Figure 5C), with *PepSep Advanced*-exclusive peptides displaying slightly higher hydrophobicity and comparator-exclusive peptides tending to be more hydrophilic, suggesting modest differences in peptide selectivity between the two LC columns.

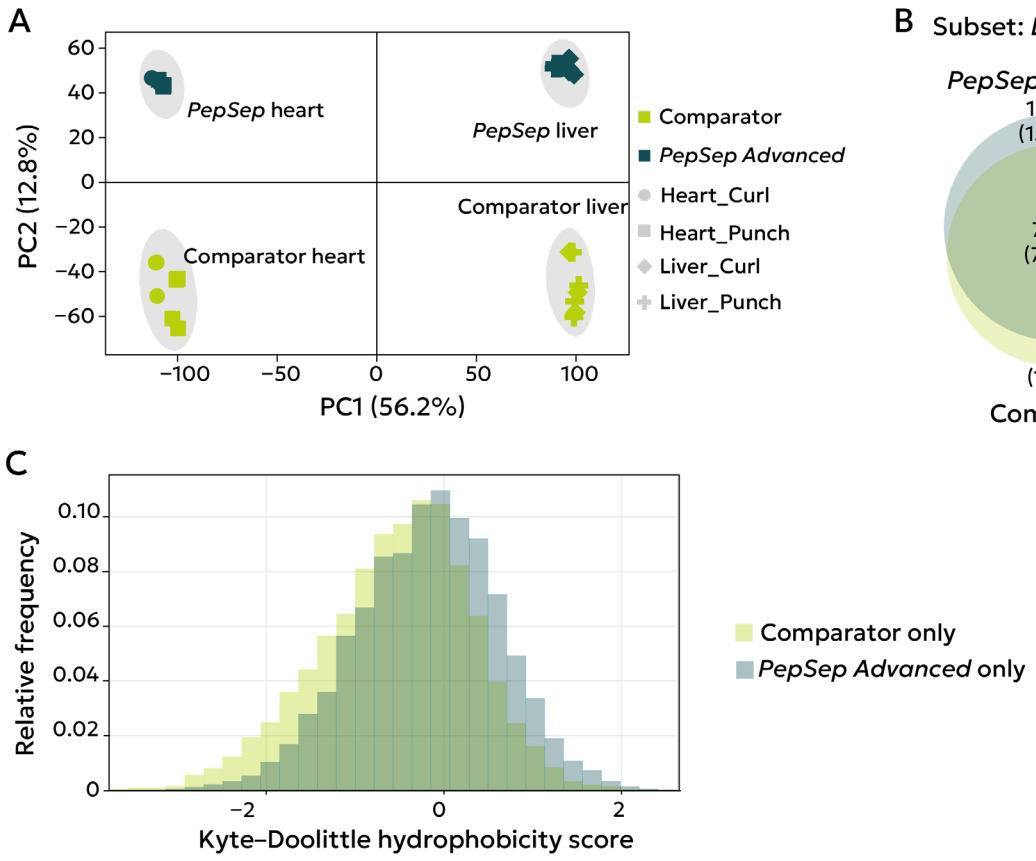


Figure 5. Complementary peptide detection between LC columns. (A) Peptide-level PCA showing LC-columns-dependent separation (PC2), *PepSep Advanced* or comparator, respectively. (B) Peptide overlap in liver punch samples. (C) Kyte–Doolittle hydrophobicity distribution of LC columns-exclusive peptides. Panels B and C are based on the liver punch dataset.

Conclusions

The *BeatBox Tissue Kit 24x* extends the established *BeatBox–iST* FFPE workflow to higher tissue inputs and multiple FFPE sampling formats. Increasing liver input from two to five curls increased total protein yield while maintaining consistent proteome depth, with both curls and punches enabling robust protein and peptide identification.

Differences between curl and punch preparations were tissue dependent: heart samples showed distinct protein profiles and subcellular enrichment patterns, whereas liver samples remained closely comparable. This suggests that tissue architecture and spatial heterogeneity may influence the proteomic composition captured by different FFPE sampling formats.

Across all evaluated conditions, *PepSep Advanced* provided lower quantitative variability than the comparator LC column while maintaining comparable proteome coverage. At the peptide level, the two LC columns showed largely overlapping coverage along with complementary peptide subsets.

Overall, the workflow combining *BeatBox* homogenization, *iST* sample preparation, *PepSep Advanced* separation, and *Spectronaut* analysis provides a flexible and reproducible approach for in-depth proteomic analysis across diverse FFPE sample formats and input amounts.

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Product	Manufacturer	Product Code
BeatBox tissue homogenizer	PreOmics GmbH	P.O.00144
BeatBox Tissue Kit 96x	PreOmics GmbH	P.O.00121
BeatBox Tissue Kit 24x	PreOmics GmbH	P.O.00128
iST 8x	PreOmics GmbH	P.O.00001
iST 96x	PreOmics GmbH	P.O.00027
PepSep Advanced 25 cm x 75 µm x 1,5 µm	Bruker	1919826
CaptiveSpray 2 Emitter 10 µm, Pack 2x	Bruker	1811112
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