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A Method for Label-Free, Differential Top-Down Proteomics

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Abstract

Biomarker discovery in the translational research has heavily relied on labeled and label-free quantitative bottom-up proteomics. Here, we describe a new approach to biomarker studies that utilizes high-throughput top-down proteomics and is the first to offer whole protein characterization and relative quantitation within the same experiment. Using yeast as a model, we report procedures for a label-free approach to quantify the relative abundance of intact proteins ranging from 0 to 30 kDa in two different states. In this chapter, we describe the integrated methodology for the large-scale profiling and quantitation of the intact proteome by liquid chromatography-mass spectrometry (LC-MS) without the need for metabolic or chemical labeling. This recent advance for quantitative top-down proteomics is best implemented with a robust and highly controlled sample preparation workflow before data acquisition on a high-resolution mass spectrometer, and the application of a hierarchical linear statistical model to account for the multiple levels of variance contained in quantitative proteomic comparisons of samples for basic and clinical research.

Keywords

Top-down proteomics; Top-down quantitation; Label-free quantitation; Quantitative mass spectrometry; Proteoform; Differential expression

1 Introduction

Mass spectrometry has emerged over the past few decades as a powerful tool for untargeted protein analysis in the clinical lab. The vast majority of proteomics research relies on the bottom-up approach, where proteins are digested with a protease, such as trypsin, prior to peptide detection and sequencing using tandem mass spectrometry [1, 2]. Precursor mass measurements, along with MS/MS fragmentation information, allow inference of the protein composition of the sample via these peptides. Top-down proteomics describes the process for identification and characterization of intact proteoforms [3] without the use of a protease. In doing so, top-down proteomics can fully characterize the composition of individual proteoforms, including proteolysis products, signal peptide cleavage, sequence variants, and PTMs co-occurring on the same molecule. A typical top-down workflow consists of single- or multi-step protein separations, such as RPLC [4] and GELFrEE [5], and the resulting protein fractions are then further separated by liquid chromatography in line with a mass spectrometer. Advances in MS instruments and protein separations have allowed top-down proteomics to become a robust technique for the identification and characterization of

~2000–3000 proteoforms [4–6]. Due to technical and analytical challenges inherent in the acquisition and bioinformatic analysis of top-down MS data, however, *quantitative* top-down methods analogous to those used by bottom-up proteomicists have lagged in terms of development and application.

While proteome-wide quantitation has remained elusive for proteoform-resolved measurements, several laboratories have published targeted studies that quantify whole proteins within a mixture of limited complexity. For example, two recent studies [7, 8] accomplished relative quantitation of multiple, coeluting proteoforms simply by measuring their intensity ratios in order to address relevant biomedical research questions. However, this kind of intraspectrum quantitation becomes less applicable to large multisample quantitative experiments in the face of ever growing biological variation among samples and technical variation among discrete data files as studies grow in scope. Several researchers have successfully applied various labeling techniques, like those often seen in quantitative bottom-up workflows, to intact protein analysis, including a study by our own group that quantified over 200 intact protein pairs by $^{14}\text{N}/^{15}\text{N}$ labeling in an anoxic yeast model [9]. In addition, studies have applied in vitro differential cysteine labeling [10] and tandem mass tag (TMT) workflows to perform MS1 and MS2-based quantitation of intact proteins [11]. However, these in vitro labeling methods are limited in their application to large-scale top-down quantitation studies, as differential labeling was found to alter chromatographic retention time and the existence of multiple precursor charge states in top-down measurements greatly complicates isobaric labeling experiments.

This chapter introduces a completely label-free method for high-throughput, quantitative profiling of intact proteins recently developed by our group [12]. We deferred from employing metabolic labeling, even though such an experiment circumvents the aforementioned chromatography challenges and requirement for single precursor ion selection. The preclusion of in vivo labeling techniques is necessary, as our method was developed to meet the growing demand for proteoform-resolved biomarker discovery in clinical research, where cells and tissue samples usually cannot be metabolically labeled. Statistical validity is also of prime importance in clinical studies, as they require great confidence in conclusions that may inspire expensive validation experiments and affect patient health in the future. While the label-free quantitation of proteoforms has been presented in various recent studies, including the introduction of “differential mass spectrometry” (dMS) by Yates and Hendrickson [13, 14], these analyses have been limited to just a few proteoforms and supporting statistical assessments were performed by the traditional Student’s *t*-test. These statistics are insufficient to address the myriad, complex sources of technical variation implicit in large-scale, comparative proteomic workflows employed by clinical, quantitative, top-down proteomics in discovery mode.

The label-free method for the comparative quantitation of proteoforms described here relies on a robustly controlled sample processing, multiple levels of replication at the biological and technical level, reproducible high-performance nano-flow liquid chromatography online with high-resolution FTMS mass analysis, and a statistical platform based on a hierarchical linear model that considers multiple levels of variation in the ANOVA analysis (Fig. 1). While the experiment outlined in the following methods section was performed and

validated in the model organism *Saccharomyces cerevisiae*, this proof-of-principle experiment can and has been carried out in mammalian cell lines and samples of peripheral blood mononuclear cells (PBMCs) isolated from human patients.

2 Materials

2.1 Cell Culture, Lysis, and Protein Fractionation

1. *Saccharomyces cerevisiae* S288c BY4742 and the *rpd3* (YNL330C) deletion mutant were obtained from ATCC (see Note 1).
2. Culture media: YPD broth (Sigma-Aldrich), with or without 0.2 g/L G-418 (Sigma-Aldrich).
3. Lysis Buffer: YPER (ThermoPierce, Rockford, IL) supplemented with 5 nM microcystin, 500 μ M 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 100 mM sodium butyrate, and 100 mM dithiothreitol (DTT).
4. Bicinchoninic acid (BCA) assay kit (ThermoPierce, Rockford, IL) for protein concentration determination.
5. GELFREE 8100 device, 8% GELFREE cartridge and GELFREE buffers (Expedeon, Cambridgeshire, UK), as supplied by the manufacturer.

2.2 LC-MS Data Acquisition

1. Dionex Ultimate 3000 RSLCnano system.
2. Mobile phase A: 0.2 % formic acid, 94.8 % water, 5 % acetonitrile.
3. Mobile phase B: 0.2 % formic acid, 4.8 % water, 95 % acetonitrile.
4. PLRP-S 1000 Å 5 μ m bulk media (Agilent).
5. Orbitrap Elite mass spectrometer (Thermo Fisher, San Jose, CA) fitted with nanospray source.
6. PicoTip emitters (New Objective, Waltham, MA).

2.3 MS Data Analysis and Quantitation

1. Xtract: MS instrument vendor supplied software for deconvoluting spectra and obtaining intensities of masses.
2. ProSight PC: search engine for protein identification and characterization.
3. SAS: statistical analysis program.

3 Methods

The typical proteomic workflow for label-free top-down quantitation is depicted in Fig. 1. The experiment includes sample preparation, molecular-weight based protein fractionation,

¹Yeast was used for the development of this protocol, but any protein source can be used instead. The lysis protocol would need to be modified but once the proteome is obtained the rest of the protocol can be followed.

LC separation and MS data acquisition, differential quantitation, statistical analysis, and identification of quantified proteoforms.

The protocol described below is designed to compare the nuclear proteome of two *Saccharomyces cerevisiae* strains, the wild type versus the *rpd3* deletion mutant. Rpd3 is a histone deacetylase and its deletion has been shown to increase acetylation levels of all core histones [15] and to have other global effects owing to a lack of epigenetic regulation [16].

3.1 Study Design

This is a protocol designed to compare the proteomes of two different biological samples (see Note 2). For each biological sample, it is recommended that three biological replicates and a minimum of three technical replicates are used to account for variability of biological and technical nature, respectively (Fig. 2).

3.2 Cell Culture, Lysis, and Protein Fractionation

3.2.1 Cell Culture

1. Single colonies of wild type *Saccharomyces cerevisiae* S288c BY4742 and the *rpd3* (YNL330C) deletion mutant (*rpd3* ::KANMX) were inoculated into 5 mL each of liquid YPD media without and with 0.2 g/L G-418, respectively. Three biological replicates of both WT and mutant strains were prepared.
2. Inoculated cultures were incubated overnight in a shaker at 250 rpm and 30 °C, and then centrifuged at $1,000 \times g$ for 10 min. Each cell pellet was gently resuspended in 1 mL YPD and then inoculated into 250 mL of YPD and YPD + G-418.
3. Cells were harvested at $OD_{600} = 0.7$ by centrifugation at $1,000 \times g$ for 20 min. The supernatant was discarded, pellets were washed once with distilled water, and pellet masses were measured before storage at $-80\text{ }^{\circ}\text{C}$.

3.2.2 Cell Lysis

1. Thawed yeast cell pellets were lysed in 2.5 mL/g wet cell weight of Lysis Buffer. The mixtures were agitated at room temperature for 20 min, and then pelleted by centrifugation at $14,000 \times g$ for 10 min.
2. To isolate nuclear proteins, each cell pellet was first resuspended in 30 mL of water and centrifuged at $18,000 \times g$ for 10 min at 4 °C, to remove excess YPER.
3. Acid/urea extraction of the histone fraction was performed by adding 2.5 volumes of 8 M deionized urea (see Note 3) with 0.4 N sulfuric acid the pellets, vortexing for 5 min, and then incubating on ice for 30 min.
4. Sample fractions were loaded onto C4 solid phase extraction columns (Baker). 30 mL of 0.1 % trifluoroacetic acid (TFA) in water was used as the wash, and sample was eluted with 3 mL of 0.1 % TFA in 60 % acetonitrile. Each eluted fraction was dried and reconstituted with 1.0 % sodium dodecyl sulfate (SDS) solution.

²It is possible to compare three or more states by adjusting the study design and statistical analysis.

³Urea was deionized using AG 501-X8 resin (BioRad) per manufacturer's instructions.

5. Protein concentration was determined by BCA assay (Thermo Pierce, Rockford, IL).

3.2.3 Protein Fractionation

1. 400 µg of total protein as measured by BCA were prepared for GELFrEE according to the manufacturer's instructions. Up to 112 µL of each sample was combined with 30 µL sample buffer, 8 µL 1 M DTT, and distilled water to a final volume of 150 µL. The vial was heated for 5 min at 50 °C.
2. Each biological replicate (*see* Note 4) was separated on a single lane of an 8 % GELFREE cartridge (six lanes total) according to the manufacturer's instructions and the first fraction (0–30 kDa range of proteins) of each sample was collected.
3. SDS removal was done by methanol/chloroform/water precipitation [17].
 - a. Four volumes of methanol were added to each fraction, and vortexed vigorously for 30 s. One volume of chloroform was then added, and vortexed again. Three volumes of water were added to each fraction, and vortexed again. The fractions were centrifuged at 14,000 rpm for 10 min (*see* Note 5).
 - b. After centrifugation, the top aqueous/methanol layer was carefully pipetted off and discarded. Three volumes of methanol were slowly added, the vial was carefully inverted to mix, and then centrifuged again for 10 min at 14,000 rpm.
 - c. The supernatant was then completely removed, leaving the protein pellet in approximately 10 µL liquid (*see* Note 6). After the supernatant was removed, residual solvent was allowed to air dry.
4. Protein pellets were resuspended in 40 µL mobile phase A.

3.3 LC-MS Data Acquisition

It is important to randomize the order of data acquisition to reduce bias in results due to instrument performance decay during data collection (*see* Note 4).

1. Trap (150 µm × 2 cm) and analytical (75 µm × 15 cm) column were packed in-house with PLRP-S media into fused silica capillaries.
2. Proteins (5 µL) were injected onto a trap column using an autosampler (Thermo Dionex). The NC pump was operated at 300 nL/min and the loading pump at 3 µL/min. The trap and analytical were operated in a vented tee setup. Table 1 outlines the LC gradient used for protein separation and elution into the mass spectrometer.
3. The Orbitrap Elite was operated under standard operating conditions. More details can be found in Table 2.

⁴Biological replicates were loaded on the cartridge in a randomized order. For a tutorial on how to randomize a list of items in an Excel spreadsheet go to <http://www.excel-easy.com/examples/randomize-list.html>.

⁵The proteins will be at the interface between the two layers after this centrifugation step.

⁶The methanol wash may be repeated once more. More methanol washes may lead to better removal of SDS, but also loss of protein.

3.4 MS Data Analysis and Quantitation

3.4.1 Proteoform Identification and Characterization—Our conventional top-down proteomics pipeline was used for proteoform identification and characterization, as previously described [5, 6, 12, 18].

1. ProSightHT within ProSightPC 3.0 was used to convert m/z data for each precursor/fragmentation scan pair to monoisotopic neutral mass values.
2. Neutral mass values were searched against a *S. cerevisiae* specific database built from UniProt release 2013_04.
3. Mass tolerance for precursors and fragments was set to 10 ppm.
4. Low confidence proteoform identifications were excluded by requiring those hits arising from an absolute mass search to have an E -value below 1×10^{-4} [5], and a more stringent E -value cutoff of 6×10^{-5} was applied to hits derived from a biomarker search as previously reported [19].

3.4.2 Proteoform Quantitation—All data files in the quantitation portion of the platform were processed using a collection of in-house tools to automate data analysis.

1. Files were analyzed for Quantitation Mass Targets (QMTs) using a moving spectral average and deisotoped with the Xtract algorithm (Thermo Fisher) at a signal to noise value of 6.
2. All QMTs were binned by mass (8 ppm) and retention time (8 min) to reduce data redundancy.
3. Intensities were normalized using the average total ion chromatogram intensity for each technical replicate.
4. The QMTs were grouped and artifactual ± 1 Da deisotoping errors were removed. Final QMTs were stored within a SQLite reporting database.
5. Intensities were aggregated across all scans and charge states, to report one intensity value for each data file and QMT. These data were provided as a text file for further statistical processing (vide infra).
6. QMTs were matched to proteoforms identified (Subheading 3.4.1) using a 10 ppm tolerance.

3.4.3 Statistical Analysis—All statistical analyses were performed within SAS 9.4 (SAS Institute, Cary, NC).

1. Intensity data on the occurrence of putative proteoforms (QMTs) were tabulated and those not occurring in at least 50 % of all data files were excluded from further analysis (see Note 7).
2. Intensity values for the remaining QMTs were $\log_2$ -transformed so that differences in estimated treatment-level intensities could be interpreted on a fold-change scale.

⁷This was done to address the problem of “missing values.”

3. Two separate ANOVA analyses were performed; ANOVA-1 and -2. For the first analysis, ANOVA-1, intensity levels for each QMT were standardized to Z-scores across all samples. ANOVA-2 used unstandardized intensity values. ANOVA-1 was used to test the statistical significance of QMT intensity changes between the wild type and *rp d3* mutant strains, while ANOVA-2 was used to estimate the size of the effect.
4. In both analyses, a hierarchical linear model was employed as the general statistical approach. The fixed effect hierarchical linear model can be expressed as

$$I_{ijk} = \mu + A_i + B_{j(ik)} + C_{k(ij)} + \varepsilon_{ijk}$$

5. In ANOVA-1, “*I*” represents the QMT intensity Z-score, while in ANOVA-2, this represents the log₂-transformed intensity. In both models, μ is the true mean, *A* is the treatment factor levels (wild type and *rp d3*), *B* is the biological replicates’ variance, *C* is the technical replicates’ variance, and ε is the residual variance (see Note 8). In ANOVA-1, all *p*-scores were corrected for multiple testing at a false discovery rate of $\alpha = 0.05$ [20].
6. To visualize the results, we created a volcano plot which represented each proteoform (i.e., QMT) as a function of estimated effect size (in log₂ fold-change) and the statistical confidence (the FDR) that there was a difference in normalized intensity between the two states “wild type” and “*rp d3*” (Fig. 3). Proteoforms identified to the right of the y-axis were upregulated in *rp d3* as compared to WT.
7. Quantitation of individual proteoforms can be visualized with box and whisker plots, as seen in the case of Histone H4 in Fig. 4.

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⁸Notice that the technical replicates are nested within the biological replicates, and that each biological replicate is treated as a random effect, thus each biological preparation is allowed to have its own mean. Analysis was done using restricted maximum likelihood (REML) estimation.

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Fig. 1.
Overall workflow for label-free quantitation of intact proteoforms

Fig. 2.
Study design for comparing differential expression of proteins in two states with three biological replicates and four technical replicates ($2 \times 3 \times 3$ design)

Fig. 3.

Volcano plot generated to compare the WT vs. *rpd3* strains of *S. cerevisiae* S288c for the nuclear proteins below 30 kDa having 54 differentially expressed proteoforms, above the 5 % FDR threshold (*dotted red line*). Datapoints highlighted above correspond to proteoforms of histone H4 (*red filled square* = diacetylated histone H4, *red filled triangle* = triacetylated histone H4) and their quantitation is explored further in Fig. 4. Reproduced from [12] with permission from the publisher

Fig. 4. Quantitation of histone H4 proteoforms. Box and whisker plots (panels at *right*) are presented for the histone H4 proteoforms with 2, 3, and 4 acetylations. The monoacetylated and tetraacetylated proteoforms were not considered within our analysis because they were observed in <50 % of the technical replicates. In all cases, mass spectra are the sums of individual scans across the full elution time of each proteoform within a single technical replicate. The symbol ** indicates significant proteoform abundance changes as reported by our platform. Reproduced from [12] with permission from the publisher

Table 1

LC gradient used for label-free quantitation of proteins

Time (min)	%B	Valve position
0	5	1_10
10	5	1_2
12	15	
62	55	
67	95	
72	95	
75	5	
90	5	

Table 2

MS method parameters for label-free quantitation of proteins

Parameter	Setting
Source	Nano-ESI
Capillary temperature (°C)	320
S-lens RF voltage	50 %
Source voltage (kV)	1.8
<i>Full MS parameters</i>	
Mass range (m/z)	500–2000
Resolution settings (FWHM at m/z 400)	120,000
Target value	1×10^6
Max injection time (ms)	1000
Microscans	4
SID (V)	15
<i>MS2 parameters</i>	
Top-N MS ²	2
Resolution settings (FWHM at m/z 400)	60,000
Target value	1×10^6
Max injection time (ms)	2000
Isolation width (Da)	15
Minimum signal required	500
Normalized collision energy (HCD)	25
Default charge state	10
Activation time (ms)	0.1
Lowest m/z acquired	400
Charge state rejection on: 1+, 2+ and 3+	enabled
Dynamic exclusion	Repeat count 1 Repeat duration 240 s Exclusion list size 500 Exclusion duration 5000 Exclusion mass width by mass ± 18 Da