
# timsTOF Pro with PASEF and Evosep One: Maximizing throughput, robustness and analytical depth for shotgun proteomics

**The timsTOF Pro powered by PASEF enables sequencing speed of > 120 Hz, high sensitivity and robustness as well as an additional dimension of separation (TIMS) for improved peak capacity**

Recently, Bruker Daltonics launched the timsTOF Pro for high speed and deep shotgun proteomics. The instrument offers a unique separation technique, Trapped Ion Mobility Separation (TIMS), and a unique acquisition method, Parallel Accumulation Serial Fragmentation (PASEF). TIMS and PASEF are exclusive to the timsTOF Pro mass spectrometer. The Evosep One is a conceptionally new HPLC, aimed at making clinical proteomics research faster and more robust. High throughput with short analytical gradients requires a very robust instrument with high sequencing speed and sensitivity, which are all inherent features of the timsTOF Pro mass spectrometer. In this application note, we demonstrate the performance of the timsTOF Pro mass spectrometer (Bruker Daltonics) in combination with the Evosep One HPLC system (Evosep) using short gradients of 5.6, 11.5, and 21 minutes. More than 1400 protein groups from 50 ng of HeLa digest were identified with the 5.6 min gradient. Increasing the gradient length to 21 min, resulted in the

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identification of more than 2800 HeLa protein groups, all with outstanding robustness and reproducibility. With short gradients and the chromatographic performance of the Evosep One, peaks are focused to ~ 2 seconds and these results are obtained with only 50 ng of digest sample injected on column. Small sample loads further contribute to the robustness of the timsTOF Pro/Evosep One coupling. The results presented here demonstrate combining the timsTOF Pro and the Evosep One, for the first time enables high-throughput measurements while maintaining analytical depth.

## Introduction

Depth of coverage in shotgun proteomics workflows is usually achieved by high resolution nano-LC peptide separation coupled to ESI-MS/MS. This technology is one of the most powerful strategies to search for and validate candidate biomarker panels in clinical research. While good analytical depth is currently achievable, reaching the throughput required to analyze hundreds or even thousands of samples needed to obtain sufficient statistical power for biomarker validation studies is unachievable with traditional nano-ESI LC-MS/MS systems. Conventional nano-LC systems suffer from long cycle times due to long loading times, nanoflow scale and the use of long gradients run on long columns. Additionally, the high backpressure in nano-HPLC systems sacrifices robustness and the suitability for the analysis of large sample cohorts. While the Evosep One minimizes overhead-time and thereby allows efficient use of short gradients, and improves robustness by minimizing the use of high pressure pumps and the use of disposable precolumn, it requires the use of a high resolution mass spectrometer equally robust and fast enough to sequence the highest proportion of eluted peptides. The timsTOF Pro, featuring trapped ion mobility spectrometry (TIMS) offers additional separation power which increases the peak capacity and for the first time simultaneously increases sensitivity and duty cycle. Fast quadrupole switching and the powerful Parallel Accumulation Serial Fragmentation (PASEF) method (Meier et al., JPR 2015 PMID: 26538118) provides very high sequencing speeds and allows the selection of 100 – 350 precursors per second. The orthogonal ion optics of the timsTOF Pro, together with its very high sensitivity requiring less sample to be injected, minimizes the contamination of the mass spectrometer, and results in a system that provides stable and reproducible results over several months without the need for in-depth cleaning of the system. Synergistically, the high chromatographic turnover rates of the Evosep One together with the speed and robustness of the timsTOF Pro achieve high depth at high throughput with applications for biomarker validation and research in clinical proteomics with very large sample cohorts.

## Results and Discussion

HeLa peptide digests (50 ng) were separated on an Evosep One system using very short gradients of 5.6, 11.5, or 21 minutes, which would correspond to 200, 100 or 60 sample measurements per day, respectively and analyzed on a high-resolution timsTOF Pro instrument. When using

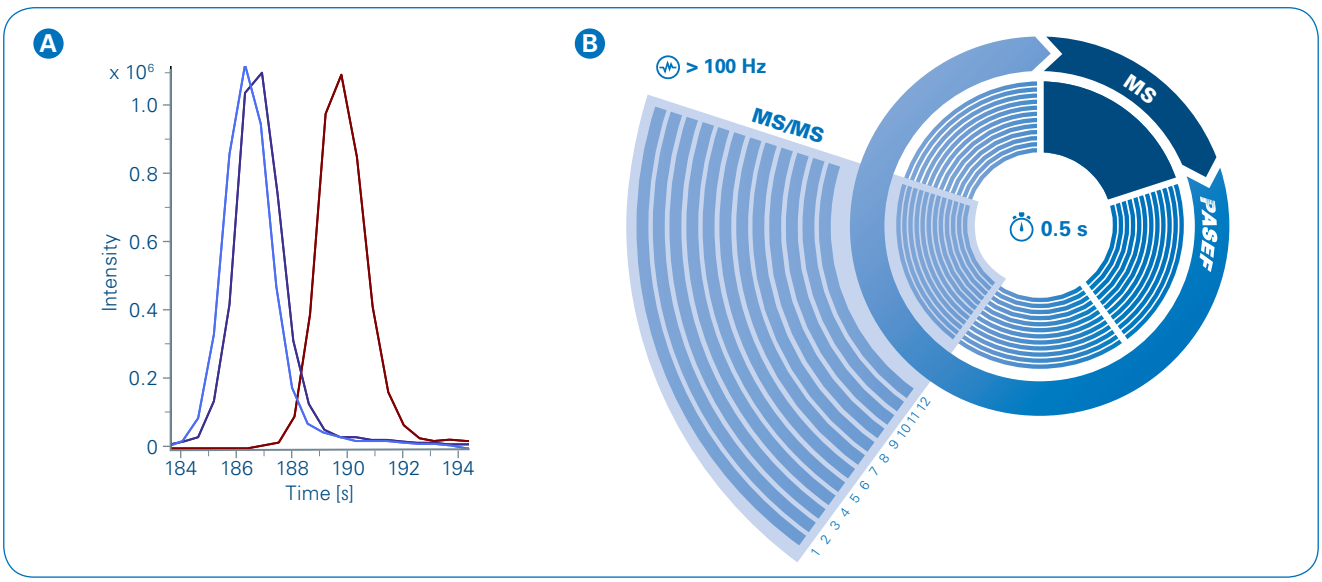


*Figure 1: High sequencing speed for short gradients provided by PASEF. (A) Extracted ion chromatograms of the same peptide in three technical replicates with similar elution time and high quantitative reproducibility from 5.6 min gradients. Using a 5.6 min gradient, peptides typically elute in ~2 s timespan (FWHM), illustrating the need for an instrument with high sequencing speed. (B) PASEF method with 0.5 s cycle time enables acquisition of 100 MS/MS spectra/second while maintaining sufficient full MS spectra for accurate quantification.*

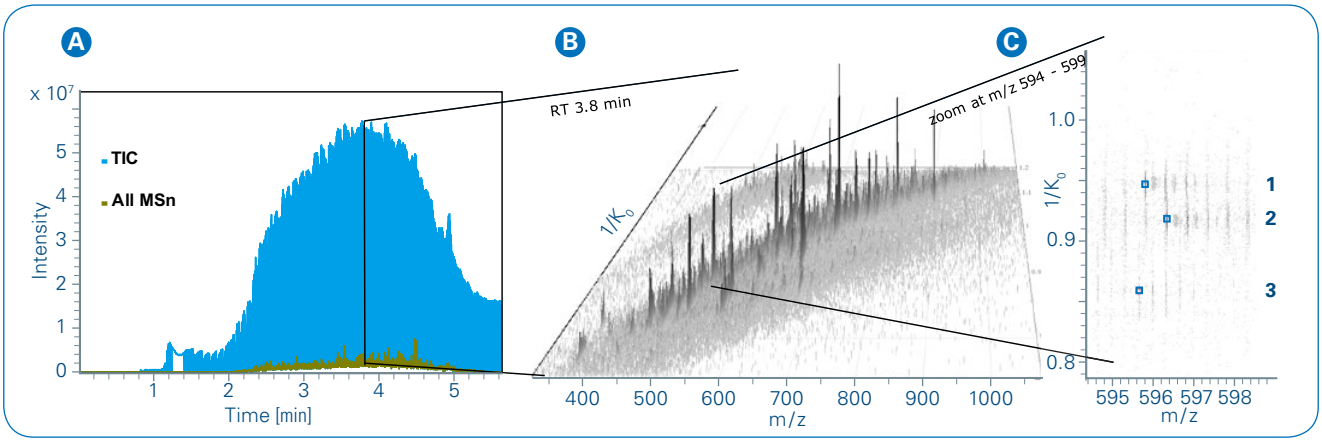


*Figure 2: Trapped ion mobility spectrometry (TIMS) increases peak capacity and produces cleaner MS/MS spectra.* **A** *Total Ion Chromatogram (TIC) from a 5.6 min gradient. With such short gradients the number of eluting peptides at any given timepoint is extremely high. TIMS provides an additional dimension of separation (space to charge (Ω/z), where Ω is the ion collisional cross section (CCS)) in addition to retention time and m/z and thereby increases the peak capacity in 3D space of RT, Ω/z and m/z.* **B** *3D view of an TIMS MS scan at RT 3.8 min, illustrating partial orthogonality of Ω/z and m/z and also the high complexity of the sample at a specific RT.* **C** *Separation of three co-eluting peptides by ion mobility in a narrow m/z range (~1Da). PASEF enables each peptide to be selected and fragmentated separately, something which is not possible with mass spectrometry alone.*

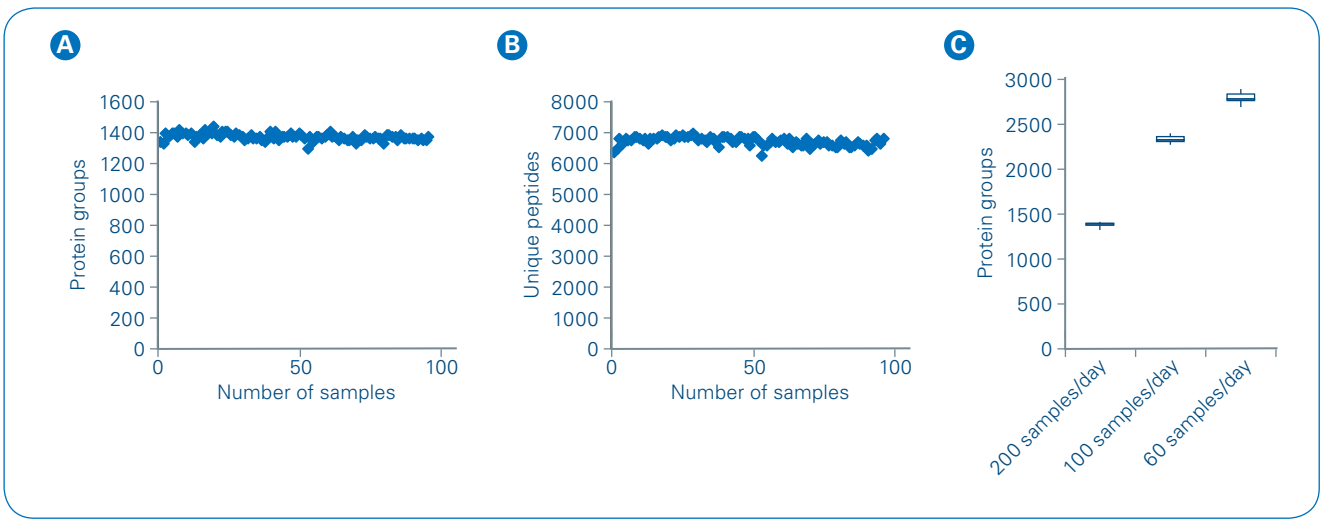


*Figure 3: Protein identifications and reproducibility of 50 ng of HeLa digest on an Evosep One system.* **A** *Approximately 1400 protein groups were reproducibly identified with a method allowing acquisition of 200 samples/day (5.6 min gradient).* **B** *Unique peptide identifications from the 200 samples/day method.* **C** *Reproducible identification of 1400 protein groups (200 samples/day, 5.6 min gradient), 2300 protein groups (100 samples/day, 11.5 min gradient) and 2800 protein groups (60 samples/day, 21 min gradient). All measurements were performed with at least 15 replicates.*

such short gradients peptides elute in a short time span of ~ 2 s (FWHM) (Figure 1A), illustrating the need for an instrument with high sequencing speed. The PASEF method which is exclusive to the timsTOF Pro instrument provides an extremely high sequencing speed. In a traditional shotgun proteomics experiment using a 90 min gradient, a cycle time of 1.1 s is used where 1 TIMS scan (100 ms) and 10 PASEF scans (100 ms each) are standard. With the very short elution time of ~ 2 s the PASEF method was modified for the fast eluting peptide features (Figure 1B). Cycle time was set to 0.5 s (1 TIMS MS scan and 4 PASEF MS/MS scans) which enables acquisition of > 100 MS/MS spectra/second while acquiring sufficient full TIMS MS scan for accurate label free quantification. Peptide and protein IDs increased with the 4 PASEF scan method when compared to the standard 10 PASEF scan method. In a standard PASEF scan method, the same peptide can be targeted for MS/MS up to 10 times to increase sensitivity by summed ion intensities. The new method targets the same precursor up to 4 times, which is more time efficient, given that there are 20 times more features eluting at a given time. From extracted ion chromatograms (EICs) of the same peptide in technical triplicates it is apparent that peptides have similar elution time and quantitative reproducibility in the 5.6 minute

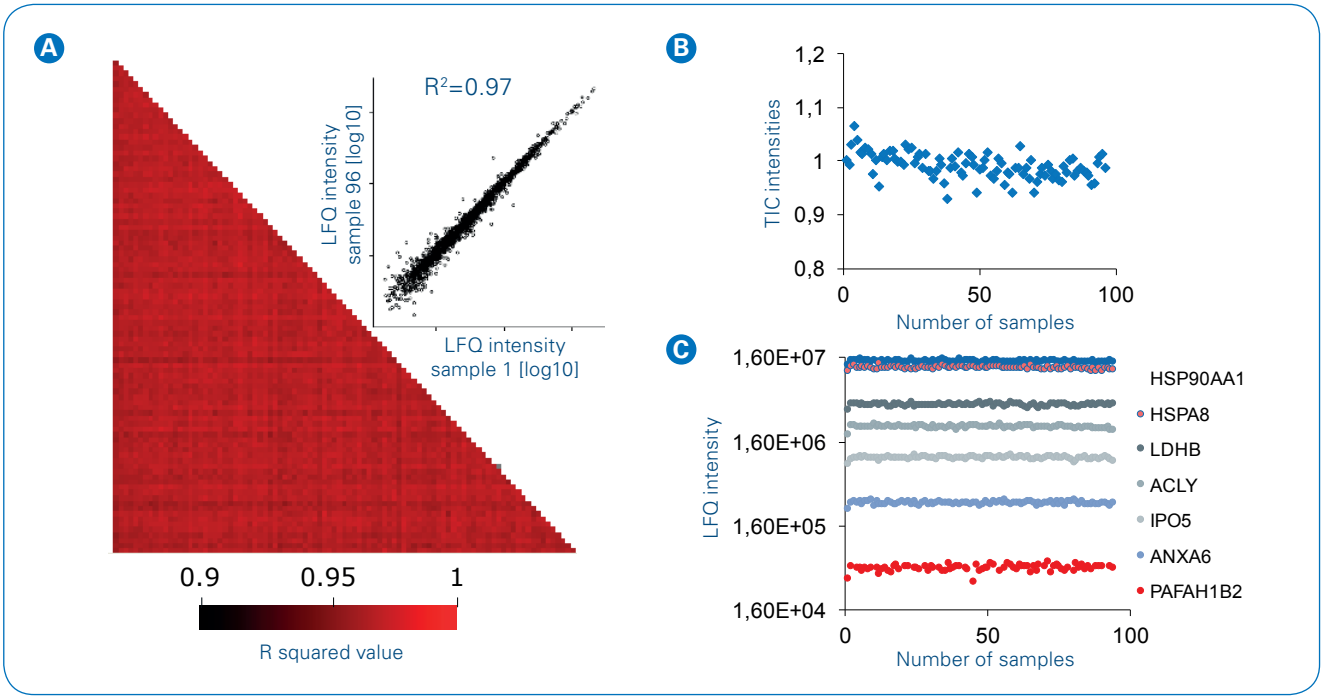


*Figure 4: Reproducible protein quantification across 96 samples of 50 ng HeLa digest. A $R^2$ values of around 0.97 across all 96 runs. B Reproducible Total Ion Chromatogram (TIC) intensities over 96 runs with a coefficient of variation (CV) of 2.3%. C Dynamic range of 3 orders of magnitude with 7 proteins selected as an example.*

gradients (Figure 1A). The number of eluting peptides at any given point in time is extremely high using short gradients (Figure 2A). The TIMS provides an additional dimension of separation (size to charge [Ω/z], Ω being the ion collisional cross section [CCS]) in addition to retention time and m/z and thereby increases the peak capacity in the 3D space of RT, Ω/z and m/z. A 3D view of an TIMS MS scan illustrates partial orthogonality of Ω/z and m/z (Figure 2B). Separation of the three peptides shown in Figure 2C using both ion mobility and quadrupole isolation produces clean MS/MS spectra which could not be obtained by isolation by m/z alone with a quadrupole. With the 5.6 min gradient and 50 ng of material more than 1400 protein groups and 7000 unique peptides were identified in 96 consecutive runs without using the match between runs functionality (Figure 3A, B). These short gradients enable the analysis of 200 injections of 50 ng HeLa with >90% MS utilization, identifying at least 1,400,000 peptides and 280,000 proteins per day. To get deeper proteome coverage longer gradients of 11.5 and 21 minutes were used. With the 11.5 min gradient more than 2300 protein groups could be identified and doubling the gradient length to 21 min more than 2800 protein groups could be identified (Figure 3C). Small error bars of the 15 measurements for the different gradient lengths indicates excellent reproducibility. Hence, the choice of predefiend LC method must reflect the sample complexity, the desired analytical depth and sample throughput. Quantitative data show $R^2$ values of around 0.97 across all 96 runs (Figure 4A) and reproducible total ion chromatogram (TIC) intensities with a coefficient of variation (CV) of 2.3% (Figure 4B). Reproducible label free quantification over a dynamic range of 3 orders of magnitude can be obtained using very short gradients of 5.6 min (Figure 4C). In the example shown here several HeLa proteins were selected to show the reproducible quantification of HSP90AA1, representing a very high abundant protein and PAFAH1B2 as an example of the low abundance.

## Experimental

A complex peptide mixture derived from a human cancer cell line (HeLa) was diluted with 0.1% formic acid in water to a concentration of 50 ng/µL. The Evosep One system (Evosep) (Figure 5A) was coupled to the timsTOF Pro mass spectrometer (Bruker Daltonics) (Figure 5 B). The Evosep One HPLC is a conceptually novel chromatography system that dramatically increases robustness and sample throughput while maintaining the sensitivity of current nano-flow LC instruments. Four low-pressure pumps run in parallel at high flow to form a gradient that sequentially elutes analytes from a disposable Evotip (Figure 5A (I)). The initial gradient containing embedded analytes is created by pump A and B, pumps C and D then modify the gradient after the elution from Evotip. This creates a gradient offset which ensures optimal focusing and maximal chromatographic performance of the analytical column. The preformed and offset gradient with the embedded and

separated peptides are first stored in a holding loop that is subsequently switched in-line with the analytical column (Figure 5A (II)). A 4 cm column was used for the 200 samples/day method and 8 cm for the 100 and 60 samples/day method. It is pumped out by a single high-pressure pump with a constant flow rate. This configuration also ensures a very low overhead time of only 2-3 minutes per sample and the virtual absence of cross contamination for crude biological samples such as body fluids. In the timsTOF Pro mass spectrometer ions are generated in an electrospray source, transferred into the vacuum system through a glass capillary and then deflected by 90° into the TIMS device where ions are accumulated and released from the device based on their size-to-charge ratio. The orthogonal deflection of ions dramatically increases robustness of the mass spectrometer while the TIMS device increases the sensitivity making measurements of low sample amounts possible. The quadruple switches mass position extremely quickly in sync with the elution time of the precursor ion packages from the TIMS device, isolating the precursors for subsequent fragmentation in the collision cell. This Parallel Accumulation Serial Fragmentation (PASEF) method significantly increases the sequencing speed to > 120 Hz and therefore is ideally suited to short gradient measurements. Data collected over a m/z range of 100 to 1700 for MS and MS/MS on the timsTOF Pro instrument using an accumulation time and ramp time of 100 ms. Data were analyzed using DataAnalysis (Bruker Daltonics) and PEAKS studio (Bioinformatics Solutions Inc.).

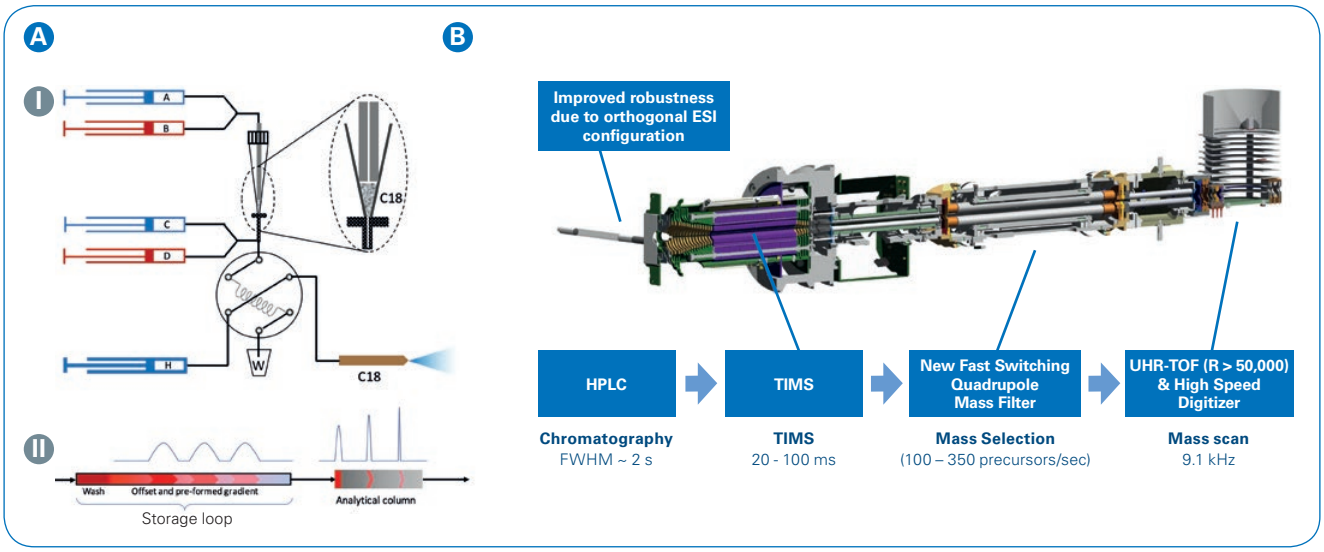


*Figure 5: Evosep One and timsTOF Pro.* **A** *Illustration of the Evosep One separation principle. Peptides are eluted from Evotips with low pressure pumps (A/B) by increasing concentrations of the eluent B (ACN). Peptides are then diluted with low pressure pumps (C/D) and transferred to a storage loop. Pre-separated peptides are further separated on a short analytical column to achieve higher chromatographic peak capacity.* **B** *timsTOF Pro with trapped ion mobility spectrometry (TIMS) further increases peak capacity. Fast quadrupole switching enables fragmentation of 100 – 350 precursors/sec in PASEF mode to manage the high complexity on short gradients.*

## Conclusion

Increased peak capacity via trapped ion mobility spectrometry as coupled to TOF MS and novel chromatographic separation technologies allows the analysis of 200 samples/day, at formidable analytical depth, with unparalleled robustness. High sample throughput results in higher statistical power, it also challenges the mass spectrometer with narrower (< 2 s) chromatographic peaks and an overall increase in the complexity of the eluted sample. The increased speed and peak capacity of the timsTOF Pro allows reproducible identification of 1400 protein groups from a 50 ng HeLa cell digest and a 5.6 minute gradient.

Reproducible quantification with $R^2 > 0.90$ over a dynamic range of 3 orders of magnitude was achieved. Moreover, with increased sensitivity and lower sample loads the timsTOF Pro is designed to maintain robust performance over large sample cohorts, representing the optimal solution for proteomics research.


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