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A quality control of proteomic experiments based on multiple isotopologous internal standards

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ABSTRACT

The harmonization of proteomics experiments facilitates the exchange and comparison of results. The definition of standards and metrics ensures reliable and consistent data quality. An internal quality control procedure was developed to assess the different steps of a proteomic analysis workflow and perform a system suitability test. The method relies on a straightforward protocol using a simple mixture of exogenous proteins, and the sequential addition of two sets of isotopically labeled peptides added to reference samples. This internal quality control procedure was applied to plasma samples to demonstrate its easy implementation, which makes it generic for most proteomics applications.

1. Introduction

Proteomics, with its ability to generate large data sets, has emphasized the necessity of comparing and integrating results across laboratories and platforms. The issue has gained acuteness as proteomics has shifted from qualitative to more quantitative studies. At present, there is a diversity of approaches and platforms that result in very heterogeneous data sets, whose integration remains very challenging. A first step toward the harmonization of proteomics results is the definition of methods and criteria to facilitate the systematic assessment of the analytical platform performance and the quality of the data generated. Furthermore, the preparation of samples using well-established procedures is necessary. These points have been widely recognized and several efforts have been undertaken in the past years toward the standardization of bottom-up proteomics LC-MS/MS analyses [1–11]. More specifically for quantitative analyses, proteomics can actually rely on the guidelines previously established in analytical and clinical chemistry [12,13]. While these recommendations relate to a single or a limited set of analytes, the general concepts outlined can be adopted in the context of proteomic quantitative LC-MS measurements. A recent workshop, focused on best practices for targeted analysis, has emphasized the necessity to define the purpose of the study (fit-for-purpose approach) [14].

In order to ensure the generation of reliable and consistent data sets, a comprehensive internal quality control procedure is required. It has to include the assessment of the sample preparation and the qualification of the instrument, which are combined in a validated analytical method. This provides a system suitability test, required prior to the analysis of actual samples [15]. The sample preparation method, which covers the sample handling, digestion, extraction and dilution, has to match the analytical question, the type of samples to be analyzed, and has to be reproducible across series of samples. The instrument and its associated operation method need to be specific and evaluated on test samples to assess the fulfillment of predefined requirements, in terms of analytical sensitivity (limits of detection and quantification), selectivity, precision (determined from replicated experiments), accuracy (based on the analysis of a reference material), and lastly robustness. Both the sample preparation and the instrument method need to be evaluated, first independently and ultimately in an integrated manner. A robust and validated protocol represents the basis for an internal quality control and its routine implementation. It allows the assessment of (i) the instrument performance, (ii) the sample preparation performance, and (iii) the system suitability.

A quantitative proteomics workflow needs to be specific, somehow addressing a well-defined analytical question. At present, most proteomics experiments are generic; nevertheless some level of systematic quality control is imperatively required. In

Abbreviations: LC, liquid chromatography; MS, mass spectrometry; SIL, stable-isotope labeled; SRM, selected reaction monitoring; PRM, parallel reaction monitoring; AUC, area under the curve; FWHM, full width at half maximum; CV, coefficient of variation; STD, standard deviation.

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an attempt to apprehend all the elements of a bottom-up LC-MS/MS proteomic workflow and monitor the different stages of the process, a simple protocol was designed. It allows the system suitability for routine proteomic analyses to be qualified while overcoming the rigidity of a full method validation. Rigorous quality controls remain required for systematic quantitative studies (e.g., preclinical), whereas relaxed constraints are applicable for initial screening experiments [14]. The protocol that was recently proposed to routinely assess the uniformity of proteomics analyses addresses this point [16]. It evaluates sample preparation and instrument performances concomitantly through the addition of isotopic variants of internal standards (peptides isotopically labeled with different motifs) at several stages of the workflow, corresponding to two peptides of each protein from the standard protein mixture. The analysis of these peptides in buffer enables to test the suitability of the LC–MS platforms, using acceptance/rejection metrics that were established based on long-term data collection. The systematic implementation of the protocol allows to monitor LC–MS performance overtime and to detect possible drifts or dysfunctions. It can also be employed to compare sensitivity levels of different platforms or analytical methods.

In an attempt to expand its applicability to clinical samples, the simple quality control procedure based on the sequential addition of multiple isotopically labeled peptides was tested on plasma samples. In this account, the standard protein mixture designed was spiked into several plasma samples, and the reproducibility of the overall workflow was assessed using control charts, which allows to define acceptance criteria. The quality control procedure is easily applicable in individual laboratories, and has shown a high level of reproducibility and robustness when applied to plasma samples, used as reference materials.

2. Material and method

2.1. Chemicals and reagents

Dithiothreitol, formic acid, iodoacetamide, Tris–HCl (Trizma hydrochloride), and urea were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sequencing grade modified trypsin was obtained from Promega (Madison, WI, USA). All solvents used were HPLC grade and purchased from Sigma–Aldrich.

2.1.1. Standard materials

The mixtures of three proteins and stable-isotope labeled (SIL) peptides were prepared as previously explained [16] (Supplementary Table).

2.1.2. Sample preparation

The unfolded standard protein mixture was either spiked in plasma samples or underwent the sample preparation procedure in buffer. Six plasma samples from deidentified human specimens were provided by Integrated BioBank of Luxembourg (IBBL) and treated as “not human subjects research” materials. Each plasma sample was individually mixed with the standard protein mixture (15 μL, volume corresponding to 7.5 μg of each protein) at a final concentration of 300 ng/μL. The reduction was performed with 20 mM dithiothreitol (5 mM final concentration) by incubation at 37 °C for 30 min. Then, the protein mixtures were alkylated with 75 mM iodoacetamide (15 mM final concentration) for 30 min at 25 °C in the dark before the addition of a first set of isotopically labeled peptides (H_A peptides) at a final nominal concentration of 50 fmol/μL. A solution of 25 mM Tris–HCl was used to dilute urea to 1 M and sequencing grade modified trypsin was added to a final enzyme:substrate ratio of 1:20. After an incubation of 4 h at 37 °C, peptides were cleaned on Sep-Pak tC18 cartridges (Waters, Milford, MA, USA) and eluted with 50% acetonitrile. The samples were lyophilized on a vacuum centrifuge and resolubilized in 0.1% formic acid. Prior to LC-MS/MS analysis, the mixtures were supplemented with a second set of isotopically labeled peptides (H_B peptides) at a final nominal concentration of 50 fmol/μL. In addition to the standard materials, a mixture of isotopically labeled peptides corresponding to 42 peptides from plasma was added before LC-MS/MS analysis on the quadrupole orbitrap instrument at a concentration close to that of their endogenous counterpart.

2.2. Liquid chromatography and mass spectrometry

2.2.1. LC separation

Analyses were carried out on a Ultimate 3000 RSLC nano system (Thermo Scientific). A trap column Acclaim PepMap 2 cm × 75 μm i.d., C18, 3 μm, 100 A and an analytical column Acclaim PepMap RSLC 15 cm × 75 μm i.d., C18, 2 μm, 100 A (Thermo Scientific) were used. The samples were loaded into the trap column at 5 μL/min with an aqueous solution containing 0.05% trifluoroacetic acid and 1% (v/v) HPLC grade acetonitrile. After three minutes loading, the trapping column was put on-line with the analytical column. The peptides were eluted 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. Separation was performed using a linear gradient of 2–35% solvent B at 300 nL/min either over 33 min for analyses performed on the triple quadrupole instrument (SRM) or over 66 min for analyses carried out on the quadrupole orbitrap instrument (PRM). One microliter of each sample was injected.

2.2.2. Analyses on triple quadrupole instrument

Selected reaction monitoring analyses were carried out on a TSQ Vantage extended mass range triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA, USA). A dynamic nano-electrospray source was used with uncoated SilicaTips, 12 cm length, 360 μm outer diameter, 20 μm inner diameter, 10 μm tip inner diameter. Ionization was obtained by using 1200 V of liquid junction voltage and 250 °C for the capillary temperature. The selectivity for both Q1 and Q3 was set to 0.7 Da. The collision gas pressure in Q2 was set at 1.5 mTorr argon. The time-scheduled SRM method targeted 6 triplets of isotopically labeled peptides/endogenous peptides in ± 6 min retention time windows by monitoring five transitions for each peptide within a cycle time of 2.5 s.

2.2.3. Analyses on quadrupole orbitrap instrument

Parallel reaction monitoring analyses were performed on a Q-Exactive HF mass spectrometer (Thermo Scientific, Bremen, Germany). The nano-electrospray source was identical to the one used for analyses performed on the triple quadrupole mass spectrometer. For ionization, 1500 V of liquid junction voltage was used. The acquisition method included a full scan event (mass range of 300–1500 m/z), using a resolution of 60,000 (at m/z 200), a target automatic gain control value of 1e6, and a maximum fill time of 100 ms. The second event consisted in a PRM scan event operating with a 2-Th isolation window, a resolution of 30,000 (at m/z 200), a target AGC value of 1e6, a maximum fill time of 120 ms, and a normalized collision energy set at 25. The time-scheduled method targeted the six triplets of standard peptides (two labeled and one unlabeled) derived from proteins of the standard mixture, and 42 pairs of endogenous/isotopically labeled peptides from plasma in ± 2 min chromatographic monitoring windows. Some experiments were replicated on a quadrupole orbitrap plus instrument.

3. Data processing

Data analysis was performed using Skyline (Vers. 2.6, University of Washington). The area under the curve (AUC) of each target