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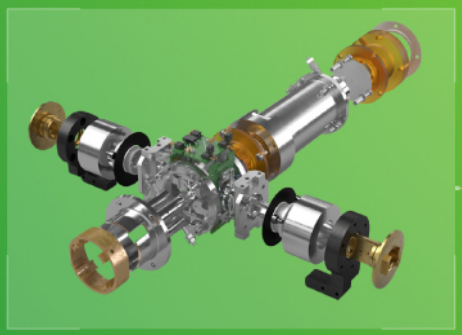

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REVIEW

Parallel reaction monitoring using quadrupole-Orbitrap mass spectrometer: Principle and applications

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Targeted mass spectrometry-based approaches are nowadays widely used for quantitative proteomics studies and more recently have been implemented on high resolution/accurate mass (HRAM) instruments resulting in a considerable performance improvement. More specifically, the parallel reaction monitoring technique (PRM) performed on quadrupole-Orbitrap mass spectrometers, leveraging the high resolution and trapping capabilities of the instrument, offers a clear advantage over the conventional selected reaction monitoring (SRM) measurements executed on triple quadrupole instruments. Analyses performed in HRAM mode allow for an improved discrimination between signals derived from analytes and those resulting from matrix interferences translating in the reliable quantification of low abundance components. The purpose of the study defines various implementation schemes of PRM, namely: (i) exploratory experiments assessing the detectability of very large sets of peptides (100–1000), (ii) wide-screen analyses using (crude) internal standards to obtain statistically meaningful (relative) quantitative analyses, and (iii) precise/accurate quantification of a limited number of analytes using calibrated internal standards. Each of the three implementation schemes requires specific acquisition methods with defined parameters to appropriately control the acquisition during the actual peptide elution. This tutorial describes the different PRM approaches and discusses their benefits and limitations in terms of quantification performance and confidence in analyte identification.

Keywords:
High resolution accurate mass / Parallel reaction monitoring / Precise quantification / Targeted proteomics / Technology

1 Introduction

MS has been established as a corner stone of proteomics for both qualitative and quantitative analyses. Two main approaches have emerged over the years: shotgun methods frequently applied to discovery experiments and targeted methodologies for quantitative applications [1]. Discovery studies performed in a data dependent acquisition (DDA) mode have allowed the identification of thousands of proteins in biological samples [2, 3]. In such experiments, a series of precursor ions selected from a survey scan based on their signal intensities undergo fragmentation and are identified by means of a database search or spectral matching procedures. The latest generation of hybrid mass spectrometers, with fast and sensitive acquisition capabilities in MS/MS mode, has increased the routinely achievable proteome coverage [4–8]. However, such DDA experiments still suffer from an under sampling problem due to the persistent mismatch between the peak capacity of LC-MS(/MS) platforms and the complexity of biological samples, resulting in an under representation of low abundance analytes [9]. In addition, only a fraction of the acquired MS/MS spectra yields a positive identification [10], typically in the range of 25–30% and at best 40% as

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Abbreviations: **AUC**, area under the curve; **DDA**, data dependent acquisition; **DIA**, data independent acquisition; **HRAM**, high resolution/accurate mass; **IS-PRM**, internal standard triggered-parallel reaction monitoring; **NSCLC**, non-small-cell lung cancer; **SIL**, stable isotopically labeled

recently reported [5]. The quantification of the analytes, mainly performed at the precursor ion level, is a limiting factor in DDA experiments especially for low abundance analytes.

More recently, data-independent acquisition (DIA) was proposed as an alternative to perform both qualitative and quantitative analyses [11]. This acquisition is unbiased and relies on the systematic and sequential isolation and fragmentation of precursor ions using wide isolation windows (e.g., around 20 *m/z* units in the SWATH implementation) to cover the entire *m/z* range and thus create a digital map [12, 13]. The identification is based on a reference spectral library and the quantification of peptides is based on the fragment ions derived from all precursor ions included in the isolation window. This technique enables large-scale quantitative studies at the MS/MS level but requires advanced data processing methods [12], including extended spectral libraries. Although DIA experiments are well suited for sensitive quantification studies, in samples of moderate complexity the convolution of signals from multiple precursors can lead to interferences, especially in complex samples [14].

To overcome some of these issues, targeted methods were developed to systematically and precisely quantify well-defined sets of peptides in complex samples. This strategy allows the consistent monitoring of peptides with a high degree of specificity and sensitivity. In contrast to unsupervised approaches, targeted methods are hypothesis-driven and require the careful selection of the peptides used as surrogates for proteins [15]. The analyses are commonly performed in selected reaction monitoring mode [16] on triple quadrupole mass spectrometers. In such experiments, predefined pairs of precursor/fragment ions (called transitions) are sequentially measured. Although well adapted to obtain precise quantification over a wide dynamic range, the low resolution of both Q1 and Q3 mass analyzers can result in interfering signals during the analyses of complex samples biasing the end results [17, 18]. Furthermore, the number of targeted peptides in an experiment is limited to ensure the specificity of the measurements (i.e., acquisition of multiple transitions) while keeping an adequate cycle time to sufficiently sample the elution profiles of the peptides. This has prompted the development of time-scheduled SRM acquisitions, which involve the segmentation of the chromatographic separation time. In this mode, peptides are only monitored during a time window around their actual elution (typically 2 to 4 min), which increases dramatically the number of peptides monitored in one analysis while not compromising the precision of measurement [19].

More recently, the emergence of fast scanning HRAM instruments also found application in quantitative studies and has led to the development of a novel paradigm for targeted assays. Taking advantage of the new features of these mass spectrometers, the parallel reaction monitoring mode was designed [20, 21]. In a PRM event, all ions resulting from the fragmentation of a single, or several, precursor ions are measured simultaneously in one MS/MS scan. The technique represents a true alternative to SRM for quantitative analyses, especially in complex samples such as bodily fluids [20, 22], and its application in the field of proteomics has progressively gained attention [21–26].

The versatility of the PRM technique has led to its implementation in various situations, which has required the development of dedicated methods in line with the purpose of the specific experiments. The key factors to be considered in designing an acquisition method include: the number of peptides to be analyzed, the desired level of sensitivity, the precision, and the accuracy of the results. The necessity of fit-for-purpose approaches to conduct targeted proteomics experiments was previously outlined [27], leading to the development of specific PRM method counterparts. First, exploratory studies aim to assess the detectability of peptides on a large scale (100–1000 analytes) in LC-MS analyses across a limited set of clinical samples; there are also used to estimate relative changes between different biological conditions or clinical statuses. Second, wide-screen experiments attempt to perform relative quantification of a large number of analytes (50 to 200) with precision using standard purity isotopically labeled peptides in samples of large cohorts. Third, precise and accurate quantification methods aim at monitoring a limited panel of peptides using calibrated amounts of high purity internal standards in a large number of samples. This tutorial provides a description of the PRM technique applied to proteomic studies and the impact of all critical parameters on quantification results, with a strong focus on the implementation on quadrupole-Orbitrap instruments. It provides the baseline to design appropriate acquisition methods to fit the purpose of the intended experiment.

2 Principle

PRM experiments are typically performed on the latest generation HRAM hybrid instruments, namely the quadrupole time-of-flight (Q-TOF) and the quadrupole-Orbitrap mass spectrometers. Such instruments exhibit a high analytical performance, especially in complex samples where selectivity is required to distinguish analyte fragment ions from background signals. The quadrupole-Orbitrap instrument offers specific trapping capabilities, which improve the analysis of low abundance peptides [28]. This tutorial will focus on the implementation of PRM on quadrupole-Orbitrap mass spectrometers, but PRM applied on a quadrupole time-of-flight instrument has been reported [24].

Targeted analyses are by nature hypothesis-driven and the experimental design of peptide- and PTM-centric PRM assays is straightforward [25, 29–31]. However, it includes additional steps for protein-based assays, which comprises: (i) the definition of the proteins of interest to answer a biological or clinical question and (ii) the selection of the best surrogate peptides representing these proteins using the same protocols established for SRM (Fig. 1).

During PRM analyses, full MS/MS spectra are acquired and only limited information regarding the targeted analytes

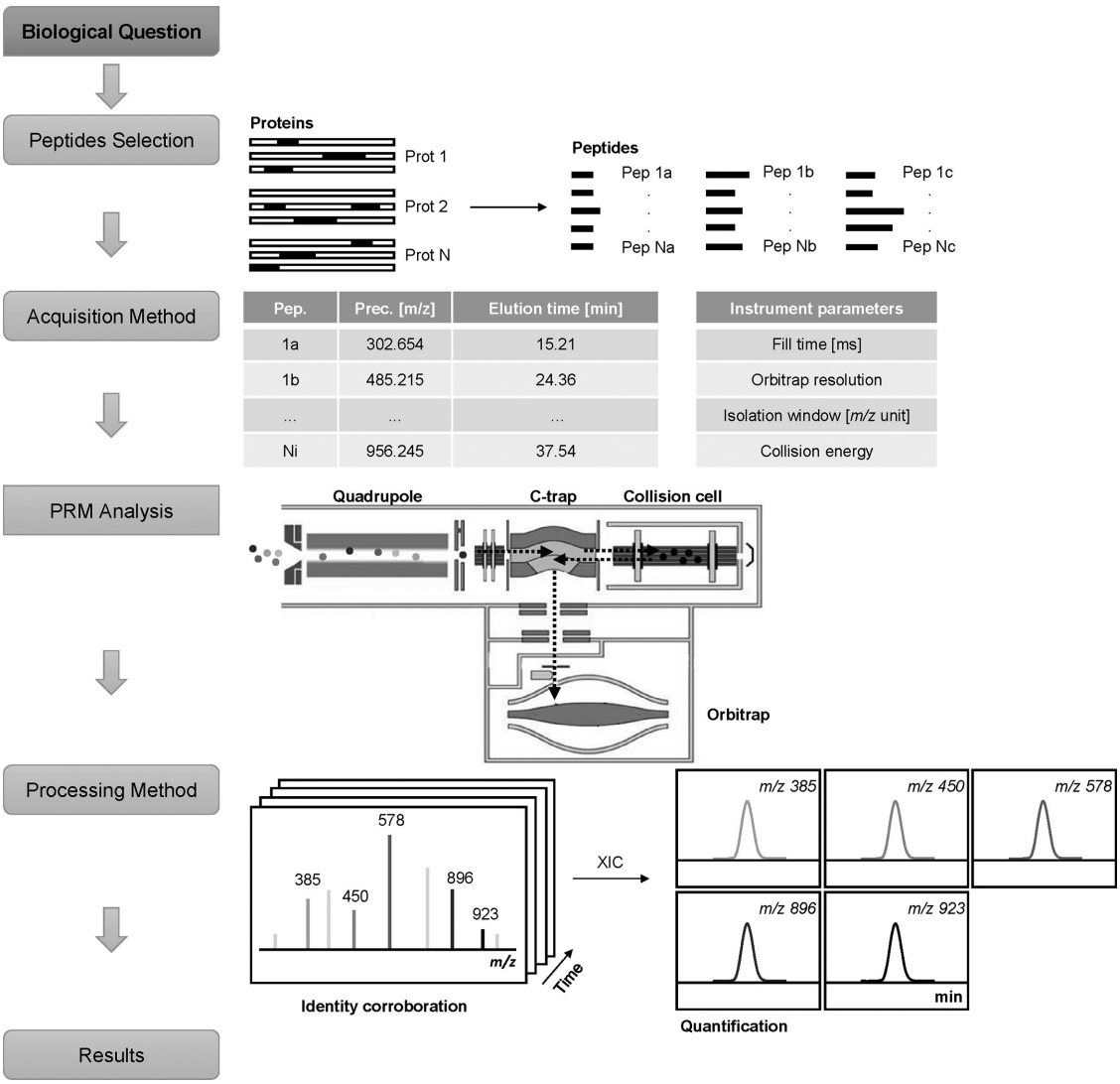

Figure 1. Parallel reaction monitoring experimental workflow. First, a set of peptides, used as surrogates for the selected proteins, is defined. Second, an acquisition method is created, including the definition of the precursor ion *m/z,* the elution time of the peptides, and the instrument parameters such as the width of quadrupole isolation window, the maximum fill time, and the nominal Orbitrap resolution. Third, the targeted LC-MS/MS analysis is carried out. Fourth, the data are processed post-acquisition to confirm the identity of the targeted peptides and to determine the abundance of the analytes based on the extracted-ion-chromatograms (XIC). Figure adapted from [37].

(precursor ion *m/z* and optionally expected elution time range) are necessary prior to acquisition. In addition, the acquisition method requires the definition of specific instrumental parameters including the quadrupole isolation window width, the maximum fill time, and the Orbitrap resolving power. These parameters are dependent on the scale of the experiment and the desired analytical performance of the study. Moreover, peptide monitoring windows are defined for time-scheduled PRM acquisition.

Similar to any targeted experiment, in PRM analysis the predefined precursor ions are isolated, during their monitored chromatographic elution, in the quadrupole mass filter based on the preset isolation window width. Then, the precursor ions are transferred into the collision cell and fragmented at the default collision energy (e.g., normalized collision energy of 25 applied to all precursors), or at their optimal collision energy (i.e., optimized for each individual precursor). The duration of this process is controlled by the automatic gain control and the maximum fill time. The resulting fragment ions are transferred back to the C-trap and pushed to the Orbitrap for mass analysis as previously described [20]. The time spent to analyze the ions in the Orbitrap, called transient time, is determined by the predefined operating resolving power. Full MS/MS spectra are systematically acquired (typically every two or three seconds) for each precursor ion fragmented during the entire elution window and

the recorded data are stored. A full MS spectrum is typically included between each cycle and used to drive the fill time actually used in PRM acquisition according to the automatic gain control and maximum fill time settings. The implementation of PRM is similar on a Q-TOF instrument but with the Orbitrap substituted by a TOF analyzer [24]. In this case, the duration of the acquisition process dedicated to each peptide is controlled by the accumulation time (i.e., the number of individual scans acquired and summed to generate the MS/MS spectra).

The data processing, which occurs post-acquisition, is an important step of PRM analyses and the comprehensive data sets acquired and stored allow flexible and iterative data interrogation. Furthermore, the MS/MS fragmentation patterns are employed to confirm the identification of the analytes in the initial stage, while quantification is performed on the traces extracted from those fragments yielding the highest sensitivity and selectivity. Therefore it represents a major shift in targeted analyses as identity confirmation is a prerequisite for quantification. The signals generated by high resolution MS are less prone to interferences and therefore do not require extensive post-acquisition data processing.

3 Acquisition parameters

The analyses in PRM take advantage of the specific features of the quadrupole-Orbitrap instrument which are the high resolution of the Orbitrap mass analyzer, the filtering aptitudes of the quadrupole at the front-end, and the multiplexing capabilities of the C-trap and the collision cell.

3.1 Quadrupole isolation window

The quadrupole isolation window width directly controls the selectivity of the measurements, especially in case of high complexity samples [32]. Narrowing the isolation window enables the removal of interferences around the precursor ions of interest but may induce a decrease in ion transmission. In practice a default value of 1 m/z unit is used on second generation quadrupole-Orbitrap instruments (i.e., Q-Exactive Plus and Q-Exactive HF) as it properly balances selectivity and ion transmission. In some instances, challenging measurements may benefit from an ad-hoc optimization, i.e., narrowing further the precursor isolation window to exclude specific matrix interferences.

3.2 Orbitrap resolving power

The high resolving power of the Orbitrap mass analyzer provides a significant improvement on the selectivity of measurements. Thus, low limits of quantification and detection are generally reached in spite of the high complexity of the samples, especially for the highest values of transient time. This is explained by the theoretical increase in S/N ratio in proportion to the square root of transient time in the Fourier transform spectra [33] but also, more importantly in complex samples, by the discrimination of nearly isobaric product ions generated from co-isolated precursors. The tight link between selectivity and the Orbitrap resolving power has been demonstrated by analyzing 122 isotopically labeled peptides spiked into a urine background with progressively increased resolution values and thus longer transient times [34]. The proportion of fragments showing interferences was decreased by a factor of two and three by increasing the resolution from 17 500 to 35 000 and to 70 000, respectively.

3.3 Fill time

The fill time settings directly impact the sensitivity and the dynamic range of PRM measurements [32]. This was illustrated by the analysis of a dilution series of eight isotopically labeled peptides spiked into various amounts of a yeast tryptic digest using fill times ranging from 10 to 500 ms and by estimating the limits of quantification of each fragment (six fragment ions evaluated for each peptide) associated with the various settings. The sensitivity was notably enhanced by sampling ions over long fill times, as reflected by the lower minimum amount of peptides required for proper measurement. For instance, 50% of the evaluated fragments allowed reliable quantification of peptides at amounts of 450, 150, and 50 amol using fill times of 30, 60–120, and 250 ms, respectively.

4 Acquisition methods

The time devoted to the actual measurement of each peptide (defined by the transient and fill times) is essential for the quality of the data generated, but this will constrain the number of peptides included in one experiment. In practice, several peptides from the full set of targets are likely to have similar elution times and are thus measured within the same PRM acquisition cycle. The cycle time is defined, based on the chromatographic separation, to collect a sufficient number of data points over the elution profile of each peptide monitored (typically eight to ten points, resulting in a 2.5 s cycle time for a 20–30 s chromatographic peak width). Thus, two distinct schemes, in which priority is given either to the scale of the experiment (number of analytes) or the intended data quality (selectivity and sensitivity of measurements), can be followed relying on the adjustment and tight control of the acquisition time or of the number of monitored peptides, respectively. Depending on the purpose of the experiment specific instrument settings are chosen.

4.1 Default acquisition method

The default method consists in the application of the above described acquisition protocol (i.e., the consecutive isolation and

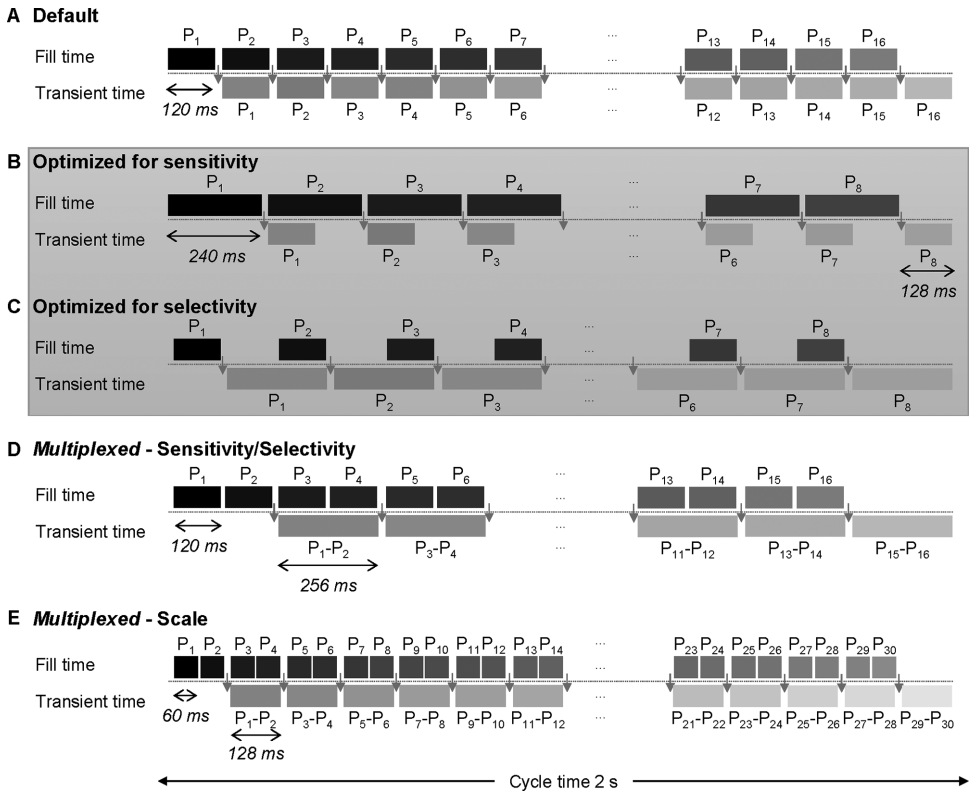

Figure 2. PRM acquisition methods. (A) The default data acquisition method relies on the sequential analysis of co-eluting peptides: One precursor ion is isolated and fragmented in the collision cell, while the fragment ions MS/MS spectrum of the previous precursor ion is collected in the Orbitrap. The maximum fill time is set to match the transient time, e.g., a fill time of 110–120 ms for a transient time of 128 ms corresponding to a 35 000 resolution. (B) An increased maximum fill time boosts the sensitivity of the measurements. (C) An increased transient time improves the selectivity. Both schemes, B and C, are not efficient due to lack of synchronization between the two events and therefore avoided in practice. (D–E) In the *multiplexed* acquisition method, the co-eluting precursor ions (duplex in this instance, e. g., endo and SIL peptide pair) are sequentially isolated and fragmented in the collision cell. They are transferred collectively to the Orbitrap to collect a composite MS/MS spectrum. Different set-ups can be considered by adjusting maximum fill time and transient time to favor either data quality (i.e., enhanced sensitivity and selectivity of measurements, D) or scale of the experiment (i.e., increased number of targeted peptides, E).

fragmentation of the precursor followed by the measurement of its product ions) to each peptide sequentially as shown in Fig. 2A. As stated previously, both fill time and transient time can be individually optimized to increase either the sensitivity or the selectivity of measurements, respectively (Fig. 2B and C). However, such optimization schemes are suboptimal and therefore avoided in practice as both events take place in parallel, i.e., the isolation and fragmentation of the precursor ion of a given peptide occur while the product ions generated from the peptide fragmented in the previous event are recorded. Consequently, the maximum fill time is typically adjusted according to the Orbitrap resolution employed and the MS acquisition time of each peptide can be approximated to the transient time. The acquisition parameters are thus specifically tuned to fulfill the purpose of the experiment by applying the simple relationship "cycle time = number of active peptides x transient time". For precise quantification experiments the fill time and transient time can be maximized but at the expense of the number of peptides targeted in the same cycle. The default parameters proposed for experiments carried out on the Q-Exactive instrument are; a maximum fill time of 100–120 ms and a resolving power of 35 000 (transient time of 128 ms). This corresponds to the acquisition of about 15 peptides for a cycle time of 2 s. With the default method the maximum number of peptides monitored within a cycle is limited by the shortest transient time available on the instrument used (32 and 64 ms for a resolving power of 15 000 and 17 500 on Q-Exactive HF and Q-Exactive Plus, respectively). This bottleneck can be overcome by using the multiplexing capabilities of the trapping device.

4.2 Multiplexed acquisition method

The *multiplexed* acquisition mode relies on the sequential isolation of co-eluting peptides using narrow isolation windows prior to their sequential fragmentation in the collision cell.

All the generated fragment ions are trapped and accumulated together and subsequently a single combined MS/MS spectrum is acquired in the Orbitrap. It is well adapted for quantitative experiments in which the endogenous and internal standard peptides are analyzed concomitantly. In such an experiment the maximum fill time for each peptide is adjusted according to the transient time and the multiplexing degree following the relationship "maximum fill time = transient time / multiplexing degree". Thus, the *multiplexed* mode enables the use of the same Orbitrap resolving power, as shown in the example of Fig. 2C, while increasing the scale of the experiment (Fig. 2D). Further expansion in the number of targets measured within one LC-MS run can be achieved in multiplexed acquisition by reducing the maximum fill time and transient time, thus at the expense of the sensitivity and selectivity of measurements (Fig. 2E). The fact that only y-type fragment ions remain exclusively associated to each form of the peptide pairs for commonly used stable isotopically labeled (SIL) peptides with ^{15}N- and ^{13}C-labeling has limited impact on quantification performance, due to their prevalence in HCD fragmentation spectra [35].

4.3 Peptide elution windows

The analyses in PRM mode rely on time-scheduled events in which the peptides are monitored over pre-defined windows around their predicted elution time. Each segment contains only a subset of peptides limiting the number of PRM events in each acquisition cycle. This time restriction has an immediate benefit on the total number of peptides measured during the entire chromatographic run, which is proportional to the elution monitoring window narrowness. Monitoring windows should ideally be set so that they match exactly the duration of peptide elution (20–30 s in the chromatographic set-up defined above) and thus capture all informative data. Nonetheless, relatively longer monitoring windows are often employed in practice (up to 3–4 min) to accommodate shifts in elution time, which are common on nano LC set ups [19]. Narrowed monitoring windows can be achieved by a tight control of the predicted peptide elution time, by an "on-the-fly" detection of landmark peptides and correction in-real time of the scheduled elution times [32].

5 Data processing

The data acquired and stored during PRM analyses on quadrupole-Orbitrap instruments are processed using proprietary software packages Xcalibur (Thermo Scientific) and Pinpoint (Thermo Scientific) or multi-platform software solutions such as Skyline [36] or SpectroDive (Biognosys AG). In the different steps of the workflow, ion chromatograms and intensities of fragment ions are extracted from tandem mass spectra using a narrow mass tolerance defined by the operator (typically 2–20 ppm) or calculated from the Orbitrap resolving power actually used in the analyses to match ion peak width (for Skyline). Some intended operations have required the development of specific tools as they were not available in standard software packages (e.g., spectral matching carried out on individual MS/MS spectra).

The data processing workflow typically uses a three-step process. First, the identity of a peptide is corroborated by evaluating the similarity between the MS/MS spectra (collected multiple times across its elution profile) and its corresponding reference spectrum stored in a library. A local generation of the reference MS/MS spectra is preferable as the spectral matching strategy required well-controlled experimental conditions to ensure reproducibility and comprehensive collection of the fragmentation patterns. The constitution of such high-quality libraries, typically achieved by the analyses of small sets of synthetic peptides (10–20 peptides), requires a substantial effort which is however recouped on the long term as they are used in a multitude of studies. The spectral matching of the fragmentation patterns (i.e., relative intensities of fragment ions) is typically evaluated using the five to eight most intense fragments. The spectral contrast angle (θ) estimates the spectral similarity and is defined as: $\theta = \cos^{-1}(\sum(I_{\exp_i} \times I_{\mathrm{ref}_i})/(\sqrt{\sum(I_{\exp_i})^2} \times \sqrt{\sum(I_{\mathrm{ref}_i})^2})$ [37] (where I_{exp_i} and I_{ref_i} are the intensities of the fragment ions in the experimental and the reference MS/MS spectra, respectively). If internal standards (SIL peptides) are present in the sample, they can be used as an additional factor to support the identity confirmation of the corresponding endogenous forms, based on their co-elution. The conventional database search used for DDA data analysis, in which experimental MS/MS spectra are compared with in silico-generated spectra is not always very effective to evaluate PRM data, as illustrated in Fig. 3. Both strategies were applied to process PRM data generated from the analysis of a dilution series of 778 SIL peptides spiked in a yeast tryptic digest at concentrations ranging from 50 amol/μL to 37 fmol/μL. At the highest concentration, a similar success in detecting the peptides was achieved by both approaches, as reflected by the 652 and 743 (95%) peptides unambiguously detected by the database search strategy and the spectral matching approach, respectively. For peptides present in low amounts the spectral matching approach was definitively more robust. At 50 amol, the identity of 108 peptides was unambiguously corroborated by spectral matching, whereas only nine of them were reliably identified using a conventional database search (Mascot). In complex samples, precursor ions are often isolated with significant amounts of background ions leading to convoluted MS/MS spectra, which can however be resolved by spectral matching.

In the second step, for the peptides successfully detected (confirmed identities), the peak purity of their pertinent fragment ions is assessed prior to quantification in order to exclude from the process those showing interferences. The evaluation typically relies on the systematic assessment of the relative intensities of the fragment ions with regard to their

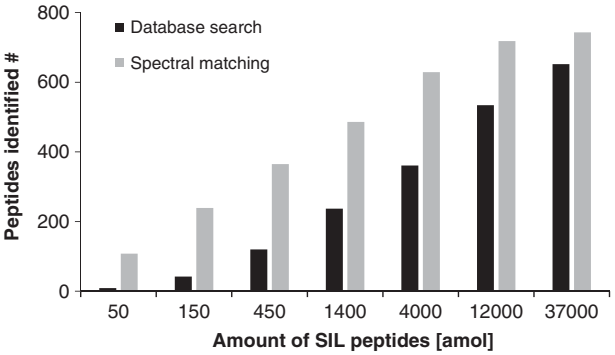

Figure 3. Comparison of approaches for detecting and assessing the identity of the peptides. Several dilution series of 778 SIL peptides, corresponding to human endogenous peptides, were spiked in a yeast lysate tryptic digest, and analyzed by PRM. The data were processed by both conventional database search using Mascot and spectral matching. The spectral matching was performed using a high-quality spectral library generated in-house. Mascot database searches were performed against a home-made database only including the sequences of the entire set of peptides targeted in the experiment (778 entries), using a mass tolerance of 0.5 and 0.01 Da for MS and MS/MS data, respectively, pertinent ^{15}N- and ^{13}C-labeled lysine and arginine residues as variable modifications, and carbamidomethylation of cysteines as fixed modification. Spectral matching and Mascot database search results were filtered using a maximum spectral contrast angle of 12° and a minimum Mascot ion score of 10, respectively, in order to maximize the number of SIL peptides detected while avoiding any false positive identifications. These criteria ensured none positive detection when processing the data of the analysis of the yeast lysate digest sample devoided of SIL peptides. The MS files have been deposited to the ProteomeXchange Consortium [48] via the PRIDE partner repository [49] with the data set identifier PXD003781.

reference over the elution of the analytes [17,34,38–42]. In the case of experiments using internal standards (SIL peptides), both peptide forms are evaluated as possibly affected by interferences. Several implementations are possible, which have been described in detail recently [37]. Briefly, one of these implementations relies on the determination of the deviation in fragment ion intensities in the MS/MS spectra collected relatively to the reference and the application of threshold values qualifying ions with acceptable peak purity. The stringency of the threshold values are adjusted according to the type of experiments, which requires different confidence levels in the quantification results (e.g., fragment ion intensity deviation systematically within +/− 30% in accurate quantification experiments).

In the last step of the data processing, which is the actual quantification, only the traces of one or several fragment ions qualified with acceptable peak purity are extracted and integrated for each peptide, including internal standards. This enables to restrict the volume of data to be processed. In exploratory studies, the peak heights can be used as a proxy of peptide abundance (discussed below). Alternatively, steps two and three can be combined by treating the signal of a more extended set of fragments and applying the quality control on the composite MS/MS spectra reconstructed from the area under the curve (AUC) of the fragment ions. This operation can be readily achieved by Skyline [36] optionally used in combination with external tools such as QuaSar [43] or mProphet [44].

6 PRM method for specific applications

The interdependence between the scale of a targeted experiment and the quality of the data generated (*vide supra*) has prompted the design of different methods to fulfill specific requirements depending on the scope and purpose of the experiment. Three scenarios for PRM have been envisioned for exploratory-, wide-screen-, and accurate quantification- experiments, which take into account scale and data quality, and the potential use of internal standards to achieve the desired level of analytical precision.

6.1 Exploratory studies

These studies aim at assessing the detectability of as many peptides as possible and at estimating the magnitude of changes between, for example, different clinical statuses or biological conditions, to ultimately define a pertinent subset to be further investigated. The detection of targets is the primary objective, ideally exceeding one thousand peptides, by adjusting the acquisition setting accordingly. It includes adequate maximum fill time and resolving power, and narrow elution time monitoring windows, which can be even shorter than the elution duration (corrected in real-time). The quantification in exploratory analyses is typically performed without internal standards and only provides an indication of the expression level of the targeted endogenous peptides. In these studies, a partial sampling of the elution profiles is tolerated as long as the apices remain captured to enable peak height determination. This approach was recently applied to urine samples and showed systematic identification and quantification of more than 80% of the targeted peptides (1586) with acceptable reproducibility (CV $<$ 30% across four analytical replicates) [45]. The PRM analytical workflow adopted for exploratory analyses is presented in detail in Fig. 4.

6.2 Wide-screen analyses

These experiments are intended to precisely quantify peptides/proteins differentially expressed in moderate-to-large sample sets, for example as observed in previous exploratory studies (e.g., drug treatment, diseased versus healthy individuals). In comparison to exploratory studies, smaller sets of peptides are targeted thus emphasizing the quantification precision. This is typically achieved by means of internal standard analogs, ^{13}C and ^{15}N isotopes, of endogenous peptides.

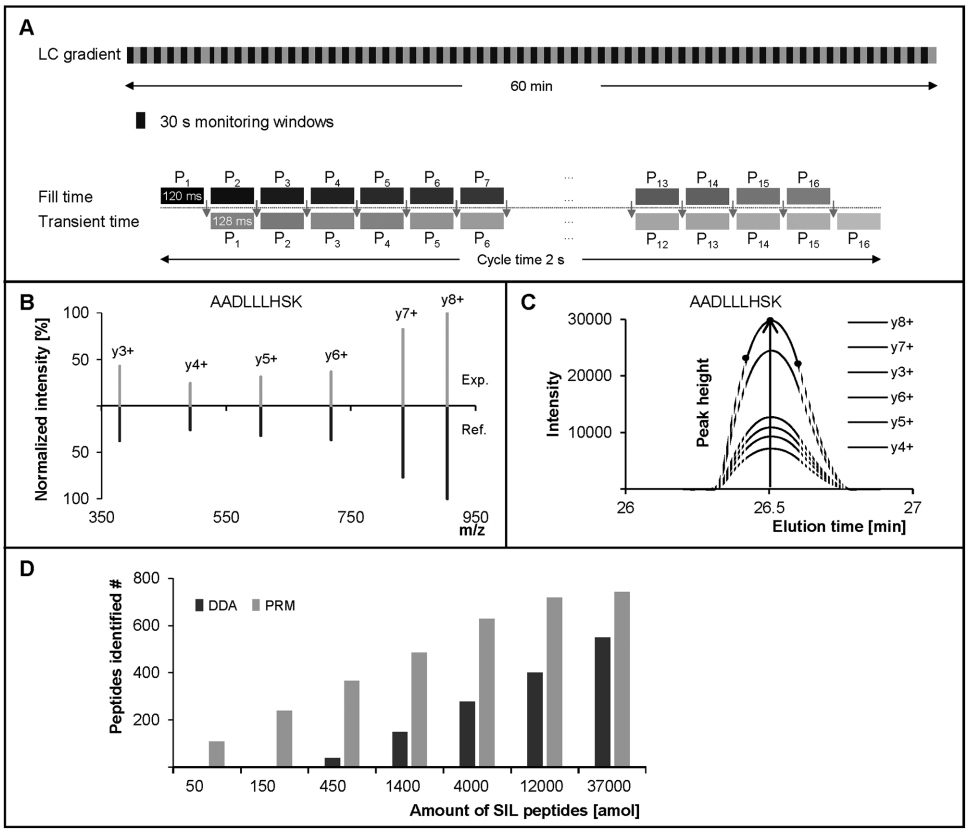

Figure 4. Example of a PRM exploratory experiment. (A) The typical setup allows monitoring thousands of targeted peptides during one hour LC separation. Very short elution monitoring windows are used (15–30 s) in order to restrict the number of co-eluting peptides measured during one cycle, thus maintaining acceptable fill and transient times to achieve the desired analytical performance. (B) The corroboration of the identity of the peptides detected is obtained by spectral matching (spectral contrast angle $<12°$, calculated using five or six fragment ions). (C) The estimation of the abundance of the qualified analytes is based on the peak height. The acquisition of at least three MS/MS spectra is necessary to properly capture the apices of the chromatographic peaks. (D) A comparison of the performance of DDA and PRM analyses for the assessment of peptide detection was conducted by measuring a mixture of 778 synthetic peptides (corresponding to human peptides) spiked in a sample of moderate complexity (yeast tryptic digest at 300 ng/μL) at various concentrations (between 50 amol/μL and 37 fmol/μL). The MS files have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD003781. The data were processed using the spectral matching approach described in Fig. 3. The PRM analyses showed superiority the detection of analytes, especially when present at low abundance. At the highest concentration (37 fmol/μL), 743 SIL peptides were detected in PRM mode, compared to only 550 peptides in DDA analyses. At lower concentrations, a three or even 100 times higher success rate was observed with PRM analyses for 1.4 fmol and 50 amol, respectively. It demonstrates that the systematic analyses in PRM are suitable for targeted peptides over a wide dynamic range thus overcoming the bias often observed in DDA.

Furthermore, the number of peptides targeted allows appropriate sampling of the elution profile of both the endogenous and SIL peptides while keeping similar acquisition parameters and therefore suitable sensitivity and selectivity. Wide-screen analyses also benefit from the dynamic correction of peptide monitoring windows (which are nevertheless somewhat wider as compared to exploratory screens) to measure the elution profiles. The presence of SIL peptides, spiked at identical concentrations in all samples, allows to correct for possible variability between technical replicates and in turn improves the quantification precision of endogenous peptides. An example of application of wide-screen analyses is depicted in Fig. 5.

6.3 Accurate quantification experiments

This type of experiment aims to precisely and accurately quantify peptides/proteins as part of validation or (pre)clinical studies. The pertinent peptides typically result from an initial screening, which has established the analytical performance and indicated discrimination between different biological or clinical statuses. The quantification of a few analytes is extended to a larger sample set while the various aspects of the assay are optimized. The MS acquisition parameters (optionally including the quadrupole isolation window width and the collision energy) are optimized to maximize accuracy, precision, and sensitivity (Fig. 6A). The analysis

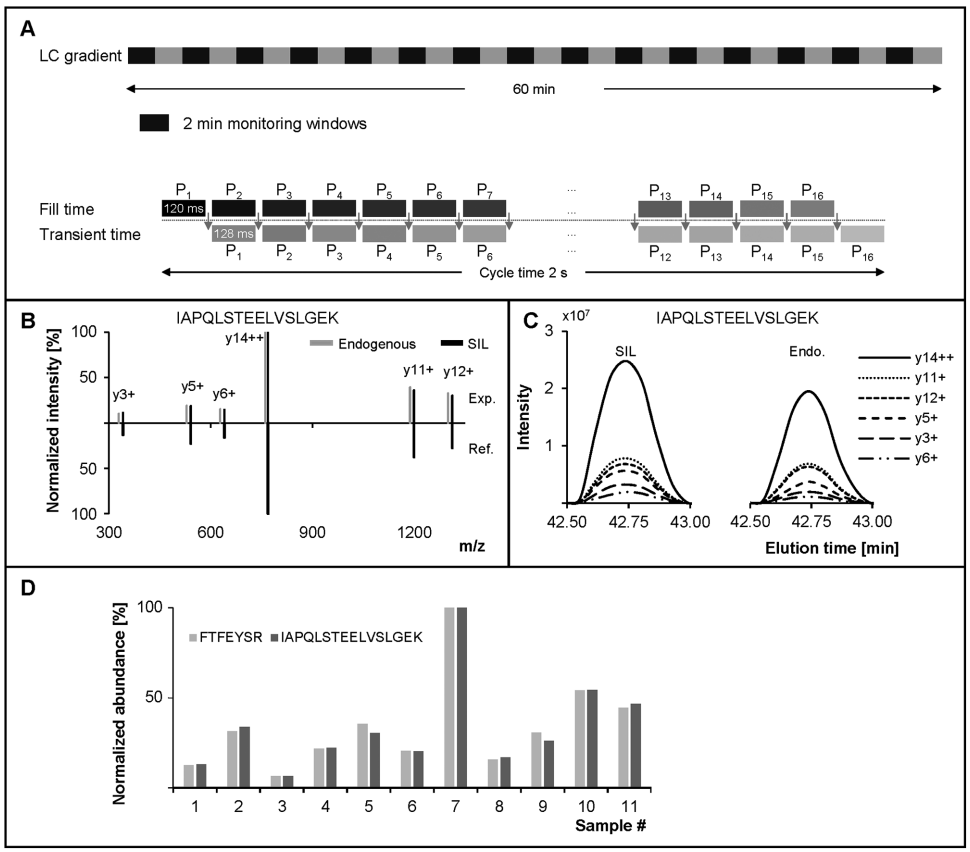

Figure 5. Example of a PRM wide-screen experiment. (A) The acquisition parameters are set to maintain acceptable sensitivity and selectivity, while enabling the measurement of hundreds of analytes during one LC-MS run. The chromatographic monitoring windows are dynamically adjusted "on-the-fly" (1–2 min) to properly record peptide elution profiles. (B) Spectral matching using a reference library is systematically performed to confirm the identity of the peptides detected (endogenous and SIL); it also allows to qualify the fragment ions to be used for subsequent quantification, i.e., absence of interferences. (C) The traces of the qualified fragment ions of the peptides are extracted and the AUCs are determined; quantification of the endogenous peptides is based on a single point calibration. (D) Endogenous and SIL peptides (197 pairs) were measured in triplicate for eleven urinary samples using PRM. The abundance of endogenous peptides was determined, an example is shown for the peptides FTFEYSR and IAPQLSTEELVSLGEK (surrogates of afamin). Highly consistent results were obtained across the eleven samples, as reflected by a low coefficient of variation (<20%) and the excellent agreement between the intensities of both peptides.

is based on internal standards of high purity added in calibrated amounts, which enable quantification based on a single-point calibration method. The assay is carefully established through the determination of the linearity range and LOQ to delineate the applicability of the method (Fig. 6B and C). For instance, accurate quantification was applied to peptides used as surrogates of ubiquitin from 0.1 fmol to more than 100 fmol [26], and to peptides representative of proteins SAA1 and SAA2 in plasma from patients diagnosed with non-small-cell lung cancer (NSCLC) and non-cancer control samples [23] (Fig. 6D). Alternatively, multiple isotopically labeled forms of the internal standards can be systematically added at different concentrations in each sample. The use of partial or full calibration curves generated in situ yields excellent accuracy and robustness, while avoiding the need for multiple analyses of individual calibrators [46].

7 PRM version 2.0: internal standard triggered-parallel reaction monitoring

As discussed above, the PRM technique presents clear advantages in quantitative analyses due to the HRAM capabilities of the instruments used. However, the technique suffers from a limitation in the number of analytes that can be included in one cycle while keeping a good performance. A new data acquisition scheme, called internal standard triggered-parallel reaction monitoring (IS-PRM), was developed to move to more precise quantification of larger peptide panels that can be of interest in studies such as extensive comparative pathway analyses. The acquisition relies on the analysis of internal standards (as SIL peptides), and uses them to actually drive the acquisition and to set the MS acquisition parameters in real-time [47]. This novel method alternates between two distinct PRM modes, called "watch" and "quantification" modes.

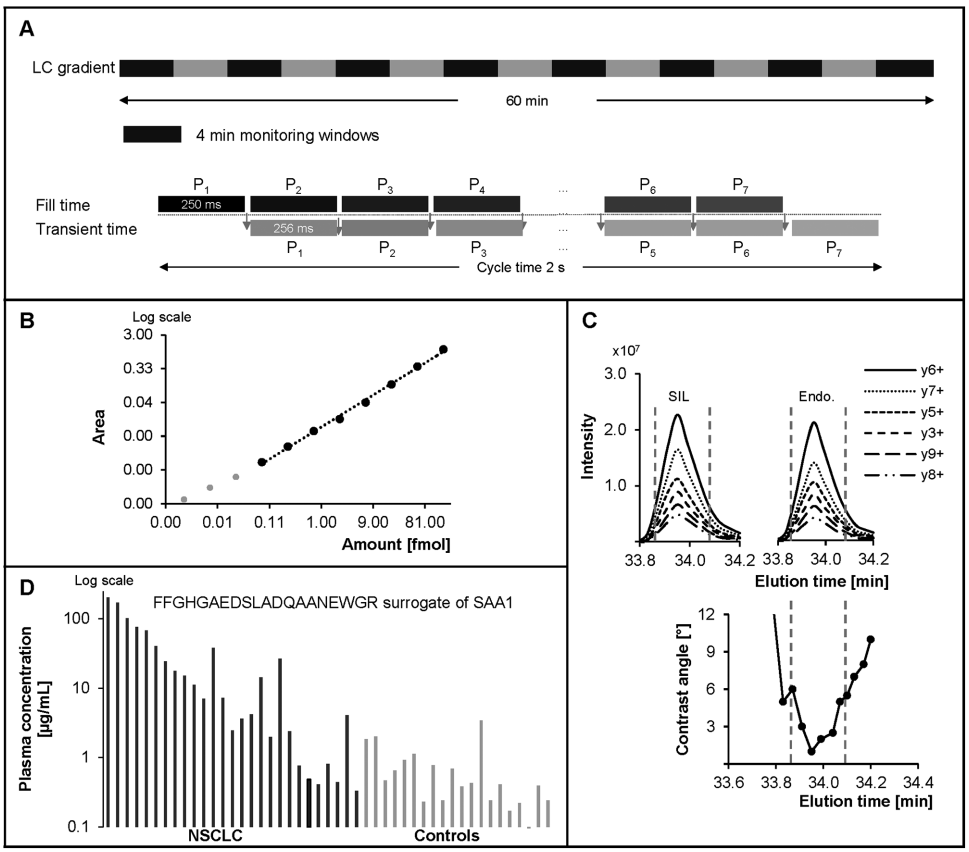

Figure 6. Example of a PRM accurate quantification. (A) The PRM technique was applied to a limited set of analytes (less than 100) using acquisition parameters favoring sensitivity and selectivity with relaxed chromatographic monitoring windows (i.e., capture of the entire peptide elution profiles). (B) The performance of the assay (linearity range, LOQ) is determined for the fragment ions of each peptide based on PRM analyses of dilution series of the SIL peptides spiked into a representative sample (for example a mixture of the samples under investigation). The fragment ions exhibiting the best performance are used for identification and quantification. The calibration curves established allow precise quantification and will guide the addition of the appropriate amount of SIL peptides in the actual samples. (C) The spectral contrast angle is confirming the presence of endogenous and SIL peptides in the samples and is an indicator of interferences [37]. The qualified fragment ion exhibiting the lowest LOQ is used for quantification based on single point calibration. (D) The accurate quantification of eight endogenous peptides (representing SAA1 and SAA2 isotypes) was performed across 47 plasma samples from healthy and diseased donors. The metrics were determined from the dilution series of the eight SIL peptides spiked in a pool of plasma samples at concentrations ranging from 3 amol/μL to 443 fmol/μL. The six fragment ions presenting the best selectivity and sensitivity were used. The quantification relied on the best-responding fragment ion, using the following equation: Conc_Endo = (Conc_SIL x AUC_SIL)/(AUC_Endo). The results are illustrated by using the peptide FFGHGAEDSLADQAANEWGR (surrogate of protein SAA1) which exhibited a higher abundance in patient samples, indicative of disease as previously reported. Figure adapted from [23].

The first one uses short acquisition times (fill time and transient time) to monitor the SIL peptides present in a defined amount within narrow, dynamically monitored, windows (using the "on-the-fly" correction described above). The detection of a SIL peptide, by performing real-time spectral matching, is used to trigger the truly quantitative measurement of both SIL and endogenous peptides over their entire elution profile, as depicted in Fig. 7A.

The strategy was applied to the analysis of a set of 606 SIL peptides, representing 338 human proteins, at concentrations ranging from 50 to 500 fmol/μL. The peptide mix was spiked into various biological samples (digests from plasma, urine, and HeLa cells) [47]. The experiment was carried out on the latest generation of quadrupole-Orbitrap instrument (Q-Exactive HF) allowing very short transient times (32 ms at 15 000 resolving power) in "watch" mode, while acquisition times more suited to precise measurement were used in "quantification" mode (128 ms for 60 000 resolving power). The technique allowed the detection and precise quantification of 525 endogenous peptides (corresponding to 300 proteins) across the different types of samples analyzed. Within one sample the peptides, systematically detected in triplicate experiments were quantified with high precision as reflected by coefficients of variation systematically below 20%. Overall,

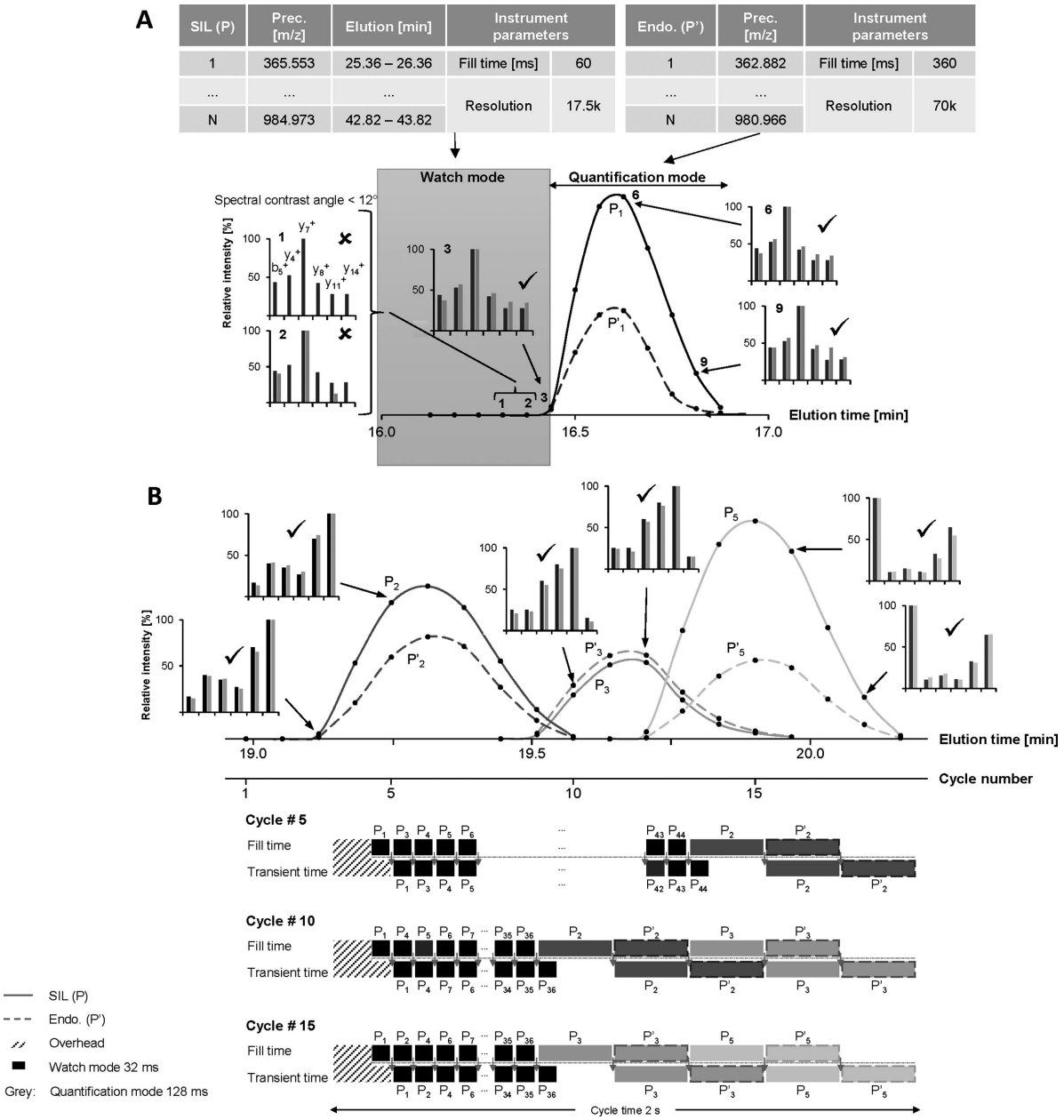

Figure 7. Principle of IS-PRM experiments. (A) Two types of acquisition are used. The "watch mode", characterized by low fill and transient times monitors the pertinent precursor ions (SIL peptides) and ensures their real-time detection. If positively identified the acquisition parameters are set to analyze the SIL (P_x) and endogenous peptide (P'_X) pairs using the "quantification mode". (B) The acquisition settings (list of targets and monitoring windows) are updated in real-time, following the "on-the-fly" data evaluation. Once a SIL peptide is positively detected (P_X), the list of targets is revised and synchronized to switch from the "watch mode" to "quantification mode" acquisition for both paired endogenous and SIL peptides. The "watch mode" is going on for all other SIL peptides on the target list. The "quantification mode" remains active for the detected peptides until the elution is completed (based on a predefined chromatographic peak width). Figure adapted from [47].

the IS-PRM acquisition method enabled an efficient management of the acquisition time, as exemplified in Fig. 7B, translating in the combination of the scale of an exploratory study and the analytical performance of a wide-screen experiment.

8 Conclusion

PRM acquisition methods applied to clinical samples have shown exquisite selectivity and sensitivity, which provide highly reliable qualitative and quantitative results.

Furthermore, compared to SRM experiments performed on triple quadrupole instruments the design of the acquisition method is straightforward. Thus, the versatility of the PRM technique allows its usage in a broad range of applications. The ability to set parameters, such as the maximum fill time and the resolving power, enables the methods to be adapted to match various types of proteomics experiments. Long fill times enhance the sensitivity of the measurements whereas the associated increase in the Orbitrap resolving power improves the selectivity. The synchronization of both parameters is defined by the number of analytes included in one acquisition cycle. It is, thus, the design of a specific acquisition method through the adjustment of the instrument parameters that reflects the intended purpose of the experiment. For instance, precise quantification based on internal standards requires maximum selectivity, while exploratory or screening experiments to detect specific peptides in a sample are geared towards speed and throughput.

Thus, several acquisition methods reflecting the intended purpose of the various targeted experiments have been designed. First, exploratory experiments were developed to obtain an initial estimation of the amount of as many analytes as possible (100 to 1000) across samples. In such experiments, the fill and transient times are set accordingly, and relaxed criteria are used to assess selectivity yielding approximate quantitative results. Second, wide-screen experiments aim at measuring relative changes in sets of analytes of moderate size (few 100 s) across a larger number of samples with a precision to derive statistically significant results. Similar fill times and resolving power are used in this case, and the analytical precision is greatly improved by using internal standards and by the systematic capture of the entire peptide elution profiles. Third, the measurement of a limited number of analytes with maximum sensitivity and selectivity allows high confidence identification and accurate quantification, which rely on high purity, calibrated internal standards. The fill times and the resolving power are set to ensure optimal analytical performance.

The recent implementation of IS-PRM has resulted in a new paradigm as the internal standards added to samples in defined amounts actually drive the data acquisition. This development is part of an effort aiming at the expansion of the scale of PRM experiments while retaining the quantification performance usually achieved in smaller scale experiments. Significant improvement has thus been achieved enabling new challenges to be undertaken by precise quantification experiments (e.g., signaling pathway monitoring). In spite of such progress, unsupervised acquisition methods (i.e., DDA and DIA) still provide superior proteome coverage, but at the expense of quantification performance.

Overall, PRM is revolutionizing quantitative proteomics. Not only the increased selectivity and sensitivity improve the analytical performance, but it sets new standards for quantification. The positive identification of the analyte and the assessment of possible interferences are critical and performed prior to the actual quantification step. This method will open avenues for biological studies requiring precise quantification to address specific questions, e.g., analysis of pathway perturbations. It can thus be anticipated that in the near future the PRM technique will be more broadly applied in quantitative proteomics studies. In addition, while the vast majority of PRM studies have been carried out on quadrupole-Orbitrap instruments the technique is not restricted to this specific instrument, as illustrated by its recent implementation on the Q-TOF platform [24]. It can be expected further expansion of its applicability to additional instrument configurations, including alternative dissociation methods (e.g., electron-transfer dissociation), provided a sufficiently fast acquisition rate is maintained.

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