Large-Scale Targeted Proteomics Using Internal Standard Triggered-Parallel Reaction Monitoring (IS-PRM)*

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Targeted high-resolution and accurate mass analyses performed on fast sequencing mass spectrometers have opened new avenues for quantitative proteomics. More specifically, parallel reaction monitoring (PRM) implemented on quadrupole-orbitrap instruments exhibits exquisite selectivity to discriminate interferences from analytes. Furthermore, the instrument trapping capability enhances the sensitivity of the measurements. The PRM technique, applied to the analysis of limited peptide sets (typically 50 peptides or less) in a complex matrix, resulted in an improved detection and quantification performance as compared with the reference method of selected reaction monitoring performed on triple quadrupole instruments. However, the implementation of PRM for the analysis of large peptide numbers requires the adjustment of mass spectrometry acquisition parameters, which affects dramatically the quality of the generated data, and thus the overall output of an experiment. A newly designed data acquisition scheme enabled the analysis of moderate-to-large peptide numbers while retaining a high performance level. This new method, called internal standard triggered-parallel reaction monitoring (IS-PRM), relies on added internal standards and the on-the-fly adjustment of acquisition parameters to drive in real-time measurement of endogenous peptides. The acquisition time management was designed to maximize the effective time devoted to measure the analytes in a time-scheduled targeted experiment. The data acquisition scheme alternates between two PRM modes: a fast low-resolution “watch mode” and a “quantitative mode” using optimized parameters ensuring data quality. The IS-PRM method exhibited a highly effective use of the instrument time. Applied to the analysis of large peptide sets (up to 600) in complex samples, the method showed an unprecedented combination of scale and analytical performance, with limits of quantification in the low amol range. The successful analysis of various types of biological samples augurs a broad applicability of the method, which is likely to benefit a wide range of proteomics experiments. Molecular & Cellular Proteomics 14: 10.1074/mcp.O114.043968, 1630–1644, 2015.

Liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) approaches have been widely acknowledged as one of the most effective methods to study complex proteomes. In particular, their preclinical, and also clinical applications, have contributed to advances in biomedical sciences. A bottom-up proteomics workflow relies on the enzymatic digestion of the proteins constituting a proteome to generate thousands of peptides, which are subsequently separated by liquid chromatography and analyzed by tandem mass spectrometry. Two main MS-based strategies have emerged from this generic process, which differ in their objectives and acquisition schemes and are commonly referred to as discovery and targeted strategies, respectively; both presenting advantages and drawbacks to study specific biological and clinical questions.

Discovery proteomics, relying on nonsupervised data dependent acquisition (DDA), is routinely used to effectively profile, with broad coverage, the proteome under investigation (1, 2). This strategy is focused on protein identification but has limitations with respect to quantitative applications. The stochastic nature of DDA sampling results in “missing values” in replicated experiments, directly affecting quantitative studies, whereas low abundance components remain largely undetected (3). By contrast, targeted proteomics has emerged to more systematically quantify peptides/proteins present in a wide range of concentrations in complex samples (4). The hypothesis-driven nature of targeted data acquisition (TDA), where the peptides used as surrogates for a preselected set of proteins are consistently measured across a multitude of

1 The abbreviations used are: LC, liquid chromatography; PRM, parallel reaction monitoring; SRM, selected reaction monitoring; IS-PRM, internal standard triggered – parallel reaction monitoring; MS/MS, tandem mass spectrometry; DDA, data dependent acquisition; TDA, targeted data acquisition; DIA, data independent acquisition; SIL, stable isotopically labeled; HR/AM, high resolution/accurate mass; AGC, automatic gain control; AUC, area under curve; LOQ, limit of quantification; CV, coefficient of variation.
samples, overcomes the undersampling issue of DDA and addresses the bias toward the most abundant components (5). Improved analytical performance (i.e. sensitivity and precision) characterizes the data generated by targeted analysis as the instrument acquisition time is focused exclusively on a predefined set of analytes (6), which are generally monitored around their predicted elution time (time-scheduled analysis). There is an interdependence between the number of peptides measured in one LC-MS/MS experiment and the time allocated to measure each of them. The necessary trade-off between the scale and the analytical performance level has prompted the development of different types of experiments, each with specific scopes where these two factors are adjusted accordingly, ranging from a screening mode to a “true” quantification mode (7). This has recently been formalized in a report defining the three tiers of targeted experiments in biology and medicine (8). In its largest scale implementation format, the screening mode, thus aiming at the detection and the estimation of the abundance of predefined peptides/proteins (relative quantification between samples), a TDA experiment presents a dramatically increased coverage but still well below that of DDA (9, 10). The latter remains the reference method to generate initial hypotheses in early-stage biological studies, to be gradually refined by TDA approaches as progress is made toward more quantitative measurements of fewer analytes, benefiting from an increased analytical performance. The use of stable isotopically labeled (SIL) peptides as internal standards for the targeted endogenous peptides is also of primary importance for the precision of the measurements. Initially limited to experiments including only a few analytes, SIL peptides are now routinely used in various experiment types as they have become available at moderate cost (in acceptable purity) with respect to their added value in the quality of measurement.

Selected reaction monitoring (SRM) performed on triple quadrupole mass spectrometers is currently the reference method to conduct targeted proteomics experiments (7, 11). However, the SRM technique presents limitations with respect to the selectivity of its measurements, substantially affected by the low resolution of the quadrupole used for both precursor ion and fragment ion selections (typically 0.7–1.0 m/z units). The two stages of mass filtering are often not sufficient to discriminate the signals of the analytes from those of background interferences having an identical chemical composition, commonly encountered in proteomic samples (12). The latest generation of quadrupole-time of flight and quadrupole-orbitrap instruments, showing fast sequencing and high-resolution/accurate mass (HR/AM) measurement capabilities, represents an alternative to perform TDA, especially when high selectivity is required (13–15). More specifically, the parallel reaction monitoring (PRM) technique, implemented on a quadrupole-orbitrap instrument, still relies on quadrupole-based precursor ion mass selection but consists in systematically acquiring full MS/MS spectra of targeted peptides with high-resolution in the orbitrap mass analyzer. The postacquisition extraction of fragment ion signals with tight tolerance (typically 10–20 ppm) facilitates their discrimination from interferences. In addition, the trapping capabilities of the instrument provide a specific advantage to enhance the signal-to-noise ratio of those peptides present at very low concentration in the complex background through the use of long fill times in the process of fragmentation/accumulation of fragments. Previous side-by-side performance comparison of both techniques applied to the measurement of peptides in complex samples (i.e. full yeast lysate or urine protein extract) showed the superiority of PRM by generating data of increased quality, thus translating in lower limits of detection and quantification (15, 16). The improved analytical performance of PRM is likely to advance targeted proteomics applications by directly increasing the reliability and consistency of biological results. However, these high performance levels were achieved when limited sets of analytes were included in the LC-MS/MS experiment (typically < 50 peptides), yielding only a few co-eluting peptides, which allowed performing measurements in the upper range of acquisition parameter settings (i.e. a maximum fill time exceeding 100 ms and an orbitrap resolving power of at least 35,000). As already mentioned, expanding the scale to include a large number of targets requires the adjustment of PRM acquisition parameters, thus altering the performance (17). There is thus a significant need to develop targeted methods for the analysis of moderate-to-large peptide numbers while retaining the high performance level of low-scale PRM experiments to benefit a wider range of targeted proteomics experiments, including early-stage biological studies.

The use of variant PRM acquisition methods (e.g. broadband mode or multiplex mode), as an alternative to the generic sequential PRM mode, only moderately alleviated the trade-off between experiment scale and analytical performance (17). A more significant increase in scale can be obtained in the extreme implementation of these variants, namely the data independent acquisition (DIA) strategy (18, 19). This technique presents specific advantages in an unsupervised set up, such as an extensive coverage of the proteome, with no a priori hypothesis. However, the method in its current implementation presents limited selectivity and sensitivity for complex samples compared with TDA methods (20). Alternatively, the modification of the chromatographic conditions of peptide separations was also explored as a mean to increase the scale of TDA experiments. In the context of SRM analyses, the use of long chromatographic columns (typically > 30 cm) in conjunction with optimized shallow gradients (typically > 3 h) was shown to allow the measurement of 400 pairs of SIL and endogenous peptides with acceptable dwell times (between 8 and 100 ms) (21). Such a set up provides a better separation of analytes from interferences in comparison with a more conventional set up (e.g. 15-cm column and 1-h gradient), and in turn improves the sensitivity level of SRM.
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experiments. For PRM analyses, benefiting from higher intrinsic selectivity, this gain will be less obvious. In addition, the increased multiplexing capability is obtained at the expense of the analytical throughput, because of the long gradients. On the other hand, considering the limited acquisition efficiency of PRM and more generally in TDA acquisition, the use of narrow monitoring windows in time-scheduled PRM acquisition has been identified as a more promising measure to include larger numbers of peptides in the experiment while keeping the same time devoted to their measurement and maintaining the chromatographic throughput (17). The proof-of-principle of such a method has been established but has also shown challenging requirements for its robustness, such as the implementation of dynamic monitoring windows, while providing limited gain.

Thus, a more effective and universally applicable method is desired. Here, in an attempt to fully optimize the acquisition efficiency of PRM, we propose a new data acquisition scheme called internal standard triggered-parallel reaction monitoring (IS-PRM). It relies on added SIL peptides to drive in real-time the PRM measurement of endogenous peptides and benefits from an in-depth revisiting of the overall acquisition time management. The dynamic control of the instrument parameters based on real-time analysis of MS or MS/MS data was previously applied in several contexts. For DDA experiments, it was used to make decision regarding the optimum settings of acquisition parameters in order to improve the quality of spectra (22, 23). It was also used to drive the selection of the activation methods (e.g. CID or ETD) (24) or fragmentation schemes (e.g. neutral-loss triggered MS²) (25, 26) to generate the most informative fragmentation spectra according to the characteristics of the peptide under investigation. In addition, “intelligent” data acquisition methods were designed to increase the MS/MS acquisition frequency of the peptides of interest in directed analyses (27), and to improve the acquisition efficiency (through a better scheduling (9, 17, 28)) and the measurement specificity of TDA (10, 29). The IS-PRM method described in this study is taking advantage of dynamic data acquisition schemes to carry out targeted proteomics experiments at an unprecedented combination of scale and analytical performance.

**Experimental Procedures**

Sample Preparation—

**Generic Sets of 10–20 SIL Peptides Supplemented with Landmark Peptides**—Low-purity synthetic isotopically labeled peptides (PEPotec™ peptides), with C-terminal ¹⁵N and ¹³C-labeled arginine and lysine residues, were provided by Thermo Fisher Scientific (Ulm, Germany) and were prepared at a nominal concentration of 50 to 500 fmol/µl in aqueous solution. In addition, a mixture of 15 synthetic isotopically labeled peptides with C-terminal ¹⁵N and ¹³C-labeled arginine and lysine residues was provided by Thermo Fisher Scientific (Ulm, Germany) and were prepared at a nominal concentration of 50 to 500 fmol/µl in aqueous solution. In addition, a mixture of 15 synthetic isotopically labeled peptides with C-terminal ¹⁵N and ¹³C-labeled arginine and lysine residues was provided by Thermo Fisher Scientific (Ulm, Germany) and were prepared at a nominal concentration of 50 to 500 fmol/µl in aqueous solution. The same set of peptides was spiked in all the samples analyzed in the present study. A subset of 13 peptides was selected to be used as external landmark peptides to recalibrate off-line the peptide monitoring windows prior to regular PRM analyses and to correct on-the-fly the dynamic chromatographic monitoring windows of internal standards in IS-PRM analyses. The list of the 13 peptides is given in Supplemental Data S6. The two discarded peptides were almost fully co-eluting with one of the 13 selected peptides.

**Mixture of 93 SIL Peptides in a Plasma Sample**—Low-purity synthetic isotopically labeled peptides (PEPotec™ peptides), with C-terminal ¹⁵N and ¹³C-labeled arginine and lysine residues, were provided by Thermo Fisher Scientific (Ulm, Germany) and were spiked at a nominal concentration ranging from 50 to 500 fmol/µl (depending on their estimated response factor) into a human plasma digest at 500 ng/µl (depending on their estimated response factor) into a human plasma digest at 500 ng/µl prepared as described earlier (30). Plasma pooled from de-identified human specimens was provided by Integrated Biobank of Luxembourg (IBBL) and treated as “not human subjects research” material for samples prepared in this study. A list of the 93 SIL peptides (with their associated concentration spiked into the plasma sample) derived from human proteins is given in supplemental Data S1.

**Molecule of Series of 93 SIL Peptides in a Plasma Sample Supplemented with Corresponding Unlabeled (“Light”) Synthetic Peptides**—High-purity synthetic isotopically labeled peptides (AQUATM peptides) with C-terminal ¹⁵N and ¹³C-labeled arginine and lysine residues, were provided by Thermo Fisher Scientific (Ulm, Germany) and were spiked at different concentrations (0.002, 0.005, 0.015, 0.050, 0.150, 0.400, 1.2, 3.8, 11.5, and 35 fmol/µl) into a human plasma digest at 500 ng/µl (depending on their estimated response factor) into a human plasma digest at 500 ng/µl prepared as described earlier (30). A list of the 93 SIL peptides is given in supplemental Data S1. In addition, a mixture of a subset of the corresponding unlabeled (“light”) synthetic peptides (49 peptides) was prepared to fortify the amount of the endogenous peptides present at low-abundance in the plasma digest. Low-purity unlabeled synthetic peptides, provided by Thermo Fisher Scientific (Ulm, Germany), were spiked into the sample at a nominal concentration ranging from 100 to 500 fmol/µl (depending on their estimated response factor and the abundance of the corresponding endogenous peptides in plasma). The identity of the 49 “fortified” peptides (with their associated supplemented concentration in the plasma samples) is given in supplemental Data S2.

**Mixture of 606 SIL Peptides in a Plasma Sample, a Urine Sample, and a HeLa Cell Sample**—Low-purity synthetic isotopically labeled peptides (PEPotec™ peptides), with C-terminal ¹⁵N and ¹³C-labeled arginine and lysine residues, were provided by Thermo Fisher Scientific (Ulm, Germany) and were spiked at a nominal concentration ranging from 50 to 500 fmol/µl (depending on their estimated response factor) into a human plasma digest at 500 ng/µl (depending on their estimated response factor) into a human plasma digest at 500 ng/µl prepared as described earlier (30), into a human urine digest at 250 ng/µl (depending on their estimated response factor) into a human urine digest at 250 ng/µl prepared as described earlier (30, and into a HeLa cell protein digest, provided by Thermo Fisher Scientific (PN 88329/Pierce HeLa protein digest standard, Pierce, Rockford, IL), resolubilized in 0.1% formic acid at a final concentration of 250 ng/µl. Urine pooled from de-identified human specimens was provided by Integrated Biobank of Luxembourg (IBBL) and treated as “not human subjects research” material for samples prepared in this study. A list of the SIL peptides derived from human proteins, which includes 79 peptides common to the previous set of 93 SIL peptides, is given in Supplemental Data S5. A list of the SIL peptides unique to this set (with their associated concentration spiked into the different samples) is given in Supplemental Data S4.

**Liquid Chromatography and Mass Spectrometry**—

**LC Separation**—All peptide separations were carried out on a Ultimate 3000 RSLCnano system (Dionex, now Thermo Fisher Scientific). For each analysis, the sample was loaded into a trap column Acclaim PepMap 2 cm × 75 µm i.d., C18, 3 µm, 100 A (Thermo Fisher Scientific) at 5 µl/min with aqueous solution containing 0.05% (v/v)
trifluoroacetic acid and 1% acetonitrile. After 3 min, the trap column was set on-line with an analytical column Acclaim PepMap RSLC 15 cm × 75 μm i.d., C<sub>18</sub>, 2 μm, 100 A (Thermo Fisher Scientific). Peptide elution was performed by applying a mixture of solvent A/B. Solvent A was HPLC grade water with 0.1% (v/v) formic acid, and solvent B was HPLC grade acetonitrile with 0.1% (v/v) formic acid. Separations were performed by applying a linear gradient of 2–35% solvent B at 300 μL/min over 66 min followed by a washing step (4 min at 90% solvent B) and an equilibration step (11 min at 2% solvent B). One microliter of each sample was injected.

Analyses on Quadrupole-Orbitrap Instrument—Parallel reaction monitoring analyses were performed using Q-Exactive, Q-Exactive Plus, and Q-Exactive HF mass spectrometers (Thermo Scientific, Bremen, Germany). A dynamic nano-electrospray source housing was utilized with uncoated SilicaTips, 12 cm length, 360 μm outer diameter, 20 μm inner diameter, and 10 μm tip inner diameter. For ionization, 1500 V of liquid junction voltage and 250 °C capillary temperature were used. A set of well-characterized peptides was analyzed on a regular basis on the different instruments to assess their calibration, with a specific focus on the parameters affecting ion fragmentation such as the pressure of nitrogen in the HCD cell, and to ensure they generate reproducible peptide fragmentations patterns over time and across instruments.

The analyses of small sets of SIL peptides (10–20 peptides) were performed on a Q-Exactive instrument. The acquisition method combined two scan events corresponding to a full scan event and a PRM event targeting the doubly and triply charged precursor ions of the SIL peptides without scheduling. The full scan event employed a m/z 300–1500 mass selection, an orbitrap resolution of 70,000 (at m/z 200), a target automatic gain control (AGC) value of 1e6, and maximum fill times of 250 ms. The PRM event employed an orbitrap resolution of 17,500 (at m/z 200), a target AGC value of 1e6, and maximum fill times of 100 ms. The precursor ion of each targeted peptide was isolated using a 2-m/z unit window. Fragmentation was performed with a normalized collision energy of 25 eV and MS/MS scans were acquired with a starting mass of m/z 100, the ending mass being automatically defined by the m/z and the charge state of the precursor ion. Isolation and fragmentation were performed similarly in the various PRM and IS-PRM acquisition methods used in the present study.

The analyses of the mixture of 93 SIL peptides in a plasma sample were performed on a Q-Exactive Plus instrument. For regular PRM analyses, the acquisition method combined two scan events corresponding to a full scan event and a time-scheduled PRM event targeting the precursor ions selected for the pairs of SIL and endogenous peptides in ±1.5 min elution time windows (based on spectral library and off-line recalibration using landmark peptides prior to analysis). The full scan event employed a m/z 300–1500 mass selection, an orbitrap resolution of 17,500 (at m/z 200), a target automatic gain control (AGC) value of 1e6, and maximum fill times of 60 ms. The same parameter settings were employed for the full scan event included in the various PRM and IS-PRM acquisition methods used in the present study (with the exception of the PRM method used to generate the spectral libraries). The PRM event employed an orbitrap resolution of 17,500 (at m/z 200), a target AGC value of 1e6, and maximum fill times of 60 ms. For IS-PRM analyses, the acquisition method can be considered as a regular method combining four scan events corresponding to a full scan event and three time-scheduled PRM events, each with its own target list. One PRM event targeted 13 external landmark peptides in elution time windows exceeding 7 min, employing an orbitrap resolution of 17,500 (at m/z 200), a target AGC value of 1e6, and maximum fill times of 60 ms. A second PRM event targeted the precursor ions selected for the 93 SIL peptides in ±0.5 min dynamic elution time monitoring windows (based on spectral libraries) for all the peptides except those eluting or starting to elute before the first landmark peptide eluted (three peptides), for which the windows were set between 10 min and 18 min. This PRM event employed an orbitrap resolution of 17,500 (at m/z 200), a target AGC value of 1e6, and maximum fill times of 60 ms. The third PRM event targeted the precursor ions selected for the 93 endogenous peptides, employing an orbitrap resolution of 70,000 (at m/z 200), a target AGC value of 1e6, and maximum fill times of 360 ms. The elution time monitoring windows were dynamically corrected and defined for the second and third PRM event, respectively, based on the principles extensively discussed in the results section. Also, some additional details are provided in the data processing part of this section.

The analyses of the mixture of 93 SIL peptides in a plasma sample were performed using the same instrument and PRM acquisition method as those used for the analyses of the mixture of 93 SIL peptides in a plasma sample. The IS-PRM analyses were performed by swapping the PRM events used to monitor SIL and unlabeled peptides.

The analyses of the mixture of 606 SIL peptides in various samples were performed on a Q-Exactive HF instrument using PRM and IS-PRM acquisition methods similar to those used for the analyses of the mixture of 93 SIL peptides in a plasma sample. The acquisition parameters were slightly modified to account for the different mass spectrometer used and the expansion of the number of targeted peptides. For regular PRM analyses, the precursor ions selected for the pairs of SIL and endogenous peptides were targeted in ±1 min elution time windows (based on spectral library and off-line recalibration using landmark peptides prior to analysis) using two variant acquisition methods. In the first method variant, called “PRM-Method A,” the PRM event employed an orbitrap resolution of 70,000 (at m/z 200), a target AGC value of 1e6, and maximum fill times of 110 ms. In the second method variant, called “PRM-Method B,” the PRM event employed an orbitrap resolution of 15,000 (at m/z 200), a target AGC value of 1e6, and maximum fill times of 20 ms. For IS-PRM analyses, the PRM event targeting the precursor ions selected for the 606 SIL peptides employed an orbitrap resolution of 15,000 (at m/z 200), a target AGC value of 1e6, and maximum fill times of 20 ms. The PRM event targeting the precursor ions selected for the 606 endogenous peptides, employed an orbitrap resolution of 60,000 (at m/z 200), a target AGC value of 1e6, and maximum fill times of 110 ms. The other acquisition parameters were kept identical to those used for the analyses of the mixture of 93 SIL peptides in a plasma sample.

The spectral libraries and the associated information pertinent to the different experiments are given in Supplemental Data S1, S2, and S4.

Analyses on Triple Quadrupole Instrument—Selected reaction monitoring analyses of the dilution series of 93 SIL peptides in plasma samples were performed using a TSQ Vantage extended mass range triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA) with identical nano-electrospray and chromatographic settings. The selectivity for both Q1 and Q3 was set to 0.7 Da (FWHM). The collision gas pressure of Q2 was set at 1.5 mTorr argon. For each peptide, the selection of the six monitored transitions required preliminary experiments that were performed as described earlier (31). The collision energy was calculated using the optimized formula CE = 0.033 × m/z of precursor ion + 1.8 and CE = 0.038 × m/z of precursor ion + 2.3 for doubly and triply charged precursor ions, respectively. The time-scheduled SRM method targeted the 93 pairs of SIL and endogenous peptides in ±1.5 min retention time windows by monitoring each selected transition with a dwell-time of 10 ms. The list of transitions monitored is included in supplemental Data S3.

The MS files have been deposited to the ProteomeXchange Consortium (32) via the PRIDE partner repository (33) with the data set identifier PXD001731.
Data Processing—Data analysis was performed using Xcalibur (version 2.2, Thermo Fisher Scientific) and/or Pinpoint (version 1.3, Thermo Fisher Scientific), and processing tools developed in-house in C# programming language and based on MSFileReader library (version 2.2, Thermo Fisher Scientific) when the intended operation was not possible with standard software (e.g., spectral matching). Ion chromatograms and intensity of fragment ions were extracted with a mass tolerance of 10–20 ppm for PRM and IS-PRM data. For IS-PRM analyses, only the data generated by the quantitative mode were processed for detection and quantification purposes.

For the generation of the spectral library of 93 SIL peptides, the areas under the curve (AUC) of singly charged and both singly and doubly charged y-and b-type fragment ions were calculated for doubly charged and triply charged precursor ions of the peptides of interest, respectively. The precursor ions for which the sum of the AUC of the six most intense fragment ions provided the highest value was retained to be used in subsequent targeted analyses (with the corresponding fragment ions). The AUC of the most intense fragment ion was used as an estimator of the peptide response factor, whereas the individual AUC of the six transitions were used to reconstruct a composite reference MS/MS spectrum for the peptide. The elution profile of the transitions was further evaluated to determine the elution time of the peptide (based on apex determination and captured together with those of landmark peptides in the same analysis), to define its chromatographic peak width and to detect the presence of peak splitting. The spectral library of the 93 unlabeled peptides was derived from that of their corresponding labeled form and adjusted to account for some differences in their purity. For the generation of the spectral library of the 606 SIL peptides, a similar scheme was applied with minor changes. To reduce manual work and speed up data processing, analyses were first submitted to mascot database searches, through proteome discoverer software (version 1.4, Thermo Fisher Scientific) against a home-made database only containing the sequences of the peptides of interest. The search results were then imported in pinpoint as a spectral library. A selection of the precursor ions and their six fragment ions (among singly and doubly charged y- and b-type ions, with a preference for y-type ions) was automatically performed by the program. Then, only this precursor ion and the corresponding six fragment ions were retained for next processing steps as described above. The process was also applied to the SIL peptides common to the previous set of 93 SIL peptides as some peptides were obtained from a different synthesis batch, where their purity may slightly vary. The spectral libraries and the associated information pertinent to the different experiments are given in supplementary Data S1, S2, and S4.

The assessment of the detection of peptides, performed postacquisition or in real-time, relied on the same process. For each peptide under evaluation, the signals of the six most intense fragment ions (as defined in spectral libraries) were extracted from each corresponding MS/MS spectrum. The MS/MS spectra with at least five out of the six fragment ions detected were submitted to spectral matching. The comparison of the relative intensities of these fragments with those defined in the reference composite MS/MS spectrum was performed based on the calculation of a spectral contrast angle $\theta$ (34, 35), defined as

$$\cos \theta = \frac{\sum_{i=1}^{n} l_{\text{exp},i} \times l_{\text{ref},i}}{\sqrt{\sum_{i=1}^{n} (l_{\text{exp},i})^2} \times \sqrt{\sum_{i=1}^{n} (l_{\text{ref},i})^2}}$$

where $l_{\text{exp},i}$ is the intensity of the fragment ion $i$ in the experimental MS/MS spectrum under evaluation, $l_{\text{ref},i}$ is the intensity in the reference MS/MS spectrum, and $n$ is the number of selected fragment ions. The high quality MS/MS spectra presenting a spectral contrast angle below 11.5° ($\cos \theta > 0.98$), based on the five fragment ions with minimal degree of interference (i.e., based on the combination of five fragments giving the highest similarity score out of the six possible combinations), confirmed the detection of the peptide under evaluation.

For the analyses of the dilution series of 93 SIL peptides in a plasma sample supplemented with unlabeled synthetic peptides, the determination of the area under the curve (AUC) of selected fragment ions (PRM and IS-PRM analyses) and of target transitions (SRM analyses), the establishment of dilution curves based on isotope dilution and the determination of the linearity ranges and limits of quantification (LOQ), were performed as previously described (13, 16); see supplementary Data S3.

For the PRM and IS-PRM analyses of the mixture of 606 SIL peptides in a plasma sample, the quantification was performed for endogenous peptides systematically detected in triplicated analyses. For these peptides and corresponding SIL peptides, the AUC of the six most intense fragment ions (as defined in spectral libraries) were calculated based on the co-elution profiles of the differentially labeled peptides and summed to obtain the AUC of the peptides. The AUC of each SIL peptide was then used to normalize the AUC of its corresponding endogenous peptide. Finally, a coefficient of variation (CV) was derived from the normalized AUC of the endogenous peptides; see supplementary Data S5.

In IS-PRM acquisition, the modification of acquisition parameters in real-time relied on the updating of the target lists and their dynamic implementation in the acquisition methods guided by the on-the-fly data evaluation. This data evaluation was performed and automated using in-house scripts (C# programming language) and an application programming interface (API). The real-time correction of the monitoring windows of internal standards based on external landmark peptides (watch mode) was performed as described earlier (17). Following the assessment of the detection of an internal standard, as described above, the target lists were modified and synchronized to stop watch mode acquisition for this peptide (internal standard removed from the active target list) while starting quantitative mode acquisition for the corresponding pair of SIL and unlabeled peptides. The elution time monitoring window for this pair of peptides measured in quantitative mode thus started from the detection time of the internal standard and continued for a period matching the predefined chromatographic peak width (based on the spectral library and associated information). The updated target lists were dynamically implemented once per cycle (if needed) via the API. In the present study, the IS-PRM method was executed using prototype software in conjunction with the API. To ensure broad dissemination, the method has been converted into an application requiring only the API. The access to API functionality will be provided by Thermo Fisher Scientific. The license is available upon request and acceptance of terms and conditions. The IS-PRM application is available as Supplementary Material. A tutorial describing the installation of the application and its usage is provided in supplementary Experimental Procedures.
RESULTS AND DISCUSSION

Principle and Implementation of Internal Standard Triggered-Parallel Reaction Monitoring (IS-PRM)—The control of the conditions and the reproducibility of peptide chromatographic separations is critical in targeted experiments. It allows narrowing down the elution time windows during which peptides are monitored in time-scheduled analyses with an immediate benefit on the number of time windows segmenting the full LC-MS/MS run, and thus on the total number of peptides that can be monitored per run under conditions favoring high-analytical performance. The current targeted acquisition methods used in proteomics monitor peptides over relatively long chromatographic elution time windows (typically 2–3 min), whereas their actual elution lasts about 30 s (Fig. 1A). Such wide monitoring windows are necessary to take into account unavoidable drifts in the peptide elution times. The monitoring of a set of well-defined external “landmark” peptides (having even distribution over the entire elution space) added to the samples enables the adjustment of the monitoring windows of targeted peptides, ideally performed on-the-fly. The proof-of-principle of such dynamic monitoring window has been established and enabled the use of one-minute windows in its optimal implementation (17). A further decrease in window width proved difficult to implement routinely, limiting the advancement of the approach. This persistent mismatch between the scheduled monitoring window and the peptide chromatographic peak width translates into an inefficient use of the mass spectrometer acquisition time, as it constrains the PRM acquisition process, and leads to suboptimal settings of acquisition parameters. The factors driving sequential PRM acquisition are the number of peptides to be monitored at a given chromatographic time, the MS acquisition time (linked to transient length and fill time) devoted to their individual measurement, and the cycle time to generate one data point for each of them (as illustrated in Fig. 1B). In practice, the cycle time is so defined to collect eight to ten data points over the peptide chromatographic peak to ensure precise quantification (i.e. 3–4 s for 30-s peak width), resulting in an interdependence between the two other factors. In general, the MS acquisition time serves as an adjustment factor enabling the measurement of the intended number of peptides in the limit of the cycle time. As the maximum fill time is normally coupled to the orbitrap resolution setting to maximize the filling of ions in parallel to the orbitrap transient acquisition, the MS acquisition time can be approximated to the transient acquisition time. Therefore, the orbitrap resolution (through transient acquisition time) and maximum fill time settings are tuned according to the number of peptides monitored at a given chromatographic time (the corresponding maximum value at the full experiment level). In the example displayed in Fig. 1B, the model reflects the PRM measurement of 12 pairs of endogenous and SIL peptides at a chromatographic time, which requires the use of a transient acquisition time of 128 ms (resolution of 35,000 m/z 200 on Q-Exactive instrument) and a synchronized maximum fill time of 110 ms to maintain the cycle time close to 3 s. These
acquisition parameters are thus significantly affected by the mismatch between the scheduled monitoring window and the peptide chromatographic peak width, which translates in a low acquisition efficiency reflected by the significant portion of time during a cycle devoted to the acquisition of noninformative PRM scans (i.e. from 85% to 50% for window widths ranging from 3 to 1 min).

Improving the acquisition efficiency by minimizing the number of noninformative PRM scans and their associated acquisition time appears as an attractive option to make extra-time available to be used to increase the number of targeted peptides and/or the MS acquisition time to measure them when they are actually eluting. This can be obtained by leveraging the presence of internal standards (SIL peptides), which are routinely used in TDA experiments, to perform scheduled PRM acquisitions. The concept of using the presence of exogenous isotopically labeled synthetic peptides to drive the MS acquisition was previously proposed to improve the frequency of the acquisition of MS/MS spectra of endogenous peptides in directed analyses (27). The method relied on the evaluation of the full MS scan signal to trigger MS/MS events; it required the addition of two exogenous synthetic peptides, generated using a demanding chemical labeling scheme.

The approach that we propose relies on the use of one exogenous marker to drive the data acquisition in targeted PRM analyses. The method, compatible with a simple and robust sample preparation workflow as typically used in TDA experiments (e.g. without fractionation and derivatization), was developed and implemented using an application programming interface (API). It relies on the real-time evaluation of the MS/MS data, which provides a reliable basis to properly control the acquisition parameters, ensuring high acquisition efficiency and quantitative measurements. A data acquisition scheme was thus developed (Fig. 2A) where the instrument operates in two alternating PRM modes: a lower resolution watch mode and a truly quantitative mode. In the watch mode, only SIL peptides are continuously monitored in their 1-min dynamic monitoring window (based on external landmark peptides, (17)). In this mode, the acquisition parameters used favor speed over data quality, as allowed by the addition of easily detectable amounts of internal standards to the samples. The quantitative mode is used to measure SIL and endogenous peptides when they are actually eluting using acquisition parameters to enhance data quality. The external landmark peptides (13 peptides in the present study evenly distributed over the entire elution space) are also measured by PRM in wide monitoring windows (typically > 7 min) to ensure universal applicability of the method while using acquisition parameters to favor high speed acquisition. The detection of SIL peptides is performed through real-time extraction and evaluation of data acquired in watch mode by spectral matching against a library of reference MS/MS spectra. The unambiguous detection of a SIL peptide triggers a switch from the watch mode, which is stopped for this peptide, to the quantitative mode to measure the corresponding pair of SIL and endogenous peptides. The acquisition in quantitative mode continues for a predefined time window matching the chromatographic peak width (typically 0.5 min in our LC set up) to fully profile the elution of the peptides. The actual quantification of peptides, performed postacquisition, is carried out on the data generated by the quantitative mode.

This internal standard triggered-parallel reaction monitoring technique (IS-PRM) minimizes the actual time acquiring PRM scans not containing pertinent information at both LC level (typically 15-s window per targeted peptide for the use of 1-min dynamic monitoring window in watch mode, (17)) and MS level. This translates into an efficient acquisition of nearly 100%, despite the overhead time necessary for the effective modification of the acquisition mode in our implementation and for monitoring the landmark peptides, estimated in total at 200 ms per cycle. The model shown in Fig. 1B was adapted for IS-PRM acquisition. The transient length was set at 64 ms in “watching mode” and 256 ms in quantitative mode (with appropriate synchronization of maximum fill times). It translated into an overall acquisition efficiency of 90% devoted to the acquisition of pertinent signals for targeted peptides (Fig. 2B), against 15% in the initial model of regular PRM acquisition. This net gain enables the monitoring of a larger number of peptides while longer transient acquisition time and fill time are allocated to the measurement of peptides in quantitative mode, with additional benefits on the spectral quality.

The improvement in acquisition time efficiency was shown using a well-controlled dynamic monitoring window (1-min width). Relaxing the chromatographic constraints to 2 min maintains the same overall benefit as the few additional noninformative PRM scans will be acquired in watch mode to track the start of elution of the internal standards. It results in only a moderate decrease from 90 to 80% in the acquisition efficiency, as estimated using the model mentioned above and considering a typical increase in the monitoring window part not containing pertinent information from 15 to 45 s (per targeted peptide in watch mode). For relative quantification experiments, such as those presented in this proof-of-principle study, the acquisition parameters used to measure internal standards in quantitative mode can be set at low values, for instance similar to those used in watch mode, to further benefit the scale and/or the quality of the data generated for endogenous peptides. Performing measurements of SIL and endogenous peptides under strictly identical acquisition parameters conditions is not a prerequisite for such experiments.

Implementation of IS-PRM Methods on Quadrupole-Orbitrap Instrument—The establishment of IS-PRM methods requires some adjustments in the conventional PRM workflow. The prerequisite to use IS-PRM is actually linked to the sample. For the set of well-defined peptides under investigation, the corresponding internal standards (SIL peptides) have to be added to the sample. In designing an IS-PRM method,
as it is common practice for a regular PRM method, the generation of a spectral library is performed from PRM analyses of small sets of SIL peptides (as detailed in the experimental procedures section). In addition to the features commonly captured in conventional spectral libraries of peptides (i.e., their reference MS/MS spectrum and reference elution time), the full elution profile of SIL peptides is also carefully recorded to estimate their response factor and to assess their...

**FIG. 2.** Data acquisition scheme of internal standard - triggered parallel reaction monitoring method. A. The instrument operated in two alternating PRM modes: the watch mode and the quantitative mode. In the watch mode, only SIL peptides (internal standards) were continuously monitored in their 1-min dynamic monitoring window (based on external landmark peptides). The detection of a SIL peptide (e.g., DADPDTFFAK in this example) was performed through on-the-fly extraction and evaluation of the MS/MS data acquired in watch mode by spectral matching against a library of reference MS/MS spectra. This detection triggered a switch from the watch mode (stopped for the given peptide) to the quantitative mode to measure the corresponding pair of SIL and endogenous peptide for a predefined monitoring window matching the peptide chromatographic peak width (typically 0.5 min). B. The acquisition parameters used in watch mode favored speed over data quality. In the model, a transient length of 64 ms (resolution of 17,500 (at m/z 200) on Q-Exactive instrument) and a synchronized maximum fill time of 60 ms were used. By contrast, the acquisition parameters used in quantitative mode, that is, a transient length of 256 ms (resolution of 70,000 (at m/z 200) on a Q-Exactive instrument) and a synchronized maximum fill time of 250 ms, enhanced data quality. In the model, such parameters enabled the PRM measurement of 15 peptides per cycle (for a cycle time of 3.5 s), including six pairs of SIL and endogenous peptides at the time of their actual elution. This resulted in an acquisition efficiency of 90% (overhead time of 200 ms estimated per cycle). An increase in the dynamic monitoring window of SIL peptides in watch mode to 2 min moderately affects the acquisition efficiency. Under this condition and keeping unchanged the other parameters in the model, nine PRM scans would be acquired in watch mode, resulting in an acquisition efficiency of 80%.
chromatographic peak width. The estimated response factor is subsequently used to determine the minimum concentration of each individual SIL peptide to be spiked in the actual samples, ensuring their robust detection in watch mode measurement. The vast majority of peptides analyzed on a given chromatographic set up show a typical elution profile (0.5-min peak width with symmetric profile in the present study) allowing the definition of a generic peak width value. In few cases, peak tailing or peak splitting because of isomeric species fragmenting under undiscernible CID pattern (e.g. proline- or Asp-/isoAsp-containing peptides, (36)) can be observed and require an increase in the chromatographic peak width value associated to these peptides (i.e. up to 0.75 or 1 min).

All the information collected for SIL peptides, together with that of external landmark peptides (limited to reference MS/MS spectra and elution times), is translated into an IS-PRM acquisition method guiding the acquisition of SIL and corresponding endogenous peptides under the two PRM modes. In addition, a set of criteria is needed for the robust and reliable SIL peptide detection in real-time in order to trigger the measurement of the corresponding endogenous peptide. In the present study, it combined: (1) the detection of at least five out of the six most intense fragment ions of the reference MS/MS spectrum of a peptide, and (2) a high similarity fragmentation pattern of experimental and reference spectra (based on the five fragment ions with minimal degree of interference) as measured by a spectral contrast angle value below 11.5° (or above its cosinus-transformed value of 0.98) (34, 35). In addition, to avoid a triggering event at an early stage of the elution of internal standards (observed for particularly high-responding SIL peptides), an individual minimum intensity threshold on the most intense fragment ion of each SIL peptide is defined based on its estimated response factor and spiked-in concentration.

A proof-of-principle experiment was performed to benchmark the IS-PRM method with the regular PRM method analyzing 93 pairs of SIL and endogenous peptides (corresponding to 55 proteins) in a plasma sample in the context of a relative quantification experiment. Following the development scheme mentioned above, the spectral library for the 93 peptides was generated from the PRM analyses of the SIL peptides (low purity grade) on a Q-Exactive instrument. All the pertinent information captured for the development of acquisition methods is included in supplemental Data S1. This information was used to define the acquisition parameters of the regular PRM method consistent with a cycle time of less than 3 s over the entire LC separation (66-min gradient) for peptides targeted within 3-min monitoring windows (resolution of 17,500 (at m/z 200), transient length of 64 ms, fill time of 60 ms). For the IS-PRM method, the MS parameters were adapted according to its improved acquisition efficiency to maintain similar cycle times. The parameters are shown in the insert of Fig. 3. Briefly, the acquisition of SIL peptides in both modes of IS-PRM used the same MS parameter values as in regular PRM. The watch mode was operated using a 1-min

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**Fig. 3. Comparison of cycle times in PRM and IS-PRM analyses of 93 pairs of SIL and endogenous peptides in a plasma sample.** The cycle times observed over the entire analyses were plotted together with the corresponding number of peptides monitored in the regular PRM analysis. The average cycle time values between 15 and 52 min, corresponding to the elution range of 90 of the pairs of peptides were also displayed, corresponding to 0.9 and 1.2 s for PRM and IS-PRM, respectively. The acquisition parameters used in each method to measure SIL and endogenous peptides on a Q-Exactive Plus instrument were detailed.

| Method       | Acquisition parameters | SIL peptides | Endogenous peptides |
|--------------|------------------------|--------------|---------------------|
|              | Orbitrap Resolving power | Fill time | Orbitrap Resolving power | Fill time |
| Regular PRM  | 17500 | 60 ms | 17500 | 60 ms |
| IS-PRM       | 17500 | 60 ms | 70000 | 360 ms |

* Conventional orbitrap analyzer at m/z 200
dynamic monitoring window. The endogenous peptides were measured with increased transient and fill times (quantitative mode; resolution of 70,000 (at m/z 200), transient length of 256 ms, fill time of 360 ms). In this instance, the maximum fill time setting was not fully synchronized with the transient acquisition time as this would have induced deviation from the initial objective to operate the two methods using similar cycle times.

The IS-PRM analyses targeting the 93 pairs of SIL and endogenous peptides were performed on a plasma sample (500 ng protein/μl) with or without supplementation of the SIL peptides using a Q-Exactive Plus instrument. In the analysis of the plasma sample devoid of the SIL peptides, it was observed that the PRM acquisition was only performed in watch mode as no SIL peptides were detected under the established detection criteria to trigger a switch between the two modes. The replication of the analysis by removing the intensity threshold on the most intense transition from the detection criteria provided the same results, confirming the reliability of the detection method. In contrast, during IS-PRM analysis of the plasma sample supplemented with the SIL peptides (at a concentration ranging from 50 to 500 fmol/μl), all the internal standards were detected and did trigger acquisition in quantitative mode in the early part of their elution, which attests the robustness of the triggering process. Over the entire analysis, the instrument alternated as expected between the two modes according to the detection of eluted peptides. The cycle times observed over the entire analysis were plotted in Fig. 3 and compared with those of a regular PRM analysis of the same sample. For both methods, the cycle time did not exceed 3 s (maximum value of 2.8 s and 2.4 s for PRM and IS-PRM, respectively). The comparison of the average cycle time between 15 and 52 min, corresponding to the elution time range for 90 of the pairs of peptides targeted, resulted in a value of 0.9 s for IS-PRM against 1.2 s for regular PRM. This slight discrepancy is explained by the adjustment of parameter settings based on the assumption that the maximum fill time is systematically reached for all the peptides (before satisfying target AGC value) whereas it is actually not. This may result in an overestimated prediction of the cycle time value when the maximum fill time setting is not fully synchronized with the transient acquisition time setting, as it is the case with the measurement of endogenous peptides in the present IS-PRM method.

Analytical Performance of IS-PRM—Following the initial analyses of the 93 pairs of peptides, an experiment was developed to estimate the benefits of IS-PRM by comparing the limits of quantification achievable with PRM and the IS-PRM methods previously developed. This evaluation was performed by analyzing the dilution series of SIL peptides in a plasma matrix supplemented with amounts of the corresponding nonlabeled (light) synthetic peptides. The IS-PRM acquisition method was modified to use the fortified nonlabeled form of the peptides as internal standards triggering the measurement of SIL peptides in quantitative mode (supplemental Data S2). The dilution series was prepared to obtain SIL peptides (high purity grade) at concentrations ranging from 35 fmol/μl to 2 amol/μl in a plasma protein digest (0.5 μg/μl), accounting for ten dilution points and one matrix blank. The samples were analyzed in triplicate on a Q-Exactive Plus instrument using regular PRM and IS-PRM methods. In another series of experiments, the same dilution series were analyzed using selected reaction monitoring on a triple quadrupole instrument. In this case, for each peptide, six transitions were selected based on an independent method development. All the peptide chromatographic separations were performed using a 66-min LC gradient.

The data obtained by each technique were used to determine the limits of quantification (LOQ) of individual transitions (558 fragment ions selected/monitored, supplemental Data S3). In the 33 IS-PRM analyses (triplicated analyses of the ten dilution points and the matrix blank), the quantitative mode was properly triggered for all the 93 peptides. Despite the fact that PRM and SRM acquisition methods were developed independently, the vast majority of selected fragment ions/transitions were common (70%, corresponding to 389 out of 558 fragment ions). To keep the comparison consistent, solely the common fragment ions were taken into account. Fig. 4 shows for each dilution point the number of transitions allowing reliable quantification. As anticipated, data of high quality were generated for the measurement of endogenous peptides in the IS-PRM mode, which is reflected in dramatically improved sensitivity as compared with the other techniques. For instance, over 70% of the transitions presented quantifiable signals for the dilution point corresponding to 50 amol of spiked SIL peptides in IS-PRM analyses, outperforming the regular PRM and SRM analyses by a factor of four or five, respectively. The regular PRM method showed a benefit over the SRM but only to a limited extent, compared with previous studies (13, 16), in which smaller sets of peptides were ana-
lized and the acquisition parameters used (i.e. transient and fill times) were better suited. A similar trend was observed for the respective performance of the three techniques when the comparison was conducted on all the 558 transitions (supplemental Fig. S1).

A typical example is presented in Fig. 5, which displays for each technique the transition/fragment ion traces extracted for the measurement of the SIL peptide DGAGDVAFVK (representing human serotransferrin) spiked at 150 amol/μl in a plasma sample. The fragment ion signals acquired in IS-PRM analysis exhibited co-eluting profiles and high signal to noise ratios whereas those acquired in regular PRM analysis showed limited ion statistics and less consistent relative intensities. Most of the SRM transitions were heavily interfered with background signals, leading to an undistinguishable elution profile.

The data set generated on 93 SIL peptides in IS-PRM mode was used to further estimate the robustness and reliability of the acceptance criteria used for peptide detection in real-time. Using the same criteria, with the exception of the intensity threshold for the most intense transition, the assessment of the systematic detection of each SIL peptide was carried out postacquisition for the different dilution points analyzed in triplicate (supplemental Fig. S2 and supplemental Data S3). The analyses of the matrix blank, thus devoid of SIL peptides, did not exhibit any identification, confirming the reliability of the acceptance criteria. The first SIL peptide (APIIAVT, representing human pyruvate kinase) was systematically detected at a concentration of 5 amol/μl and the number of detected peptides consistently increased with their spiked-in concentration to reach a total number of 64 at a concentration of 50 amol/μl; this represents 69% of the SIL peptides, perfectly matching the proportion of the transitions above LOQ at same concentration (380 out of 558 transitions). This observation was generalized for larger concentrations of SIL peptides. Thus, the acceptance criteria presently used for peptide identification assessment can be used to provide acceptable first estimation of quantifiable peptides in this proof-of-principle study. A more systematic investigation, including refined parameters and thresholds, was the object of another study to be published elsewhere.

Application of IS-PRM Method to Large-Scale Quantification Experiments—In a second experiment, the new acquisition method was tested on a larger scale to evaluate its limits. A total of 606 pairs of SIL and endogenous peptides representing 338 human proteins of potential interest in different clinical contexts were included in the target list to perform the proof-of-principle while reflecting the common constraints of screening experiments. To ensure an unbiased evaluation, the analyses were carried out in various matrices, including human bodily fluids (blood and urine) as well as a HeLa cell extract, in which the selected proteins are expected to cover a wide range of abundance. This study was performed using the latest generation of quadrupole-orbitrap instrument (Q-Exactive HF) equipped with a high-field orbitrap analyzer, which has the ability to acquire ultra-short transients (32 ms, corresponding to a resolving power of 15,000 at m/z 200). This represents an immediate benefit for the analyses of large data sets.

The IS-PRM method, as previously described, included the generation of a spectral library and the pertinent information related to the SIL peptides of interest from analyses performed on a Q-Exactive instrument (supplemental Data S4). The acquisition parameters of the IS-PRM method were optimized to minimize the time devoted to PRM scans in watch mode (resolution of 15,000 at m/z 200, transient length of 32 ms, fill time of 20 ms) while maintaining sufficient quality on data acquired for the endogenous peptides in quantitative

![Fig. 5. Comparison of the quality of data generated by SRM, regular PRM, and IS-PRM analyses of a dilution series of 93 SIL peptides spiked in a plasma sample.](image-url)
mode (resolution of 60,000 (at \(m/z\) 200), transient length of 128 ms, fill time of 110 ms) with the SIL peptides being measured using the same parameters in both modes (resolution of 15,000 (at \(m/z\) 200), transient length of 32 ms, fill time of 20 ms). Furthermore, two variants of the regular PRM method were designed to measure both SIL and endogenous peptides within 2-min monitoring windows. The first variant (PRM-Method A) used acquisition parameters to enhance the selectivity and sensitivity of measurements, that is, the same parameters as those used to measure endogenous peptides in the IS-PRM method (resolution of 60,000 (at \(m/z\) 200), transient length of 128 ms, fill time of 110 ms), which translated in a cycle time extension. The second variant (PRM-Method B) used less time consuming parameters, that is, those used to measure SIL peptides in the IS-PRM method (resolution of 15,000 (at \(m/z\) 200), transient length of 32 ms, fill time of 20 ms), to maintain an acceptable cycle time, albeit at the expense of the sensitivity and selectivity of measurements. The list of parameters is included in Table I.

A plasma sample (at 500 ng/\(\mu\)L) was supplemented with the 606 SIL peptides of interest (at a concentration ranging from 50 to 500 fmol/\(\mu\)L) and was analyzed in triplicate using the IS-PRM and the two regular PRM methods on a Q-Exactive HF instrument. For each technique, the endogenous peptides systematically detected during triplicated analyses (with 302 peptides detected in at least one analysis) outperforming the regular PRM-Method A and especially the regular PRM-Method B that resulted in the systematic detection of only 203 and 129 peptides, respectively. It is noteworthy that all the peptides systematically detected by regular PRM analyses were included in the IS-PRM dataset. More importantly, though all these quantifiable peptides were measured with good precision (CV < 20%) by IS-PRM and PRM-Method B, only 124 out of the 203 peptides presented this level of precision during the PRM-Method A analyses (Table I). Consequently, there was a twofold gain in the number of peptides reliably quantified by IS-PRM analyses as compared with the two other methods.

These significant differences in quantification performance for each technique/method is, as anticipated, explained by the specific settings of their acquisition parameters and their impact on the cycle time. On one hand, the decreased resolution and fill times used in PRM-Method B directly resulted in lower detection capabilities. On the other hand, maintaining the cycle time at an acceptable level was critical to ensure precise quantitative measurements. For IS-PRM and PRM-Method B, the average value of the cycle time observed between 15 and 52 min, corresponding to the elution time range of 578 pairs of SIL and endogenous peptides, was estimated at 2 and 2.8 s, respectively (supplemental Fig. S3). By contrast, in the analysis performed using PRM-Method A, the estimated average value of 8 s did not allow efficient LC sampling and resulted in undetected endogenous peptides compared with the IS-PRM method in addition to an altered measurement precision. A typical example is illustrated by the analysis of the endogenous peptide LTVGAAQVPAQLL-VGALR, surrogate of human monocyte differentiation antigen CD14, using each technique/method (Fig. 6). The peptide was systematically detected during triplicated analyses using IS-PRM and PRM-Method A but did not satisfy the acceptance criteria during analyses using PRM-Method B because of the low signal-to-noise ratio or the nondetection of its fragment ions. However, only IS-PRM analyses generated quantitative data of sufficient precision (CV of 5%). The results obtained by PRM-Method A were significantly affected by a long cycle time (more than 6 s) translating into the collection of only five data points over the elution profile of the peptide, which is insufficient for a proper description, resulting in a substantial variation between replicates (CV of 23%), despite the use of internal standards.

### Table I

Detection and quantification results obtained for triplicated IS-PRM and regular PRM (PRM-method A and PRM-method B) analyses of 606 pairs of SIL and endogenous peptides in a plasma sample with associated acquisition parameters.

| Method       | SIL peptides | Endogenous peptides | Number of peptides systematically identified\(^a\) | Number of peptides reliably quantified\(^b\) |
|--------------|--------------|---------------------|-----------------------------------------------|-----------------------------------------------|
|              | Orbitrap resolving power\(^c\) | Fill time | Orbitrap resolving power\(^c\) | Fill time |                       |                       |
| IS-PRM       | 15000        | 20 ms               | 60000                                       | 110 ms                          | 251                       | 251                       |
| PRM-Method A | 60000        | 110 ms              | 60000                                       | 110 ms                          | 203                       | 124                       |
| PRM-Method B | 15000        | 20 ms               | 15000                                       | 20 ms                           | 129                       | 129                       |

\(^a\) Endogenous peptides in triplicated analyses of plasma sample.
\(^b\) Coefficient of variation below 20%.
\(^c\) High field orbitrap analyzer at \(m/z\) 200.
Additional analyses were performed to further confirm the broad applicability of the large-scale IS-PRM method and to assess that the nondetected portion of the endogenous peptides set corresponded to extremely low-abundance plasma proteins. These IS-PRM analyses were performed using the same acquisition method in a urine sample and a HeLa cell digest, supplemented with the same mixture of 606 SIL peptides. As for the plasma sample, all 606 endogenous peptides were monitored in both samples, as triggered by real-time detection of their internal standards. The triplicated analyses corroborated the robustness of the acquisition method. The success in detecting the endogenous peptides was similar as in plasma sample analyses. A total of 312 (349 in at least one analysis) and 309 endogenous peptides (335 in at least one analysis) were systematically detected in the urine and HeLa cell samples, respectively. However, combining the data sets for the three samples, 525 peptides were detected (corresponding to 300 proteins), among which only 130 were common to all the samples as illustrated in supplemental Fig. S4A. The limited overlap between the sets of peptides present in the various samples is actually not surprising considering their different nature, and that proteins can exhibit different abundances in these samples. This is illustrated by the large differences in the peak areas of the endogenous peptides measured in the different samples (supplemental Data S5), as shown in the heatmap in supplemental Fig. S4B. In the examples presented in supplemental Fig. S4C, the peptides exhibited a decrease in their peak area by several orders of magnitude (up to 5000-fold decrease) across the samples, translating for the most unfavorable cases into a reliable detection limited to a single sample. The detection of a larger portion of the targeted endogenous peptides in a single run was shown through an additional IS-PRM experiment applied to a chimeric sample, prepared by mixing the three initial samples in equal proportion. The results of the triplicated IS-PRM analyses were in close agreement with expectations. A total of 487 endogenous peptides were detected in at least one analysis (455 peptides systematically detected), including most of the peptides initially detected in a single sample, which covered 92% of all the peptides from the combined data sets of individual sample analyses. In addition, the peptide peak areas generated from this experiment were very similar to the values predicted from the results obtained by analyzing individual samples (supplemental Data S5) as shown in the heatmaps in supplemental Fig. S5. This provides definitive evidence of the robustness and the efficiency of the IS-PRM method.

CONCLUSION AND OUTLOOK

A novel PRM acquisition method, called internal standard triggered – parallel reaction monitoring, was designed to expand the number of targeted peptides included in a single experiment, whereas retaining the high analytical performance usually achieved in a conventional, small-scale, PRM study. The new acquisition scheme was developed to overcome the inefficiency of current time-scheduled targeted methods by using internal standards to drive in real-time the measurement of endogenous peptides and in turn to optimize the acquisition parameters. This translated into an alternating operation of the instrument between a watch mode and a quantitative mode according to the actual elution of the targeted peptides, using different sets of acquisition parameters. The former mode favored speed and the latter mode data quality.

The new method maximized the efficient use of the instrument time (close to 100%), which was leveraged to increase both the number of endogenous peptides targeted.
in one experiment and the MS acquisition time devoted to their measurement, and thus the data quality. Applied to the measurement of moderate-to-large peptide sets in complex samples, the improved data quality obtained by IS-PRM analyses was reflected by lower limits of detection and quantification (typically in the low amol range) as compared with those obtained with SRM and conventional PRM analyses. In its largest-scale format, the IS-PRM method took advantage of the increased scanning speed of the new quadrupole-orbitrap instrument equipped with a high-field orbitrap analyzer to target more than 600 endogenous peptides in a single experiment using a 66-min LC gradient.

The implementation of the IS-PRM technique was performed following a well-defined scheme focused on robustness. In the present account, the method included the generation of a spectral library and the associated attributes pertinent to an IS-PRM experiment, whereas ensuring portability across multiple instruments. This ultimately ensures that analyses can be replicated over periods of several months across different types of proteomic samples, without the need to adjust the acquisition methods.

The robust analytical performance of the technique, based on the efficient use of the instrument time and combined with its generic applicability to a variety of samples, will improve targeted quantitative proteomics, including a wide range of applications (from large scale screening experiments to precise quantification of a limited panel of peptides). For instance, the IS-PRM method has the potential to improve quantitative experiments in the context of biomarker development, in which large panels of peptides can be explored and significant coverage of proteins achieved (to also detect isoforms or modifications). Also, the method will find application in biological studies where targeted analyses are used to perform directed discovery experiments to complement DDA or DIA methods, with the immediate benefit of obtaining precise quantitative results to address a specific biological question (e.g. pathway monitoring), difficult to achieve by other means.

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