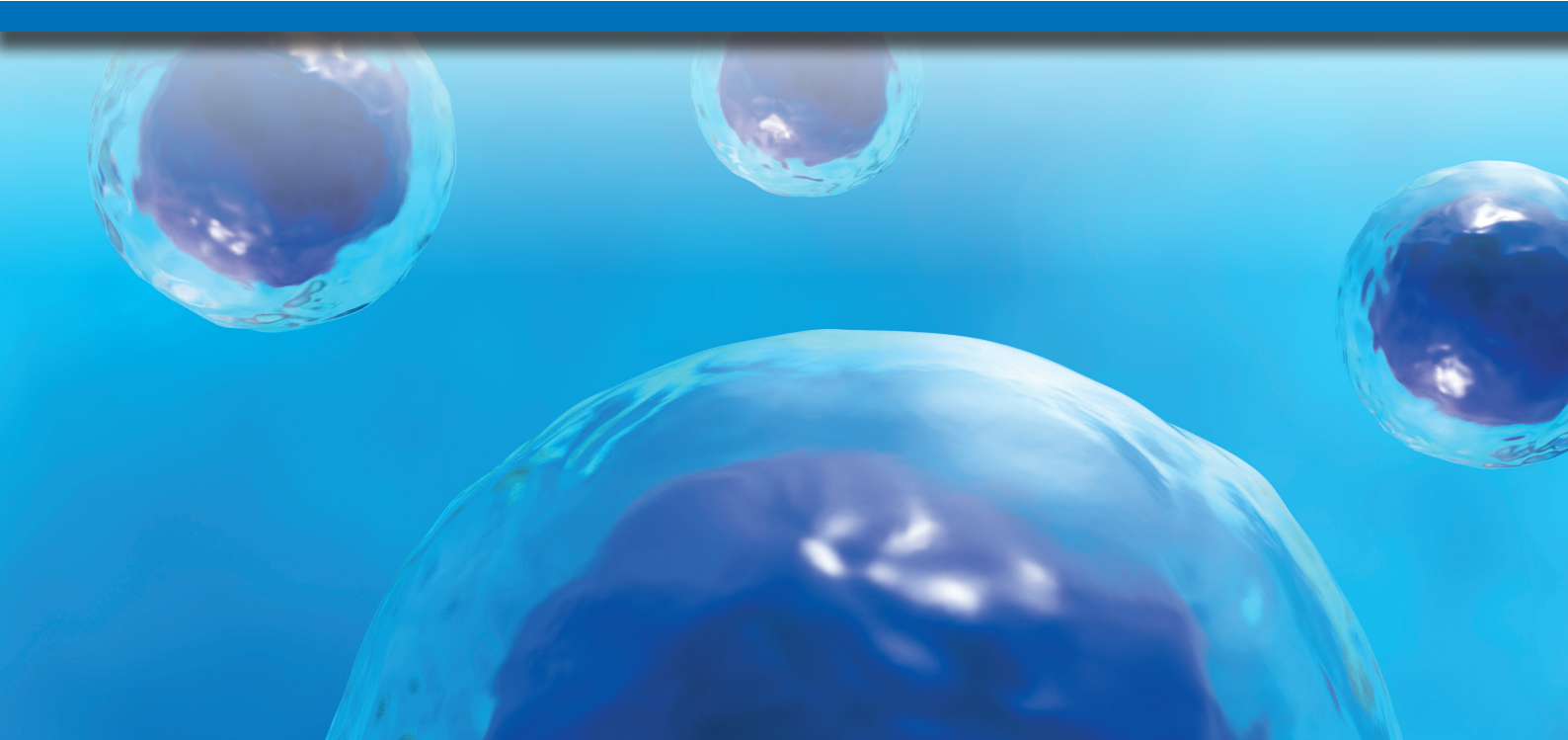


An abstract background image featuring several translucent, glowing spheres in shades of blue and purple, set against a gradient blue background. The spheres have a textured, almost crystalline appearance with internal light patterns.

● Short gradients with Bruker's nanoElute coupled to the timsTOF Pro with PASEF — Throughput and deep proteome measurements

The nanoElute optimized for high throughput in chromatographic separation together with the timsTOF Pro powered by PASEF and the additional dimension of separation (TIMS) for improved peak capacity and analytical depth on measurements of 50 runs/day.

High sample throughput in proteomics, like that in genomics, is highly desirable. Moreover, the highest analytical depth in proteomics is achieved on fractionated samples, requiring subsequent analysis of the individual fractions with short

gradients to achieve reasonable overall measurement time per sample. The timsTOF Pro with trapped ion mobility spectrometry (TIMS) offers additional separation power and increased peak capacity over instruments without trapped ion mobility separations.

TIMS enables the powerful Parallel Accumulation Serial Fragmentation (PASEF) method [1,2] for very high sequencing speed that is perfectly suited for proteome analysis on short gradients. We have optimized MS conditions, column

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lengths and LC overhead times to obtain runs of 28.8 min injection to injection (allowing for the measurement of up to 50 samples/day) on the nanoElute (Bruker Scientific). TIMS gas phase separation for high peak capacity and fast acquisition allows identification of more than 4000 protein groups from 250 ng of a proteolytic digest of a human cancer cell line (HeLa) in 28.8 min injection to injection, enabling the analysis of large sample cohorts with a reasonable proteome depth of coverage and short measurement time. To demonstrate the ultimate analytical depth achievable with this short method, offline LC-fractionation was performed on a HeLa digest and digests of murine cerebellum, and the fractions analyzed using the short column and short LC method described here. Samples were fractionated on high pH reversed-phase columns into 24 concatenated samples. Subsequent measurement allowed the identification of more than 100,000 unique peptides and 9052 protein groups in less than 12 h of measurement time on a complex sample. We conclude that analysis on short gradients with the timsTOF Pro

provides high throughput for a large number of samples without sacrificing proteome depth. Running short gradients on the timsTOF Pro with pre-fractionated samples provides an optimized strategy for proteome wide identifications of peptide collisional cross sections (CCS) and enables expedient generation of libraries for DDA and DIA.

Introduction

Nano-LC peptide separation coupled to high resolution ESI-MS/MS is one of the most powerful strategies in proteomics and is used e.g. to search for and validate candidate biomarkers. Analytical depth is important to decipher as much of the proteome as possible and to maximize the chance of identifying biomarkers, but maximum depth usually comes at a cost of long analysis times. In proteomics, nano-LC separations are usually applied for 1-3 h to achieve reasonable analytical depth in acceptable times, and for very high depth, pre-fractionation is required. The recently introduced timsTOF Pro with its trapped ion mobility separation (TIMS) together with PASEF provides new possibilities

in terms of peak capacity and fast precursor selection in the ion mobility to m/z dimension. These features enable analytical results from 20 minute gradients that previously took more than one hour to achieve. We have optimized nano-LC cycle times by minimizing equilibration and loading times with high flow rates. Additionally, short columns lower gradient dead times, and the lower backpressure improves the robustness of the nano-HPLC systems. High chromatographic turnover with short gradients, together with the speed and robustness of the timsTOF Pro achieves unparalleled proteome depth with high throughput for applications of biomarker validation and research.

Experimental

250 ng of an in-house HeLa digest or 200 ng HeLa digest (Pierce) were delivered to a 100 mm fritted column of ID 75 μm (Bruker TEN, Bruker Daltonics) packed with ReproSil 1.9 μm C18 beads, pore diameter 120 $\text{\AA}$. To achieve a high throughput solution for shotgun proteomics, the nanoElute business logic was modified in a way that turnover times were reduced

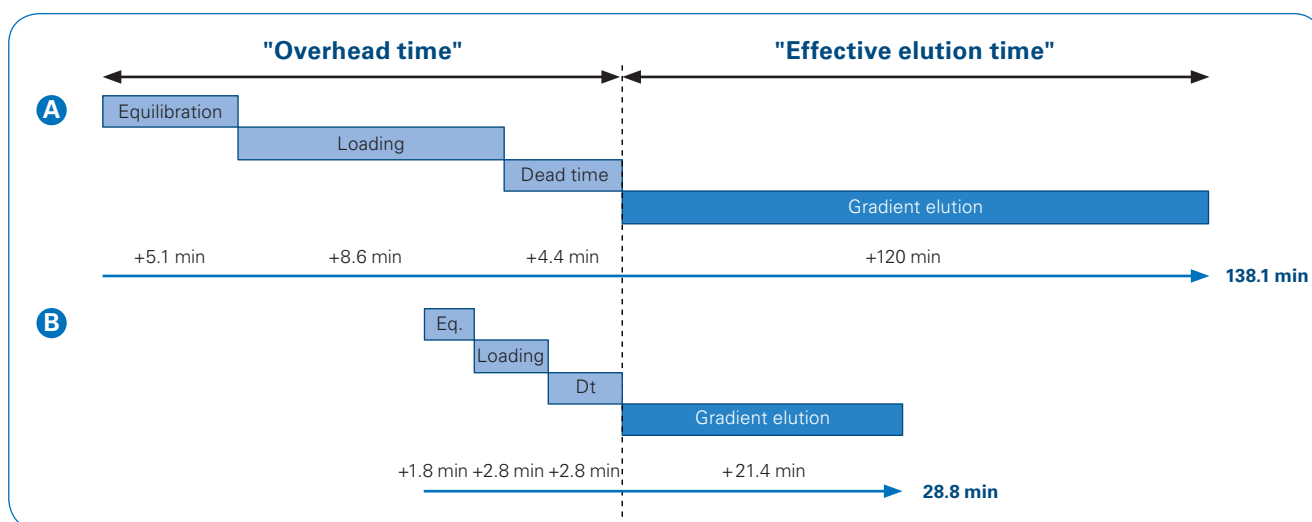


Figure 1: Reduction in overhead time for better time utilization on short nano LC-MS/MS gradients. **A** On usual 120 min LC gradients overhead times from the end of a previous run to elution of the next run take 18 min which is acceptable on long runs. **B** Overhead times were significantly reduced for short gradient elution by running at high flow rates and adjustment of equilibration steps. Reduced overhead times allow running 50 samples/day with efficient use of time for peptide elution.

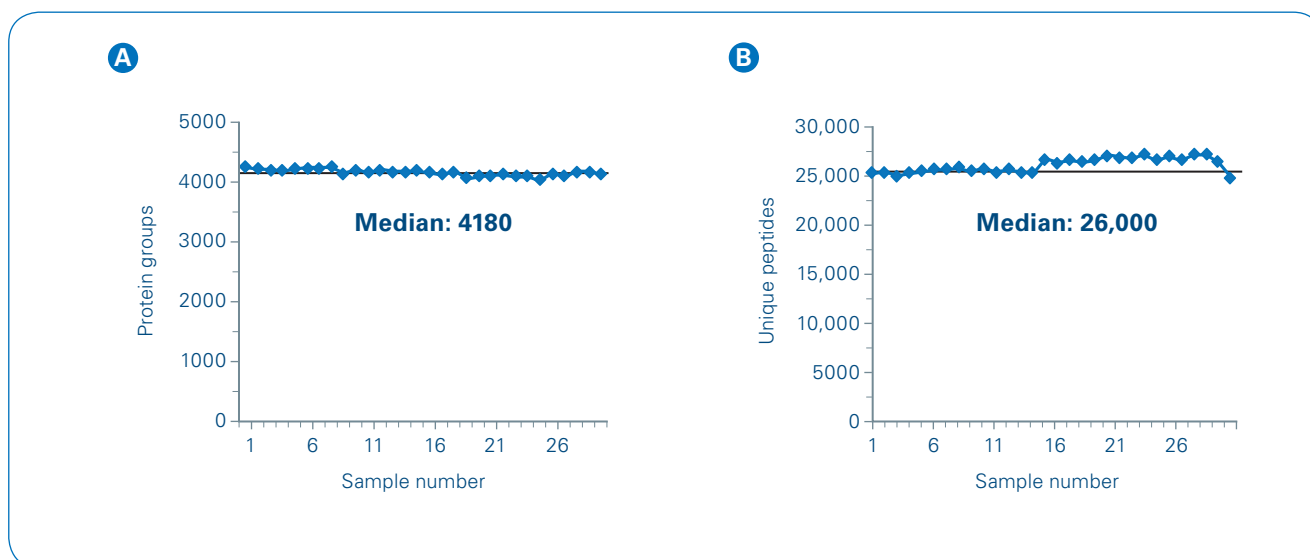


Figure 2: High reproducibility of IDs for running 250 ng tryptic in-house HeLa digest at 50 samples/day. **A** 4180 Protein identifications and **B** 26,000 unique peptide identifications across 30 consecutive runs (1% FDR).

to achieve a 21.4 min gradient time and 28.8 min inject to inject time (Figure 1). Separation was performed at 500 nl/min and loading was performed at a maximum pressure of 500 bar. This method allows up to 50 samples per day to be measured, maximizing throughput. Moreover, operation at low backpressure with short columns increases robustness of the system which means larger sample cohorts can be run without changing the nano-LC column. For offline fractionation, 25 µg of peptides from HeLa or murine cerebellum were fractionated at pH 10 on a reversed phase column

(Waters Acquity CSH C18 1.7 µm 1 × 150 mm) into 48 fractions that were concatenated into 24 fractions for subsequent analysis in 12 h of MS measurement time. In the timsTOF Pro mass spectrometer ions are generated in a captive spray source and 20 µm tapered emitter, 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 quadrupole 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 PASEF method [1,2] significantly increases the sequencing speed up to > 120 Hz and therefore is ideally suited to short gradient measurements. Data was collected over an 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. We have optimized data acquisition for short gradients with short cycle times of 0.5 s to achieve a good MS1 sampling rate for quantification on short gradients. PEAKS studio (Bioinformatics

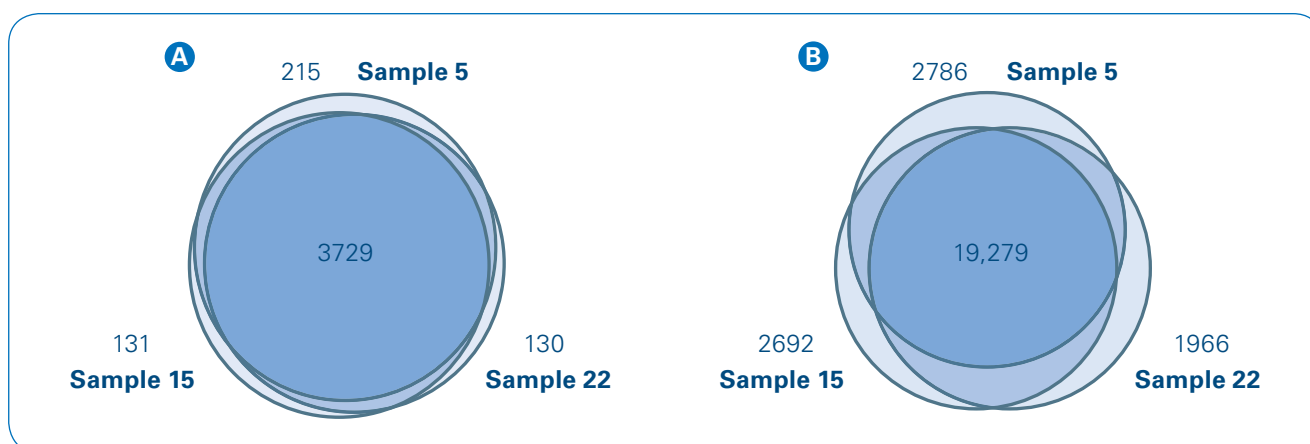


Figure 3: High reproducibility of IDs for running 250 ng tryptic in-house HeLa digest at 50 samples/day. **A** Overlap of protein IDs of three randomly selected runs and **B** Overlap of unique peptides of the same runs.

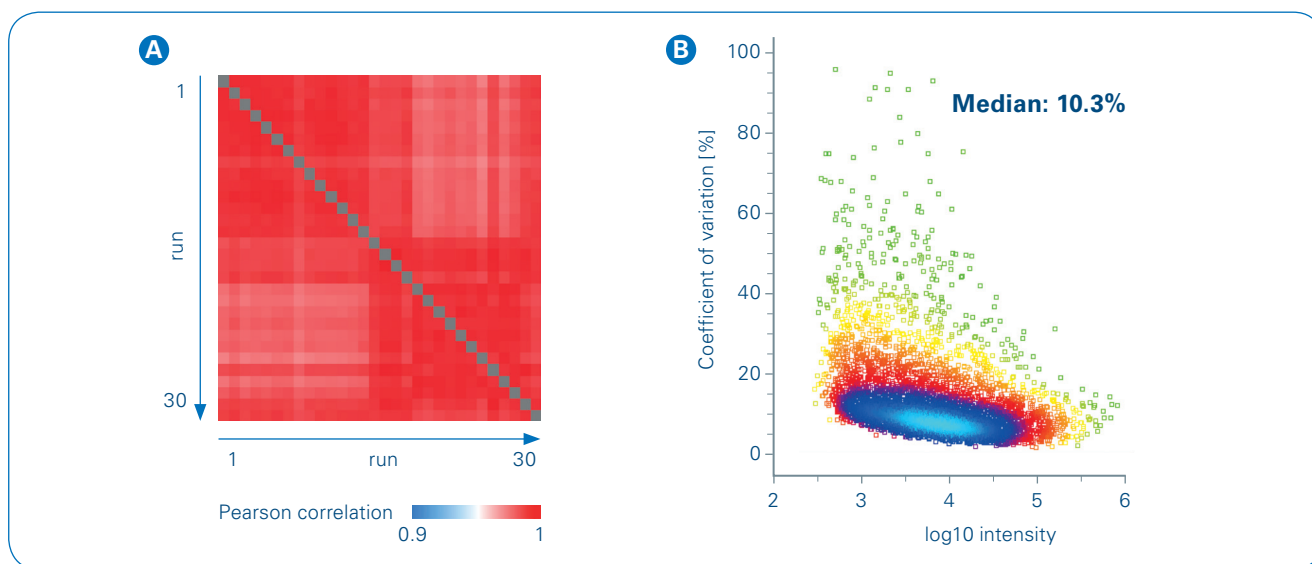


Figure 4: Quantitative reproducibility for running 50 samples/day. **A** Correlation coefficients for label-free quantification between 30 replicates. **B** Coefficient of variation across 30 replicates for each single protein quantified across all conditions.

Solutions Inc.) was used for data processing and results were corrected to 1% peptide spectrum matches false discovery rate (PSM FDR).

Results and Discussion

The high analysis speed of the timsTOF Pro and additional gas phase separation makes this instrument perfectly suited for short gradient analysis. For both, direct column loading and longer column length (25 cm), there is considerable time necessary for column equilibration, loading and elution of peptides (18.1 min) which reduces the effective measurement time of peptide elution and acquisition (Figure 1A). On short runs e.g. 20 min runs, this so called "overhead time" can easily take the same time as the gradient itself, reducing the effective gradient time to 50% of the overall method time. The modified business logic of the nanoElute reduced overhead times to achieve a 21.4 min gradient time and 28.8 min inject to inject time (Figure 1B). In looking at the protein group identifications in only 21.4 min we were able to consistently identify 4180 protein groups over 30 consecutive runs with a high ID

reproducibility (CV = 1.3%) (Figure 2A). These protein group identifications are derived from ~26,000 unique peptide identifications (CV = 2.7%, Figure 2B) which correspond to a peptide ID rate of more than 1200 unique peptide identifications per minute. To our knowledge, this is the highest peptide ID rate reported on this gradient length for proteomics and is solely possible by the execution of PASEF, QTOF speed, the high peak capacity derived from additional TIMS separation and the seamless nanoElute integration with the modified business logic. At this point it is important to add that these high ID rates can only be achieved on the in-house HeLa digest which shows a comparably high number of missed cleavages. With having more missed cleavages, more peptides can be sequenced. On 200 ng of a commercial Pierce HeLa digest, we were still able to reproducibly identify 3702 protein groups and 16423 unique peptides. The protein overlap between randomly selected runs is high (~90%), especially considering the runs are short and no match between runs is applied (Figure 3A) where the same holds true for peptides (Figure 3B). For protein quantification, good pearson correla-

tion coefficients (~ 0.97, Figure 4A) and small protein CV values (Figure 4B) are achieved. While high throughput with good depth is often very useful, some applications require deep proteome profiling (e.g. when a similar coverage to genomic approaches is desired) or for library generation for DIA approaches. We have conducted high pH reversed phase offline fractionation to investigate the proteome coverage that can be achieved in 12 h of measurement time. We were able to identify 9052 protein groups from in-house HeLa digest and could identify 10,299 protein groups in murine cerebellum (Figure 5A) using this approach. Measurement of deep proteomes provides access to generation of large peptide CCS libraries of 103,012 peptide IDs in 12 h measurement time (Figure 5B). Recently, only slightly higher peptide identifications (129,110 peptide CCS values) were achieved by investing more than 96 h of measurement time [2]. The approach described here provides an alternative to effectively use instrument time for DIA library generation and targeted analysis when these workflows are available.

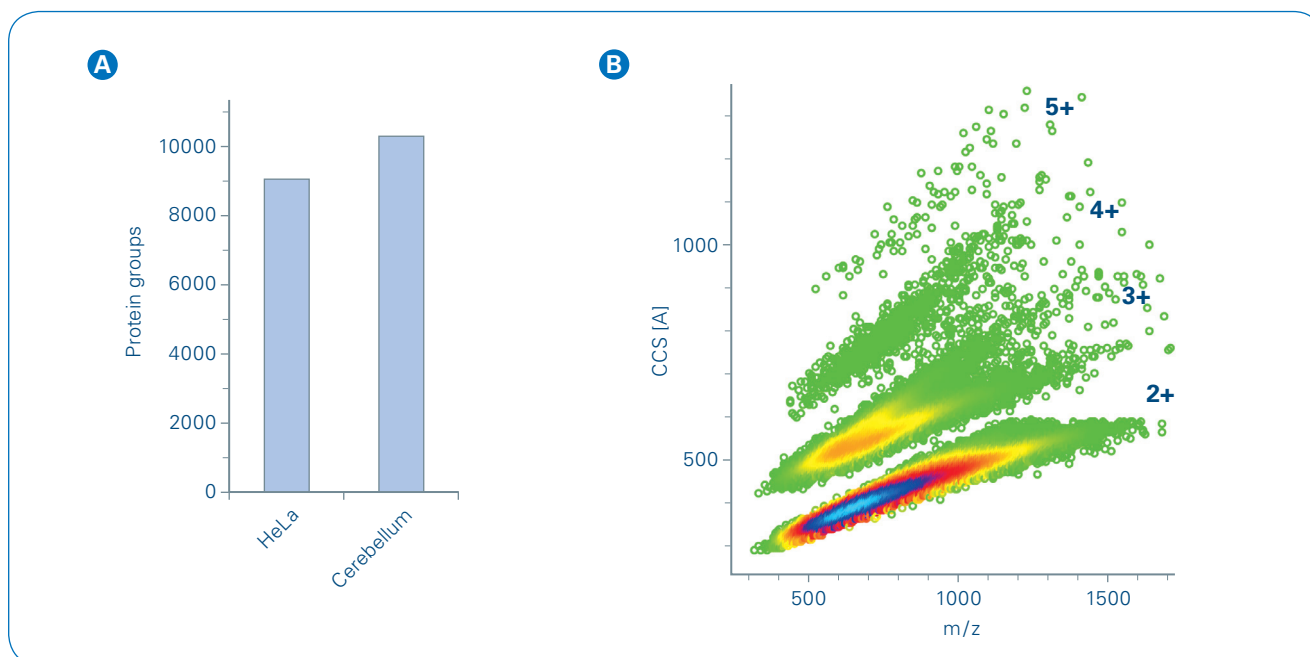


Figure 5: Deep proteome analysis on fractionated samples. **A** High pH reversed phase fractionation into 24 fractions corresponding to < 12 h measurement time and identifications of >9000 protein groups from HeLa and > 10,000 protein groups from murine cerebellum. **B** Large scale representation of 100,000 collisional cross sections from HeLa peptides.

Acknowledgements

We gratefully acknowledge the Protim Core Facility (INSERM Rennes, France) for providing the proteolytic digests of murine cerebellum.

Conclusion

- Reduced overhead times allow efficient use of acquisition times in short gradients.
- Increased peak capacity via trapped ion mobility spectrometry, coupled to TOF MS with PASEF allows the efficient use of short gradients, and provides analytical depth (> 4000 protein group IDs) that was previously only accessible on methods requiring more than an hour.
- Pre-fractionation for subsequent analysis on short gradients allows identification of more than 10,299 protein groups from murine cerebellum in 12 h of measurement time and generation of peptide libraries for DIA and DDA experiments with a depth of > 100,000 peptides and their CCS values.



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