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Detection and quantification of proteins in clinical samples using high resolution mass spectrometry

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ABSTRACT

Quantitative proteomics has benefited from the recent development of mass spectrometers capable of high-resolution and accurate-mass (HR/AM) measurements. While targeted experiments are routinely performed on triple quadrupole instruments in selected reaction monitoring (SRM; often referred as multiple reaction monitoring, MRM) mode, the quadrupole-orbitrap mass spectrometers allow quantification in MS/MS mode, also known as parallel reaction monitoring (PRM). This technique is characterized by higher selectivity and better confidence in the assignment of the precursor and fragment ions, and thus translates into an improved analytical performance. More fundamentally, PRM introduces a change of the overall paradigm of targeted experiments, by the decoupling of the acquisition and data processing. They rely on two distinct steps, with a simplified acquisition method in conjunction with a flexible, iterative, post-acquisition data processing.

This account describes in detail the different steps of a PRM experiment, which include the design of the acquisition method, the confirmation of the identity of the analytes founded upon a full MS/MS fragmentation pattern, and the quantification based on the extraction of specific fragment ions (selected post-acquisition) using tight mass tolerance. The different types of PRM experiments, defined as large-scale screening or precise targeted quantification using calibrated internal standards, together with the considerations on the selection of experimental parameters are discussed.

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1. Introduction

Protein quantification in clinical samples, and in proteomics in general, remains a very challenging endeavor. The complexity of the samples (*e.g.*, the tryptic digest of a full or partial proteome) is enormous and accounts for hundreds of thousands of distinct peptides, including post-translationally modified species. This tremendous number of analytes exceeds the actual peak capacity of any LC–MS system currently available, unless multi-dimensional fractionation is performed [1]. Second, the concentration of proteins present in bodily fluids or tissue samples spans eight to ten orders of magnitude [2], hampering the detection of low abundant components of similar hydrophobicity and mass-to-charge ratio (*m*/*z*), as they become obscured within the biochemical background. Third, the analyses performed in the context of clinical studies require a workflow allowing high throughput and a robust platform (to measure a large number of samples in a timely fashion), while producing reliable and precise quantitative results [3]. In addition, a high degree of multiplexing is desired in order to measure several hundreds of peptides within the same analysis. This is necessary to assess the specificity and sensitivity of individual, or a panel of, markers, which could ultimately be transposed to routine clinical assays [4]. A requirement for such an analytical workflow is a simple sample preparation protocol, with high recovery and scalability. Multi-step fractionation, which can result in the partition of the peptides in several fractions and associated with increased sample losses, should be avoided. The systematic use of internal standards (isotopically labeled peptides, or whenever possible isotopically labeled proteins) is highly desired to ensure precise quantification [5–8].

Abbreviations: PRM, parallel reaction monitoring; SRM, selected reaction monitoring; MRM, multiple reaction monitoring; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; TDA, targeted data acquisition; DDA, data dependent acquisition; SIL, stable isotopically labeled; HR/AM, high-resolution and accurate-mass; AGC, automatic gain control; *m*/*z*, mass-to-charge ratio; S/N, signal-to-noise ratio; Q-TOF, quadrupole-time of flight; Q-OT, quadrupole-orbitrap; AUC, area under the curve.

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However, the ultimate quantitative results are intimately connected to the analytical system, and more specifically to the chromatographic separation and the mass spectrometry. Both are critical elements for successful analyses. The reproducibility of the chromatography is paramount to generate results comparable across a large number of samples. It is the cornerstone of time-scheduled targeted data acquisition (TDA) [9], which has been broadly adopted for quantitative proteomic analyses. Initially, analyses were performed on triple quadrupole instruments operated in selected reaction monitoring (SRM; often referred as multiple reaction monitoring, MRM) mode [10]. Briefly, the first and third quadrupoles of the triple quadrupole instrument isolate a predefined pair of precursor and fragment ions (called transition) while the intermediate quadrupole serves as a collision cell. This SRM technique offers selectivity and sensitivity, due to the two stages of mass selection and the static operation mode of the quadrupoles in the ion filtering process, respectively, in conjunction with a wide dynamic range of measurements [11,12]. More recently, hybrid mass spectrometers capable of high-resolution and accurate-mass (HR/AM) measurements and high-frequency acquisition, such as the quadrupole-time of flight (Q-TOF) and quadrupole-orbitrap (Q-OT) instruments, have been used for quantitative proteomics analyses [13,14]. Quantitative measurements performed on a quadrupole-orbitrap instrument operated in parallel reaction monitoring (PRM) mode were shown to yield a similar level of sensitivity, or even better for high-complexity samples, as compared with SRM [15,16]. Briefly, this operation mode still relies on filtering the precursors by a quadrupole, but for each precursor, full MS/MS spectra are acquired in the high resolution orbitrap mass analyzer, *i.e.*, all fragment ions are concomitantly analyzed. The Q-OT instrument, initially designed to accelerate data dependent acquisition (DDA), and thus improve peptide and protein identification in shotgun experiments [17], turned out to be well suited for targeted quantitative measurements. It provides immediate benefits over conventional SRM analysis in terms of selectivity, resulting from the superior resolving power of the orbitrap mass analyzer, which allows a better discrimination of the signal of the analytes from that of the matrix [15]. In addition, the accurate mass measurements provide higher confidence in the assignment of the precursor and fragment ions. Furthermore, the trapping capability can be used to detect low levels of analytes by selecting and storing specific precursors for longer periods of time (up to a second) [13,18]. In addition to improved data quality, the acquisition of full MS/MS spectra also translates into a simplification of the experimental design [4,19].

This article describes the parallel reaction monitoring technique, and the different steps of the analytical workflow. A typical procedure for the implementation and the execution of a PRM analysis is provided, together with considerations concerning the acquisition parameters associated with two types of application, *i.e.*, initial screening experiments and precise quantification experiments.

2. Material and methods

2.1. Sample preparation

For the generation of a spectral library, the synthetic peptides are typically prepared in small sets (10–20 peptides) at a nominal concentration of 50–500 fmol/μL in aqueous solution. The use of standard-purity peptides is typically sufficient. In addition, a set of well-characterized peptides evenly covering the entire chromatographic peptide elution space can be spiked into the mixtures (typically at 30 fmol/μL) to be used as landmark peptides to normalize the elution time of the reference peptides (*e.g.*, Pierce Retention Time Calibration Mixture, PN 88321, Pierce, Rockford, IL).

Clinical samples are typically prepared from bodily fluids, such as plasma or urine, at a concentration of proteins between 0.2 and 1 μg/μL, as previously described [3,13]. They are generally supplemented with stable isotopically labeled internal standards (SIL peptides) corresponding to the targeted endogenous peptides in standard-purity (screening experiments) or high-purity (quantification experiments) at a concentration ensuring their detection and favoring quantification accuracy. In addition, the mixture of landmark peptides described above can also be spiked in the clinical samples.

2.2. Liquid chromatography and mass spectrometry

2.2.1. Liquid chromatographic separation

For the experiments mentioned in the present account, all the peptide separations were performed on an Ultimate 3000 RSLCnano system (Thermo Fisher Scientific). The samples were loaded into an Acclaim PepMap 2 cm x 75 μm i.d., C_{18}, 3 μm, 100 A trap column (Thermo Fisher Scientific) operated at 5 μL/min with an aqueous solution containing 0.05% (v/v) trifluoroacetic acid and 1% acetonitrile. After three minutes, the trap column was put online with a PepMap 15 cm × 75 μm i.d., C_{18}, 2 μm, 100 A analytical column (Thermo Fisher Scientific). The mobile phase was a mixture of solvents A and 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. Gradient elution was performed by applying a linear gradient of 2–35% solvent B at 300nL/min over 66 min (or over 20 min for spectral library generation) followed by a washing step (4 min at 90% solvent B) and an equilibration step (11 min at 2% solvent B). One microliter of sample was typically injected.

2.2.2. Analyses on quadrupole-orbitrap instruments

Parallel reaction monitoring analyses are typically performed using *Q-Exactive*, *Q-Exactive Plus*, or *Q-Exactive HF* mass spectrometers (Thermo Scientific, Bremen, Germany). The acquisition method combines two scan events corresponding to a full scan MS and a PRM event. For the generation of the spectral library, the PRM event targets the precursor ions of the peptides at their relevant charge states (*i.e.*, all charge states between two and the number of free amine groups included in the peptide sequence) without scheduling. For the analyses of clinical samples, the PRM event targets the precursor ion selected for each peptide in ±0.5–1.5 min monitoring windows (depending on the method used to calculate their expected elution time [20,21]). Typical settings of

Table 1
Typical values of instrument parameters used in PRM experiments performed on a quadrupole-orbitrap mass spectrometer. Adapted from [4].

Instrument parameters[a]	Experiment type		
	Spectral library	Screening	Quantification
Resolution (at m/z 200)	35,000	17,500–35,000	70,000–140,000
Maximum fill time [ms]	100	50–100	250–500
Isolation window [m/z units]	2	2	1–2[d]
Normalized collision energy (nCE)	25 (10–35[b])	25	10–35[d]
Elution monitoring window [min]	Unscheduled (2[c])	1–3[e]	1–3[e]

[a] Based on a *Q-Exactive* instrument.
[b] If optimization is performed using collision energy ramp.
[c] Optimization of collision energy carried out in a two-stage procedure.
[d] Optimized value, analyte-specific.
[e] Depending on the method used to re-calibrate peptide elution time.

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