# High sensitivity limited material proteomics empowered by data-independent acquisition on linear ion traps

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## ABSTRACT:

In recent years, the concept of cell heterogeneity in biology has gained increasing attention, concomitant with a push towards technologies capable of resolving such biological complexity at the molecular level. While RNA-based approaches have long been the method of choice, advances in mass spectrometry (MS)-based technologies have led to the ability to resolve cellular proteomes within minute sample quantities and, very recently, even down to a single cell. Current limitations are the incomplete proteome depth achieved and low sample throughput, and continued efforts are needed to push the envelope on instrument sensitivity, improved data acquisition methods, and chromatography.

For single-cell proteomics using Mass Spectrometry (scMS) and low-input proteomics experiments, the sensitivity of an orbitrap mass analyzer can sometimes be limiting. Therefore, low-input proteomics and scMS could benefit from linear ion traps, which provide faster scanning speeds and higher sensitivity than an orbitrap mass analyzer, however, at the cost of resolution. We optimized and improved an acquisition method that combines the orbitrap and linear ion trap, as implemented on a tribrid instrument, while taking advantage of the high-field asymmetric waveform ion mobility spectrometry (FAIMS) pro interface, with a prime focus on low-input applications.

First, we compared the performance of orbitrap- versus linear ion trap mass analyzers. Subsequently, we optimized critical method parameters for low-input measurement by data-independent acquisition (DIA) on the linear ion trap mass analyzer.

We conclude that linear ion traps mass analyzers combined with FAIMS and Whisper™ flow chromatography are well-tailored for low-input proteomics experiments. They can simultaneously increase the throughput and sensitivity of large-scale proteomics experiments where limited material is available, such as clinical samples, cellular sub-populations, and eventually, scMS.

**KEYWORDS:** peptide identification optimization, mass spectrometry, ultrasensitive proteomics, data acquisition, low-input applications, FAIMS-MS

## INTRODUCTION

Deep proteome profiling of single cells and low-input material is an attractive discovery-based, hypothesis-free data generation tool to study biological heterogeneity in health and disease. Shotgun proteomics has achieved initial milestones using the latest advances in nanoflow liquid chromatography-tandem mass spectrometry (LC-MS/MS)[1–5]. However, technical challenges remain in the study of scMS and low-input approaches. A variety of aspects such as sample preparation, experimental throughput, instrument sensitivity, and computational tools still require further optimization[6].

To maximize the efficiency of low-input proteomics experiments, many sample processing methods were developed to minimize sample losses and surface contact during sample isolation, preparation, and transfer[7–11]. While deep proteome profiling comes with the tradeoff of requiring longer LC gradients, we here use a Whisper™ stepped pre-formed beta gradient[2] eluting the peptides at a 100 nL/min flow rate and a 40 or 20 "samples-per-day" (SPD) method to balance robustness and sensitivity. Brunner *et al*.[5] included a similar platform during their recent demonstration of advances in instrument development to analyze single-cell proteomes on a trapped ion mobility mass spectrometer with diaPASEF[12]. Currently, most single-cell proteomics and low-input proteomics studies were performed on an orbitrap mass analyzer instrument[4,13] or a time-of-flight instrument[5,14]. Linear ion traps stand as an attractive alternative mass analyzer to orbitraps for low-input applications in mass spectrometry-based proteomics[15] thanks to their increased sensitivity and efficient scanning speed. To enhance the results of data acquisition for low-input quantitative proteomics experiments, data-independent acquisition (DIA) is an attractive approach, as precursor ions are fragmented and acquired independently from their intensity, which makes this acquisition method less biased and reduces missing values compared to data-dependent acquisition (DDA)[16].

Here we present DIA-LIT, a data acquisition method that combines the utilization of an orbitrap (OT) for high-resolution MS1 scans with the linear ion trap (LIT) for low-resolution but high-sensitivity MS2 scans[17]. We also integrate FAIMSPro ion mobility, which has been shown to decrease chemical background noise, increase specificity[18], and improve protein coverage in low-input proteomics experiments[19]. We demonstrate the utility of using the LIT as a mass analyzer for ultra-low input samples, simulated by carefully controlled dilution series, and determine at which sample load the LIT starts outperforming the OT. We evaluate the impact of gradient length, DIA window schemes, and MS2 injection times (ITs) and present an evaluation of these parameters. This work provides a resource for a comprehensive assessment of DIA-LIT, allowing researchers to implement tribrid instruments in their DIA-based interrogation of low-input biological samples.

# EXPERIMENT PROCEDURES

## HeLa culture and sample preparation

HeLa cells (ECACC: 93021013, Sigma-Aldrich) were maintained in an $H_2O$-saturated atmosphere in Gibco Advanced DMEM (Thermo Fisher Scientific) supplemented with 10% FBS (Gibco, Thermo Fisher Scientific), GlutaMax (Gibco, Thermo Fisher Scientific) and penicillin-streptomycin (Gibco, Thermo Fisher Scientific) at 100 µg/ml at 37 °C and 5% $CO_2$. Cells were passaged at 90% confluency by removing the medium, washing with DPBS (Gibco, Thermo Fisher Scientific), and detaching the cells with 2.5 ml of Accutase solution (Gibco, Thermo Fisher Scientific).

Cells were harvested at 80% confluence and lysed in 5% sodium dodecyl sulfate (SDS), 50 mM Tris (pH 8), 75 mM NaCl, and protease inhibitors (Roche, Basel, Switzerland, Complete-mini EDTA-free). The cell lysate was sonicated for 2 × 30 s and then was incubated for 10 minutes on ice. Proteins were reduced and alkylated with 5 mM TCEP and 10 mM CAA for 20 minutes at 45 °C. Proteins were diluted to 1% SDS and digested with MS grade trypsin protease and Lys-C protease (Pierce, Thermo Fisher Scientific) overnight at an estimated 1:100 enzyme to substrate ratio quenching with 1% trifluoroacetic acid in isopropanol.

C18 EvoTips were activated by adding 25 µL buffer B (80% Acetonitrile, 0.1% Formic acid), followed by centrifugation at 700 xg, and then they were bathed in isopropanol for 1 minute. Then, 50 µL buffer A (0.1% Formic acid) was added to each EvoTip followed by centrifugation at 700 xg for 1 min. The sample was loaded into the EvoTip, followed by centrifugation at 700 xg for 1 min, and two centrifugation steps after adding 50 µL buffer A. Lastly, 150 µL buffer A was added to each EvoTip and spun for 10 sec at 300 xg.

## LC-MS/MS analysis

For all proteome analyses, we used an EvoSep One liquid chromatography system and analyzed the benchmark experiments with diluted tryptic HeLa with a 31- and 58 minutes stepped pre-formed gradient eluting the peptides at a 100 nL/min flow rate. We use a 15 cm x 75 µm ID column (PepSep) with 1.9 µm C18 beads (Dr. Maisch, Germany) and a 10 µm ID silica electrospray emitter (PepSep). Mobile phases A and B were 0.1% formic acid in water and 0.1% formic acid in Acetonitrile. The LC system was coupled online to an orbitrap Eclipse™ Tribrid™ Mass Spectrometer (ThermoFisher Scientific) via an EasySpray ion source connected to a FAIMSPro device. The scan sequence began with an MS1 spectrum (Orbitrap analysis, resolution 120,000, scan range 400–1000 Th, automatic gain control (AGC) target of 300%, maximum IT 50 ms, RF lens 40%). The precursor's mass range for MS2 analysis was set from 500 to 900 Th, and the scan range from 200 - 1200 Th. MS1 precursors were fragmented for MS2 analysis using higher-energy collisional dissociation (HCD) at NCE (normalized collision energy) of 33%, and MS2 AGC target values set to 1000%. Nanospray

ionization voltage was set to 2300 volts, FAIMSPro gas flow set to static (3.6 L/min), and the temperature of the ion transfer tube was set to 240 °C. FAIMSPro ion mobility was applied (standard resolution), and its compensation voltage was set to -45 volts. The isolation window was 10 m/z for benchmarking experiments except for the comparison of the windowing scheme. Resolution and maximum IT were set for each experiment as described in the supplementary (Supporting information: Acquisition Parameters for each method).

## Data analysis

### Raw data analysis and downstream data analysis

AlphaPept (version 0.4.5)[20] was used to analyze DDA data for tryptic HeLa quality control. Spectronaut (version 15.5.211111.50606 and 16.0.220606.53000) (Biognosys)[21] was used for raw data quantification, and raw data were searched against the 9606 *homo sapiens* database obtained from Uniprot (Swiss-Prot with isoforms was downloaded on 07/11/2020) in a directDIA™ analysis. False-discovery rates (FDR) were set at 1% on peptide spectral match (PSM), peptide, and protein levels. Enzyme specificity was set to trypsin/P and lysC. The maximum allowed peptide length in search space was set to fifty-two, and the minimum was set to seven. The maximum allowed number of missed cleavages in search space was set to two. Cysteine carbamidomethyl was set as a fixed modification. N-terminal acetylation and methionine oxidation were set as variable modifications. Default settings were applied for other parameters.

Spectronaut output tables were imported into RStudio (Version 2021.09.2+382) for proteomics data analysis and visualization. This study analysed results based on the number of identified peptides. Briefly, peptides found in less than 65% of replicated experiments were removed from the analysis. Log-transformation was performed on the peptide level to visualize its distribution between mass analyzers at various concentrations. Lastly, identified peptides from each method at various concentrations were shown as stacked bar charts with CV ranges between < 10%, 10-15%, and > 15%.

# RESULTS AND DISCUSSION

## Comparison between an orbitrap mass analyzer and linear ion trap mass analyzer on low-input proteomics

To evaluate the impact of using a LIT instead of an OT for MS2 readouts in DIA, we compared the two mass analyzers on an Eclipse™ Tribrid™ Mass Spectrometer (ThermoFisher Scientific). We focused primarily on the performance of these mass analyzers on low-input samples by measuring a dilution series ranging from 100 ng down to 1 ng. In line with previous results[15], we find that DIA-LIT starts to outperform DIA-OT on samples below 10 ng load (Fig.1A). Conversely, above 10 ng loads, the number of identified peptides from DIA-LIT does not improve substantially, while the number of

identified peptides from DIA-OT increases proportionally with the increasing concentration of input material. This is likely due to the lower specificity of LIT measurements, hindering the deconvolution of very complex DIA spectra. Interestingly, in our setup, we were able to identify 5x more peptides at 1 ng than Borras *et al*.[15], using a 4x shorter gradient.

To investigate the precision of peptide quantification across replicate measurements, we calculated the coefficient of variation (CV) (Fig. 1B). We find that a high fraction of peptides have CVs below 10% for all methods. Interestingly, this fraction decreases in DIA-OT 30k, likely due to the longer cycle time (Table 1), resulting in fewer points per peak (PPP) and thus unreliable peak area estimation. Subsequently, we used the measured cycle times for each method (Table 1) to match methods with comparable cycle times. We calculated Pearson correlations of peptide abundances between the pairs of OT- and LIT-based methods (Fig. 1C). We find that peptide abundances are well correlated, indicating good reproducibility between OT and LIT quantification, except for the comparison between DIA-LIT *Turbo* and DIA-OT 7.5k on 1 ng input material, where due to the limited sensitivity of OT at such low ITs, only 104 overlapping peptides were found. Similarly, we examined the overlap between identified peptides in DIA-OT and DIA-LIT with increasing input material (Fig. 1D). The results show that for lower inputs, peptides measured by both LIT and OT tend to be higher abundant than peptides measured by LIT only, further supporting the higher sensitivity of the LIT. Conversely, at 100 ng input material, almost all peptides that were identified in DIA-LIT were also identified in DIA-OT.

| Mass Analyzer | Scanning Mode | Injection Time (ms) | Cycle Time (s) | Windowing Scheme (m/z) |
|---|---|---|---|---|
| Linear ion trap | Turbo | 16 | 1.4 | 10 |
| Linear ion trap | Rapid | 23 | 1.7 | 10 |
| Linear ion trap | Normal | 38 | 2.4 | 10 |
| **Mass Analyzer** | **Resolution** | **Injection Time (ms)** | **Cycle Time (s)** | **Windowing Scheme (m/z)** |
| Orbitrap | 7500 | 11 | 1.5 | 10 |
| Orbitrap | 15000 | 22 | 1.9 | 10 |
| Orbitrap | 30000 | 54 | 3.2 | 10 |

**Table 1. Parameters for method comparison between DIA--LIT-based methods and DIA-OT-based methods on 40 SPD LC method.**

## Balancing an optimized windowing scheme and injection times

After testing the different mass analyzers, we evaluated the impact of varying windowing schemes. We focused on improving peptide identification for low-input proteomics and reproducibility of the workflow while maintaining acceptable cycle times. We tested LIT-DIA methods with 34, 40, and 45 windows with constant ITs set to auto (38 ms for LIT *Normal* scanning mode), covering a scan range of 500-900 m/z. We find that the windowing scheme reported previously[15] also gave the best results in our study in terms of the number of peptides identified and quantification thereof (Fig. 2). Based on

our results, we opted to use the 40 windows scheme as a standard setup for all the evaluations in our study.

Next, we repeated experiments to test the effect of using higher ITs at similar cycle times, which comes at the cost of fewer, but larger isolation windows to cover the same m/z range during the cycle time (Supporting information: Sup. Fig. 1). Interestingly, this strategy resulted in fewer identified peptides for a 1 ng sample, indicating that the increasing complexity of MS2 spectra derived from wider isolation windows presents a significant challenge for DIA-LIT. Nonetheless, increasing ITs can be desirable to increase the number of ions sampled for subsequent fragmentation. Thus, we subsequently tested the effect of using higher ITs while using a constant 40 DIA windows, resulting in increased cycle times (Fig. 3). Up to 80 ms IT, the results indicate not only a steady increase in peptide identification but also the quantitative precision of those identifications. However, this improvement plateaus when moving past 80 ms, likely due to increased cycle times affecting efficient sampling of both MS1 and MS2 spectra.

## Comparison of injection time on linear ion trap mass analyzer

To enhance the data quality for both identification and quantification, we tested DIA-LIT methods at different scanning modes and ITs. For a gradient flow of 100 nl/min with the 31 minutes method (Whisper™ 40 SPD), we evaluated DIA-LIT methods at *Turbo*, *Rapid,* and *Normal* scanning modes. For a gradient flow of 100 nl/min with the 58 minutes method (Whisper™ 20 SPD), we evaluated DIA-LIT methods at *Rapid*, *Normal*, *Enhanced* and *Zoom* scanning modes. In all cases, ITs were set to auto to ensure optimal parallelization of ion accumulation and scan times in each scanning mode. We find that setting DIA-LIT-based methods with auto IT and 40 isolation windows covering a scan range of 500-900 m/z is a good compromise between cycle time, reproducibility, and sensitivity for benchmarking low-input proteomics experiments based on our study. One exception is the DIA-LIT-based method using *Turbo* scanning mode, where besides the auto IT (Fig. 4), we evaluated limiting IT to 8 ms, and we find that its performance is almost caught up with the DIA-LIT-based method on *Turbo* scanning mode at auto-IT (16 ms) with 100 ng input-material. In contrast, with increasing ITs, it is possible to identify more peptides but at a cost to quantitative precision due to increased cycle times.

## Improvement of the number of points across chromatographic peaks

To reach a sufficient number of data points across chromatographic peaks for accurate quantification[22], we tested different scanning modes (*Turbo*, *Rapid*, and *Normal* on Whisper™ 40 SPD and *Rapid*, *Normal*, and *Enhanced* on Whisper™ 20 SPD) to find a compromise between scanning mode and a number of data points across their chromatographic peak (i.e. points-per-peak, or PPP) for ultra-sensitive routine analysis.

For the low-input proteomics analysis with the DIA-LIT-based method, the '*Rapid*' scanning mode on 40 SPD appears to be the best fit because its Gaussian peaks have seven PPP as its median. On 20 SPD, the '*Normal*' scanning mode resulted in eight PPP as its median (Fig. 5A), suggesting in both

cases that the quantification should be reliable[22]. Our results show that we can identify more peptides with a number of points across a peak lower than 6 by using the "*Normal*" scanning mode on 40 SPD, and the "*Enhanced*" scanning mode on 20 SPD (Fig.5B). However, as visualized by the lower number of precursors with PPP > 6 at those scan rates, it is likely to affect the peak shape determination, leading to measurement errors.

### Comparison of low-input protein identifications between Spectronaut™ version 15 and 16

Regarding the inherent tradeoff of sensitivity over signal resolution from the DIA-LIT-based method, software with powerful scoring functions is required to avoid false-positive signals from noise. We used Spectronaut™ software to analyze raw data in directDIA™ mode due to its reliability and robustness. At the onset of our experimental evaluations, we analyzed our data with Spectronaut™ version 15. With the recent release of version 16, we decided to test the impact of new implementations and benchmark the performance of the new machine learning framework and Artificial Intelligence (AI)-based peak identification feature when deployed on DIA-LIT raw data. Raw files were reanalyzed with version 16 using identical parameters as used in version 15 to make results comparable. Spectronaut version 16 demonstrates clear improved performance in terms of IDs, with an average of 20% improvement in our study (Fig. 6). These results underline the performance enhancements that can be gained when a computational interpretation of complex spectra is improved through advanced machine learning.

### Current Limitations

One of the bottlenecks for low-input proteomics experiments is that we do not have sufficient material to perform e.g. high-pH offline fractionation[23] to reduce the complexity of biological samples, or gas-phase fractionation to efficiently generate a spectral library[24]. However, our results demonstrate that library-free quantification is capable of establishing a benchmark experiment on a complex tryptic lysate. We expect that the number of identified proteins and peptides could be further improved by searching against spectral libraries to increase the identification performance and quantification precision of the library-free quantification. Compared to an OT, LIT mass analyzers are superior in terms of the sensitivity needed for low-input experiments. However, a tradeoff of using LIT is its noise level, affecting subsequent raw data analysis. Future tailor-made software development for DIA-LIT data will likely further increase the number of identified peptides and proteins.

## CONCLUSIONS

This manuscript describes a DIA method tailored toward ultra low-input sample analysis, relying on the combination of an OT mass analyzer for MS1 scans and a LIT mass analyzer for MS2. To reduce background contamination, we include a FAIMSPro interface and demonstrate the ability to quantify representative proteomes from very limited input material. Our results show the LIT mass analyzer to

be a powerful detector for low-input material proteomics for loads of 10 ng and below and is well-suited for DIA-based analysis.

Through a series of optimization steps, we find that the sensitivity of a LIT allows us to use very short ITs. While the slightly longer ITs used in conjunction with higher resolution LIT scans (e.g. "*Normal*" or "*Enhanced*") produced slightly more protein IDs, the quality of those protein quantifications was hampered, which we attribute to the longer cycle times required for higher resolution LIT scans, thereby reducing the points across a chromatographic peak for quantification.

This study compares proteome depth using pre-defined LC methods on the Evosep One instrument (20 SPD and 40 SPD), which is the persistent tradeoff between sample throughput and proteome depth. With the 20 SPD chromatography method, more peptides can be identified and quantified than in the 40 SPD method, but the proteome and peptide coverage does not scale linearly. Hence, it depends on the biological question at hand which method should be preferred, and the data from our analyses can help guide such decisions. Our data suggest the main advantage of LIT over OT mass analyzers to be their higher sensitivity at similar ITs on low load samples. We envision their extreme scan speeds, thereby resulting in shorter cycle times, to potentially be well suited also for higher sample loads. However, our comparison at 100 ng suggests that dedicated efforts will be required to offset the resolution benefits of OT, and the requirement for other acquisition advancements such as e.g. BoxCar and MSX acquisitions or multi-CV FAIMS DIA [18,25,26].

In this study, Spectronaut™ performed well on our DIA-LIT-based methods and did not seem to be hampered by the inherent tradeoff of sensitivity over signal resolution from LIT versus OT. Furthermore, we demonstrate that the Evosep One[27] can increase the throughput and the sensitivity of low-input proteomics experiments. Future improvements to this method could include improved sample throughput by chemical multiplexing methods such as e.g. TMT labelling or Ac-IP tag[14,28–30]. Especially in the case of the latter, the additive signal effect on MS2 scans is likely to prove fruitful for ultra-low input applications such as laser capture microdissection (LCM) or scMS, thereby not only increasing sample throughput but also improving sensitivity and proteome depth.

We hope this study will serve as a valuable resource for low-input proteomics studies, inspire dedicated data processing improvement efforts, and provide relevant starting points for implementing DIA-LIT on compatible instrument platforms worldwide.

# ASSOCIATED CONTENT

## Data availability

All mass spectrometry raw data and search engine files from Spectronaut versions 15 and 16 from this study have been deposited to the ProteomeXchange Consortium via the MassIVE repository. Project accession: PXD034862 (ftp://MSV000089718@massive.ucsd.edu)

All of the code used to generate and analyze MS output is available in the Schoof lab GitHub repository (https://github.com/Schoof-Lab/LITDIA).

### Supporting information

- Acquisition parameters for each method.
- Supplemental Figure SF1: optimizing a balance between Windowing Schemes and ITs.
- Supplemental Tables ST1-7: The number of identified peptides and protein groups in each method, its cycle time, and the version of the search engine (Spectronaut) are listed.

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### Author Contributions

TP and EMS conceived and designed the project. TP performed the experiments and method optimization. LRW and SG performed the data analysis and visualization. BF contributed with input to the method design and data evaluation. TP and EMS drafted and revised the manuscript, which has been read and approved by all authors. EMS supervised the work.

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### Notes

The authors declare no competing financial interests.

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## ABBREVIATIONS

CV, compensation voltage; DDA, data-dependent acquisition; DIA, data-independent acquisition; FAIMS, high-field asymmetric waveform ion mobility spectrometry; IT, injection time; LC, liquid chromatography; LIT, linear ion trap; OT, orbitrap; scMS, MS-based single-cell proteomics; SPD, samples per day; TMT, tandem mass tag

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# Figure 1

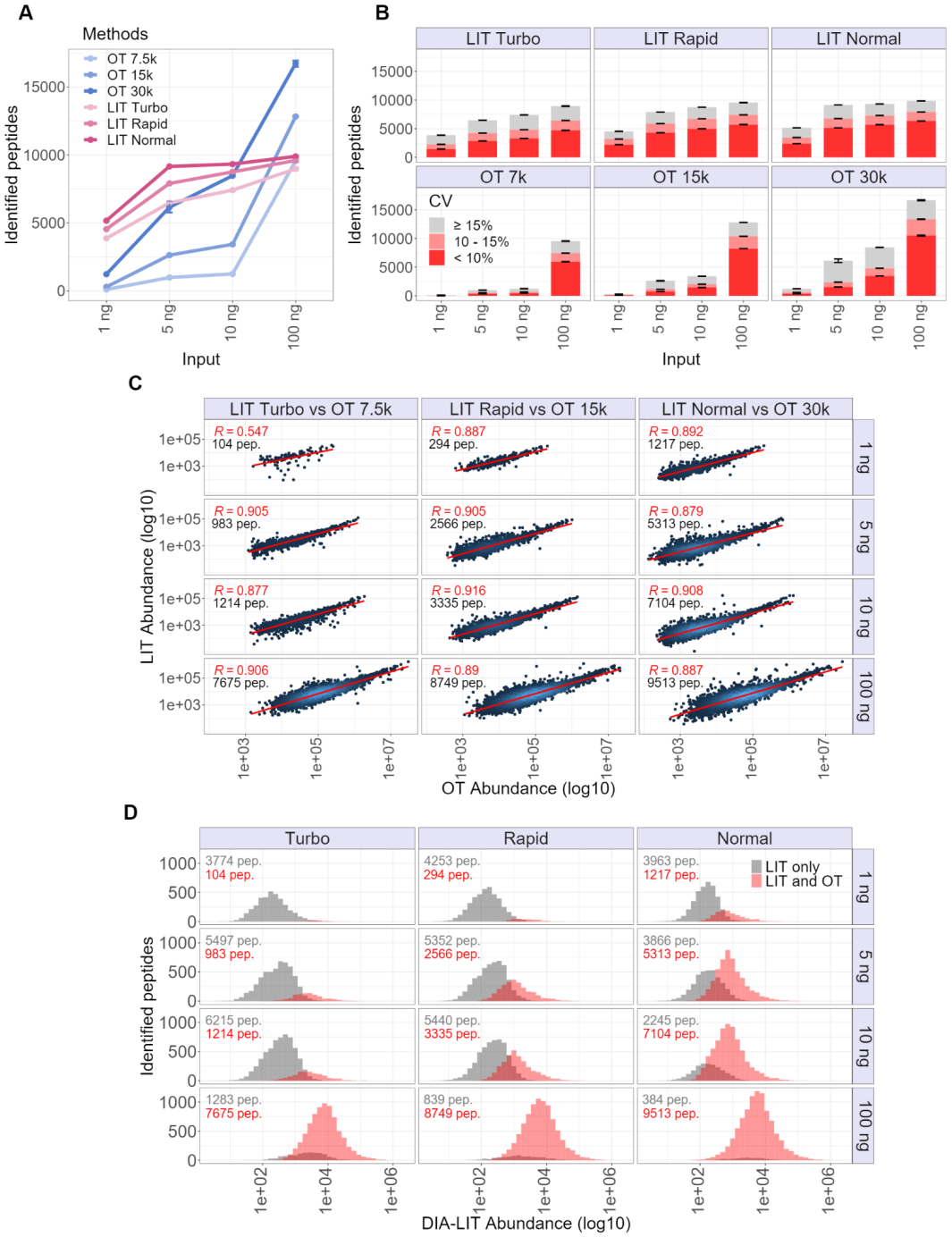


Fig. 1 Comparison between an orbitrap mass analyzer and linear ion trap mass analyzer on low-input proteomics. A. Comparison of the number of identified peptides in serial dilution (1, 5, 10, and 100 ng) of HeLa tryptic digest between DIA-OT-based methods with different resolution scans (7.5k, 15k, and 30k) and DIA-LIT-based methods with different scanning modes *(Turbo*, *Rapid*, *Normal* on 40 SPD). B. Comparison of identified peptides between the DIA-OT-based methods and the DIA-LIT-based methods. Identified peptides with a coefficient of variation (CV) between 10% and 15% are colored with light red and those with a CV below 10% with dark red. C. Pearson correlation of identified peptides between DIA-OT-based and DIA-LIT-based methods when 1, 5, 10, and 100 ng of HeLa tryptic digest were analyzed in quadruplicate. D. Distribution of the range of detection between the DIA-OT-based method and DIA-LIT-based methods and overlap of identified peptides based on their intensities.

# Figure 2

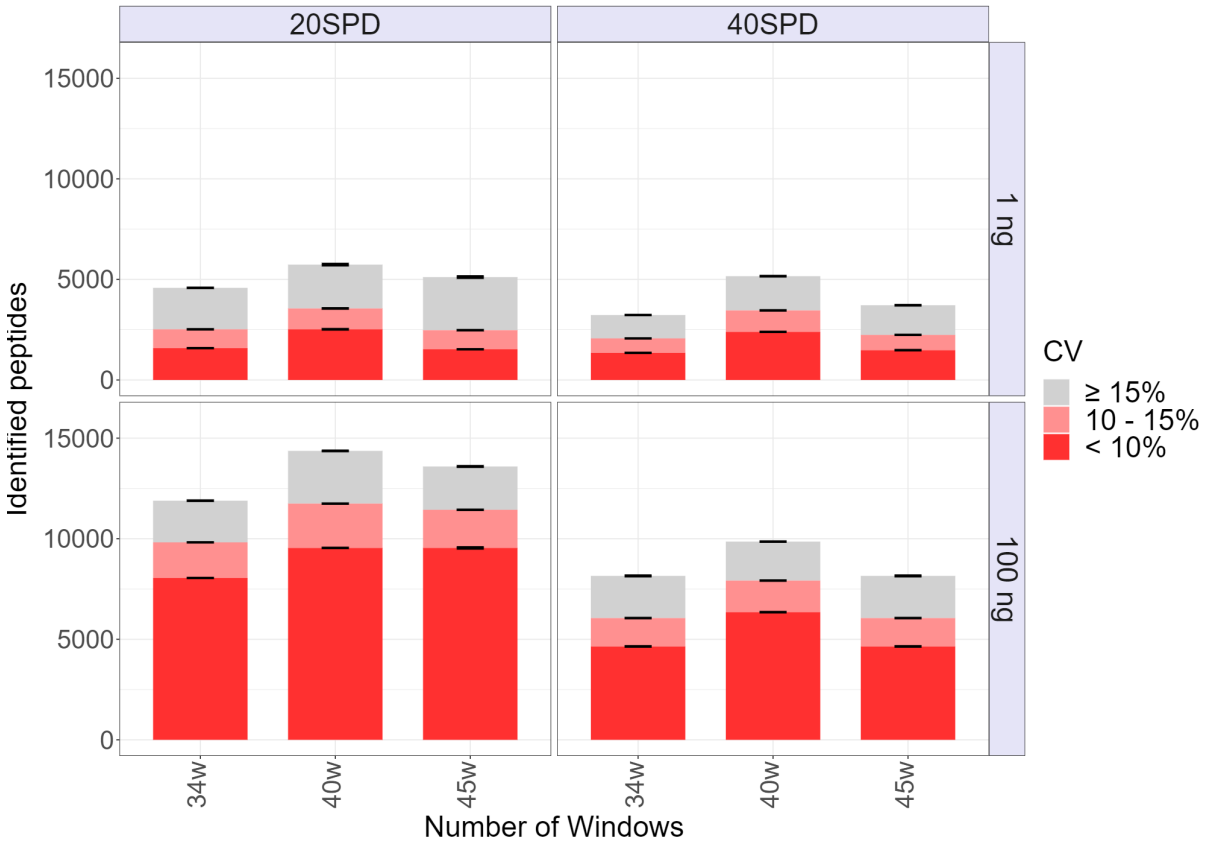


Fig. 2 Comparison of windowing scheme. Comparison of the number of identified peptides on DIA-LIT-based method on *Normal* scanning mode with constant auto-IT and different numbers of windows (34, 40, and 45 windows) and input material (low, and high-input tryptic HeLa digest) on Whisper™ for 40 SPD and 20 SPD. Identified peptides with a coefficient of variation (CV) between 10% and 15% are colored with light red and those with a CV below 10% with dark red.

# Figure 3

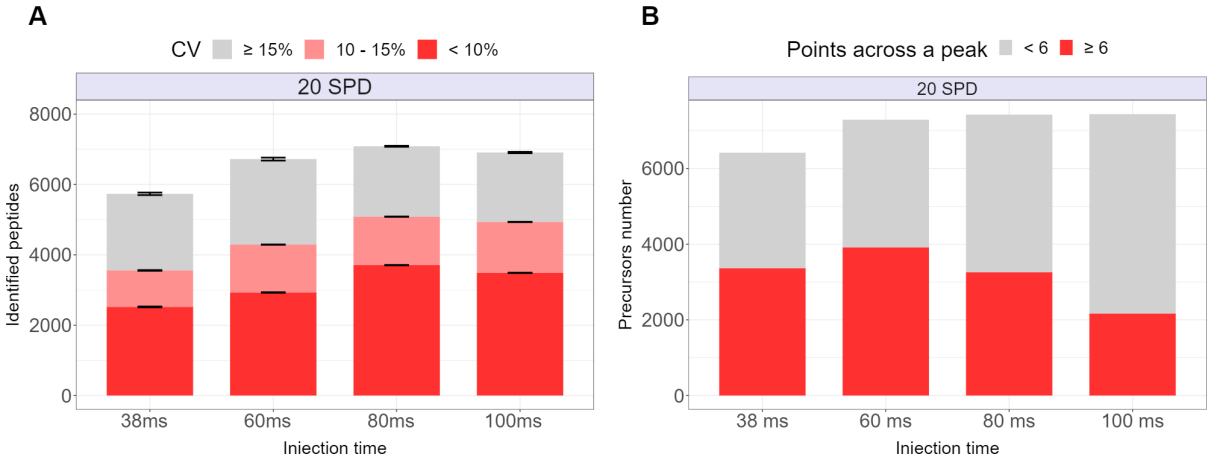


Fig. 3 Increasing injection time on DIA LIT methods. A. Comparison of the number of identified peptides on DIA-LIT-based method on *Normal* scanning mode for Whisper™ 100 20 SPD from 1 ng of tryptic HeLa digest with different ITs at fixed 40 isolation windows. Identified peptides with a coefficient of variation (CV) between 10% and 15% are coloured with light red and those with a CV below 10% with dark red. The cycle times for the methods are indicated by their injection times: 38 ms 2.4 s, 60 ms 3.28 s, 80 ms 4.09 s, and 100 ms 4.91 s. B. Comparison of the number of precursors with a number of points across a peak equal to or greater than 6 on DIA-LIT-based method on *Normal* scanning mode from 1 ng input of tryptic HeLa digest on Whisper™ 100 20 SPD with different ITs (38 ms, 60 ms, 80 ms and 100 ms) at fixed 40 isolation windows.

# Figure 4

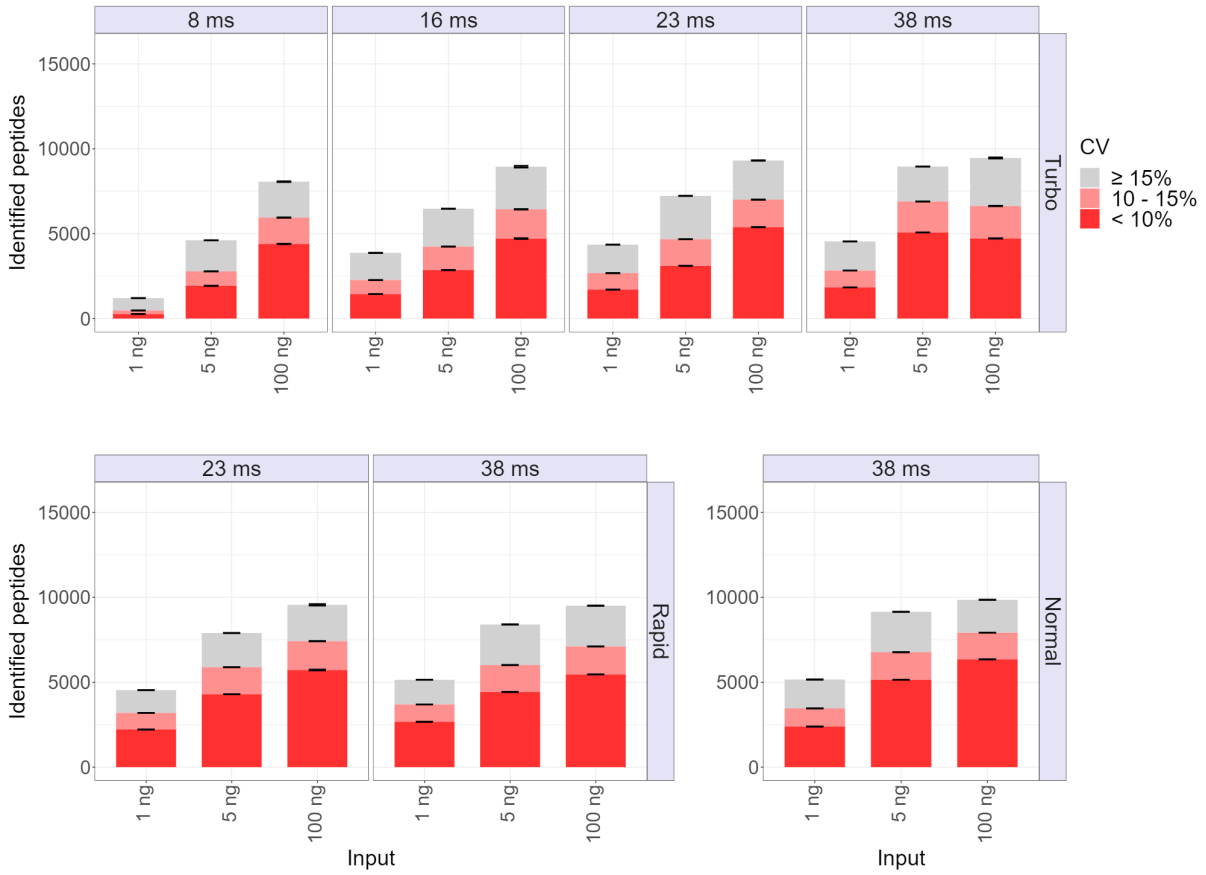


Fig. 4 Comparison of Injection time on linear ion trap mass analyzer. Comparison of the number of identified peptides on DIA-LIT-based method on *Turbo*, *Rapid*, and *Normal* scanning mode for Whisper™ 40 SPD from 1, 5, and 100 ng of tryptic HeLa digest with different ITs at fixed 40 isolation windows. For *Turbo* scanning mode auto IT (16 ms), half of its auto-IT (8 ms), and the auto IT of *Rapid* (23 ms) and *Normal* (38 ms) were applied to determine the compromise between ion filling time and scanning speed of LIT on this mode. For *Rapid* scanning mode, the ITs of 23 ms and 38 ms suffice. On *Normal* scanning mode, only its auto-IT was applied for this comparison. Identified peptides with a coefficient of variation (CV) between 10% and 15% are colored with light red and those with a CV below 10% with dark red.

# Figure 5

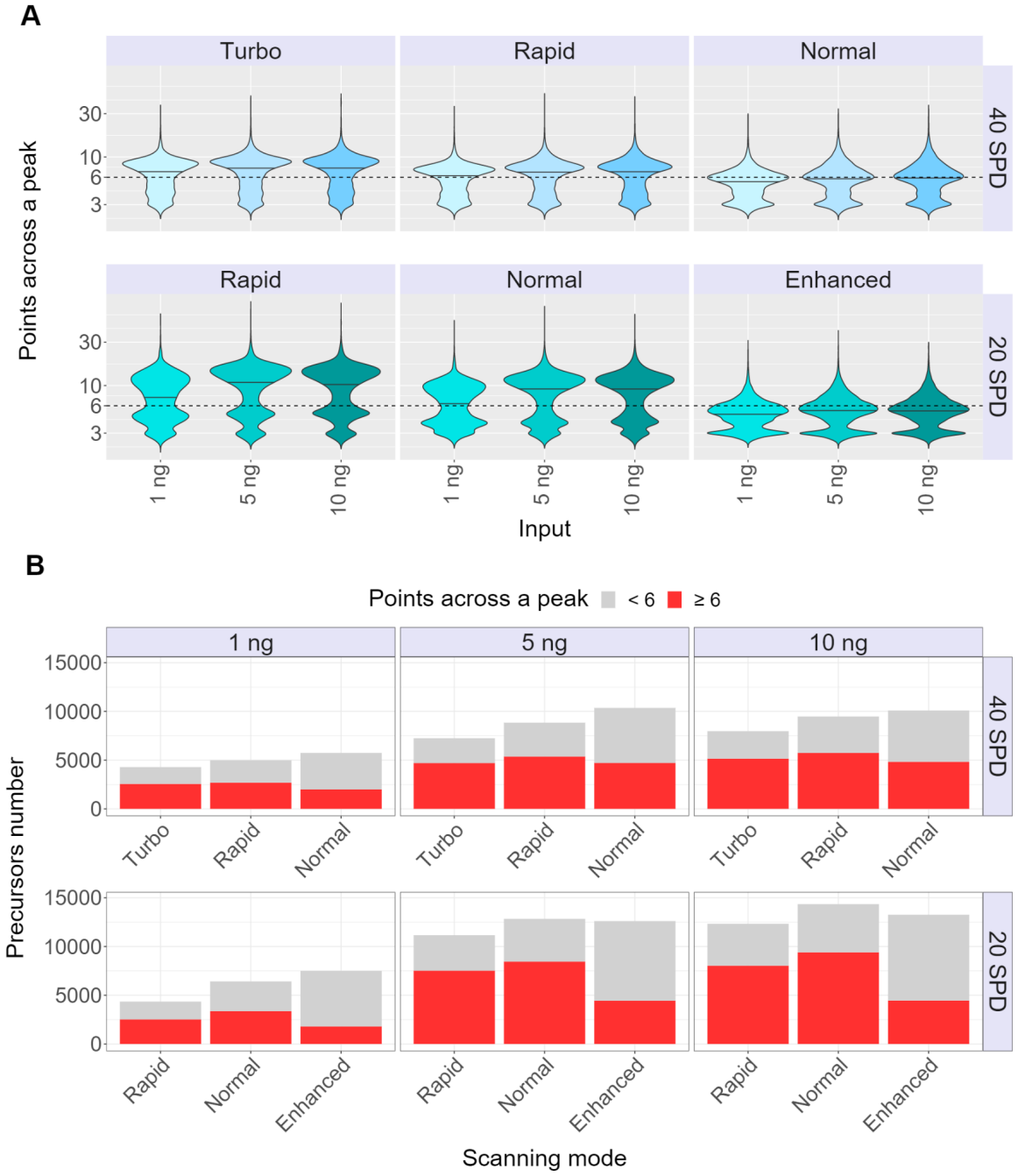


Fig. 5 Improvement of the number of points across chromatographic peaks A. Distribution of numbers of points across a peak by using Whisper™ 20 SPD with DIA-LIT-based methods on *Rapid*, *Normal*, and *Enhanced* scanning modes and Whisper™ 40 SPD with DIA-LIT-based methods on *Turbo*, *Rapid*, and *Normal* scanning modes on 1, 5, and 10 ng input material. B. Comparison of the number of identified peptides with a number of points across a peak equal to or greater than 6 on DIA-LIT-based method on *Turbo*, *Rapid*, and *Normal* scanning mode with different input material (1 ng, 5 ng, and 10 ng of tryptic HeLa digest) on Whisper™ 40 SPD and on *Rapid*, *Normal*, and *Enhanced* scanning mode with different input material (1 ng, 5 ng, and 10 ng of tryptic HeLa digest) on Whisper™ 20 SPD.

# Figure 6

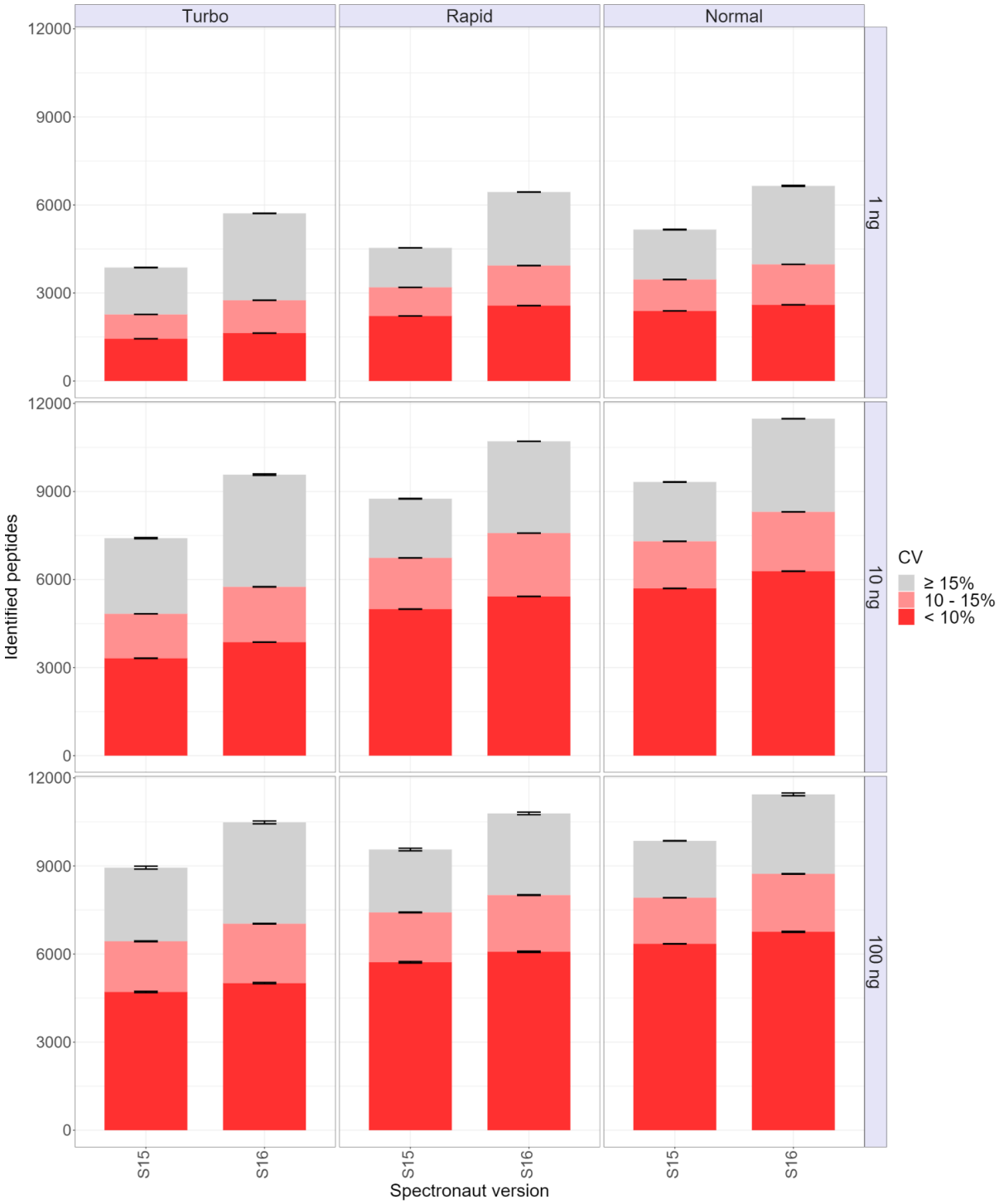


Fig. 6 Comparison of low-input protein identifications between Spectronaut™ version 15 and 16. Comparison of the number of identified peptides in serial dilution (1,10, and 100 ng) of HeLa tryptic digest analyzed by DIA-LIT-based methods with *Normal* scanning mode on 40 SPD between Spectronaut version 15 and 16.