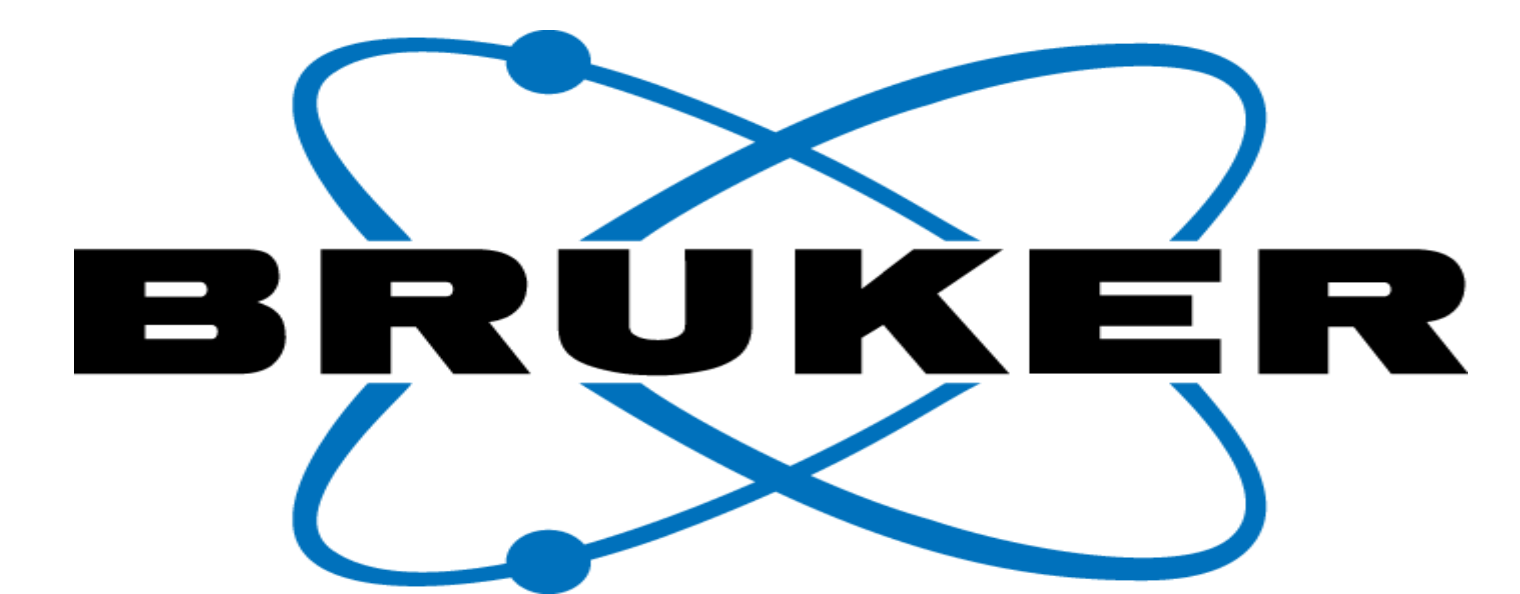


# Real-time search of Ubiquitin diGLY modified peptides and PaSER acquisition control on the timsTOF PRO



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## Introduction

Recently we introduced PaSER (Parallel Search Engine in Real-time) which performs a database search in parallel with the 120Hz sequencing speed of the timsTOF Pro mass spectrometer. The PaSER box sits adjacent to any timsTOF Pro or flex instrument. Biological processes are influenced by the PTM state of proteins, one such PTM (ubiquitination) drives protein catabolism. Phosphorylation is a critical signaling molecule acting at the time scale of seconds. Here we show PaSER has the computational power to real time assign ubiquitinated peptides by identification of the remnant GlyGly tag on lysine residues. The search engine is fast enough to perform variable modification searches (Ox-M, Deam-NQ, Ubq-K) under semi-tryptic search conditions allowing for 3 miscleavages and multiple modifications per peptide. Commonly, these search parameters come at a high computational time and costs.

## Methods

A nanoElute (Bruker Daltonics) nano-flow LC was coupled to a high-resolution TIMS-QTOF (timsTOF Pro, Bruker Daltonics) with a CaptiveSpray ion source (Bruker Daltonics). The peptide mixtures (< 200 ng) were loaded onto a 150 mm pulled emitter column (IonOpticks). Chromatographic separation was carried out using a linear gradient of 2-35% buffer B (100% ACN and 0.1% FA) at a flow rate of 500 nl/min over 21 min. The sample was a di-GLY enriched lysate using a modified protocol of the K-ε-GG kit from Cell Signaling Technologies and a QC sample of K562. Using a "if then that" logic, a user defined metric (number of protein or peptide IDs) is used to inform the Hystar (Bruker) sequence acquisition software as connected to PaSER.



**Figure 2: PaSER real-time ID of 50 samples per day Ubq enriched mouse liver:** 21 minute Base-peak chromatograms of 3 biological replicates of Ubq enriched mouse liver. Real-time database searches are performed by PaSER with non-specific enzyme settings and multiple variable modifications. Deep proteome coverage and high enrichment efficiency using CST Ubq-scan kit.

## Results

PaSER combined with low sample loads and short gradients (21 min.) resulted in deep ubiquitination coverage. Ubiquitin enrichments varied from tissue to tissue with liver showing the highest degree by percentage. PaSER showed the ability to perform strenuous searches even at very difficult search parameters keeping up with the 120Hz sequencing speed of the mass spectrometer.

Importantly, real-time ID by PaSER was able to distinguish between instrument and sample inconsistencies where the third biological replicate of the brain sample showed high protein and peptide identifications consistent with biological samples 1 and 2 but the number of Ubq modifications were inordinately low. This observation led to an immediate re-injection at a longer gradient where improved numbers of protein and peptide observations but a low number of Ubq modified peptides explained the enrichment was poor. PaSER and acqu-

## 21 minute LCMS 50SPD runs

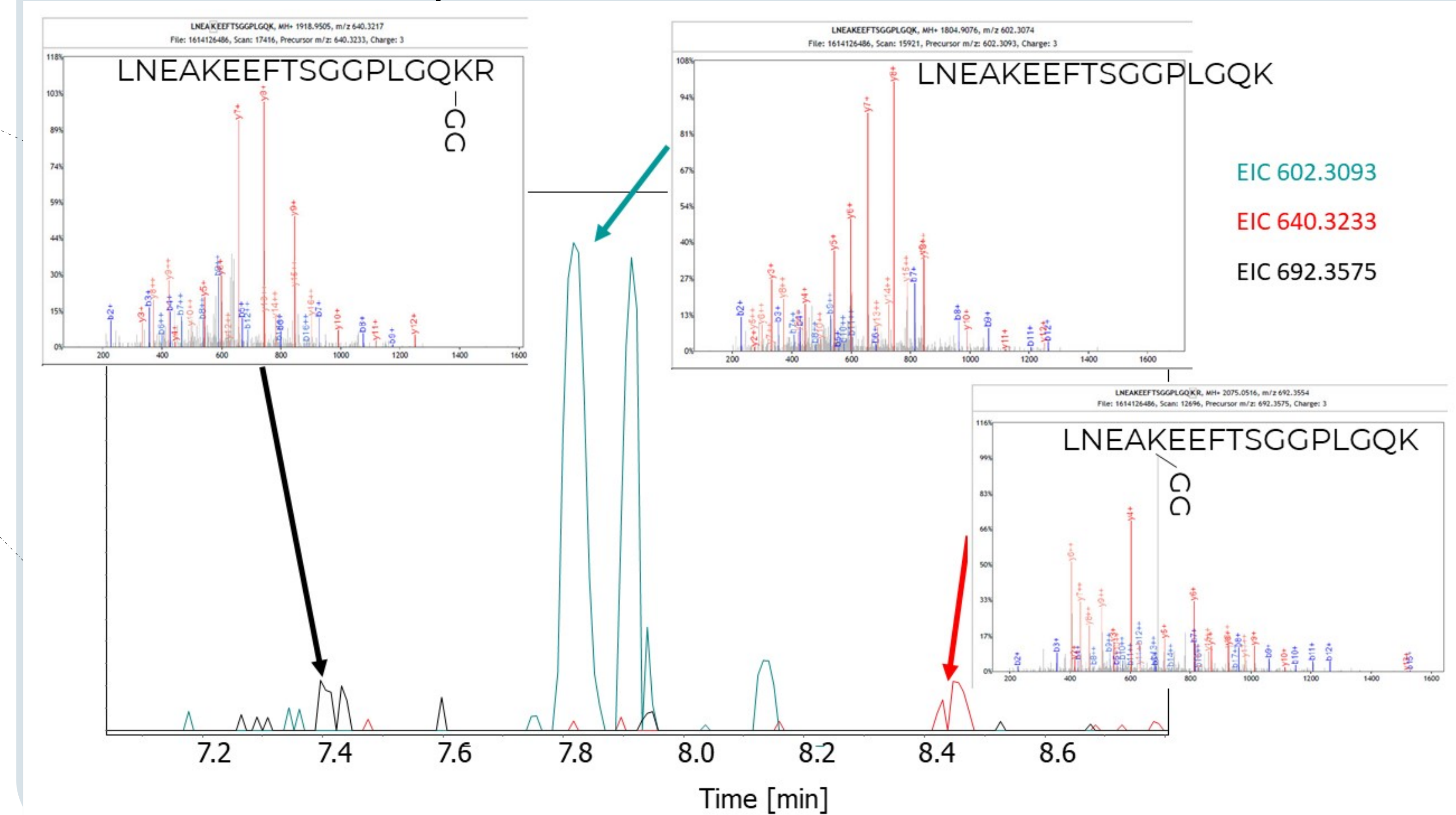
Liver	Bio Replicate	Proteins	Peptides	UBQ	% UBQ
Bio Enrichment 1	2040	11484	3792	33	
Bio Enrichment 2	2089	11939	3385	28	
Bio Enrichment 3	2239	15569	3991	26	
<b>Brain</b>					
Bio Enrichment 1	3403	22981	2222	10	
Bio Enrichment 2	3522	25486	1038	4	
Bio Enrichment 3	3565	25246	113	0	
<b>Embryo</b>					
Bio Enrichment 1	2997	16504	2080	13	
Bio Enrichment 2	3337	18960	1411	7	
Bio Enrichment 3	1361	4138	706	17	

Real-time results for acquisition control Sample or Instrument? Re-injection

Re-injection

45 min gradient  
5240 Protein  
47682 Peptides  
203 UBQ  
Sample

## LIS1 Ubq and WT state in real-time

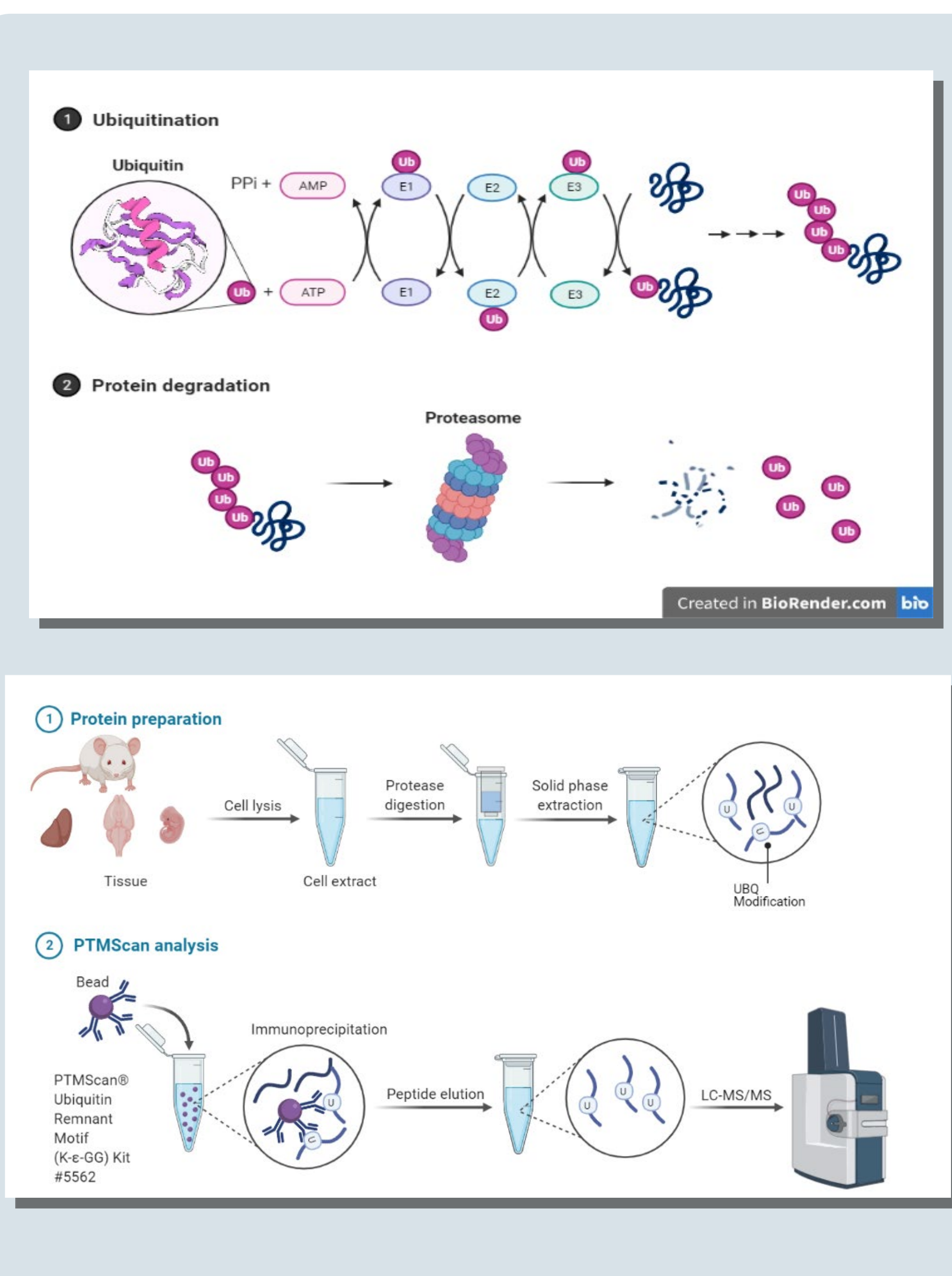


**Figure 3: High-throughput combined with real-time search for difficult PTM analysis:** Three tissue types including liver, brain and embryo from mouse were prepared as explained in Figure 1. Peptide and protein identification in real-time for each tissue type and biological replicates where the PTM findings can inform if the sample or instrument is at fault in real-time is demonstrated. LIS1, an important player in the regulation of Dynein and the Ubq states as identified and viewed by PaSER spectra in real-time. Ubq modifications at K76 and K88 are displayed, in addition to the non-modified form of the peptide. Relative quantitative information is derived by these data.

sition control can aid in saving precious samples, instrument time and overhead. PaSER, even in the most difficult search parameters offers uncompromised real-time search ability as demonstrated with Ubq modified peptides enriched with anti-Ubq enrichments.

## Conclusions

- Ubiquitin modified peptides are observed in real time
- Real-time search for even the most arduous PTM search parameters are capable using PaSER technology.



**Figure 1: Protein degradation driven through the proteasome and ID by state-of-the-art bottom up proteomics with Real-time search and 4D-proteomics:** Top panel describes protein ubiquitination and proteasome degradation. Bottom panel describes the sample processing including protein digestion and Ubq-scan enrichment from cell signaling technology (CST)

timsTOF Pro