

Protein Quantitation of the Developing Cochlea Using Mass Spectrometry

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Abstract

Mass spectrometry-based proteomics allows for the measurement of hundreds to thousands of proteins in a biological system. Additionally, mass spectrometry can also be used to quantify proteins and peptides. However, observing quantitative differences between biological systems using mass spectrometry-based proteomics can be challenging because it is critical to have a method that is fast, reproducible, and accurate. Therefore, to study differential protein expression in biological samples labeling or label-free quantitative methods can be used. Labeling methods have been widely used in quantitative proteomics, however label-free methods have become equally as popular and more preferred because they produce faster, cleaner, and simpler results. Here, we describe the methods by which proteins are isolated and identified from cochlear sensory epithelia tissues at different ages and quantitatively differentiated using label-free mass spectrometry.

Key words Cochlea, Label-free quantitation, LC-MS/MS, Mass spectrometry, Proteomics

1 Introduction

Mass spectrometry (MS) is an important tool in proteomics that can provide identification and quantitation of proteins. However, mass spectrometry is not inherently quantitative due to the fact that peptides have a wide range of physiochemical properties, such as size, charge, and hydrophobicity [1]. These differences lead to a significant difference in mass spectrometry response. Therefore, for mass spectrometry-based quantitation there are two approaches, absolute and relative quantitation, which involves labeling or label-free approaches (Fig. 1). MS-based absolute quantitation involving labeling, uses a known amount of isotope-labeled standard that is mixed with the analyte and the absolute amount of the analyte is calculated from the ratio of ion intensity between the analyte and its standard [2] (Fig. 2a). In contrast, absolute quantitation using the label-free approach uses a modified spectral counting method known as absolute protein expression (APEX) [3]. The absolute

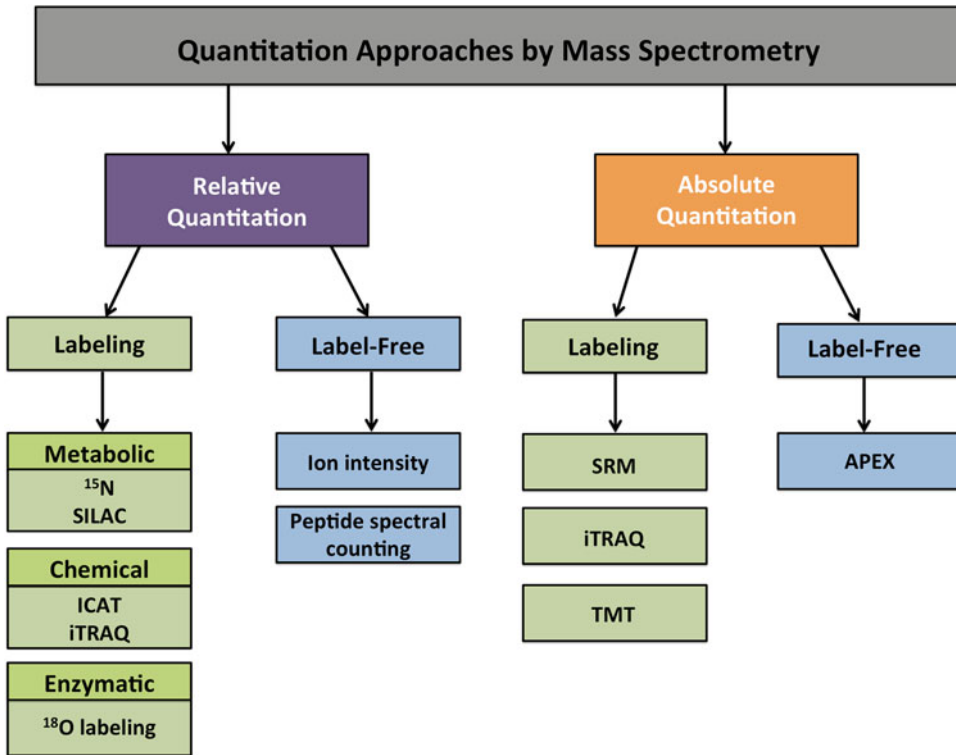


Fig. 1 An overview of the different mass spectrometry-based quantitative approaches in proteomics

protein concentration of each protein in a sample is calculated based on the empirical relationship between the number of spectra or peptides identified for a protein and the protein abundance in the sample [4]. Most commonly used is relative quantitation involving labeled or label-free methods. In the labeled approach, proteins from different samples are labeled differently and the peptides from the different samples are mixed and identified in the same spectra with different masses. Commonly used labeling techniques include metabolic labeling, chemical mass tagging, and enzymatic labeling (Fig. 1). In metabolic labeling, stable isotopes are incorporated into the protein sequence *in vivo*. Stable isotope labeling by amino acids in cell culture (SILAC) [5] is a commonly used metabolic labeling method, however it does not work well for all cell types and limited on the number of samples that can be analyzed per experiment [1]. Chemical mass tagging involves tagging proteins or peptides with stable isotopes *in vitro*. Isobaric tags for relative and absolute quantitation (iTRAQ) [6] and isotope-coded affinity tags (ICAT) [7] are commonly used chemical mass tagging methods. However, these methods are limited by their susceptibility to side reactions that can lead to unwanted products, which can adversely affect the quantitation [1]. Another labeling method is enzymatic addition of stable isotopes, which involves

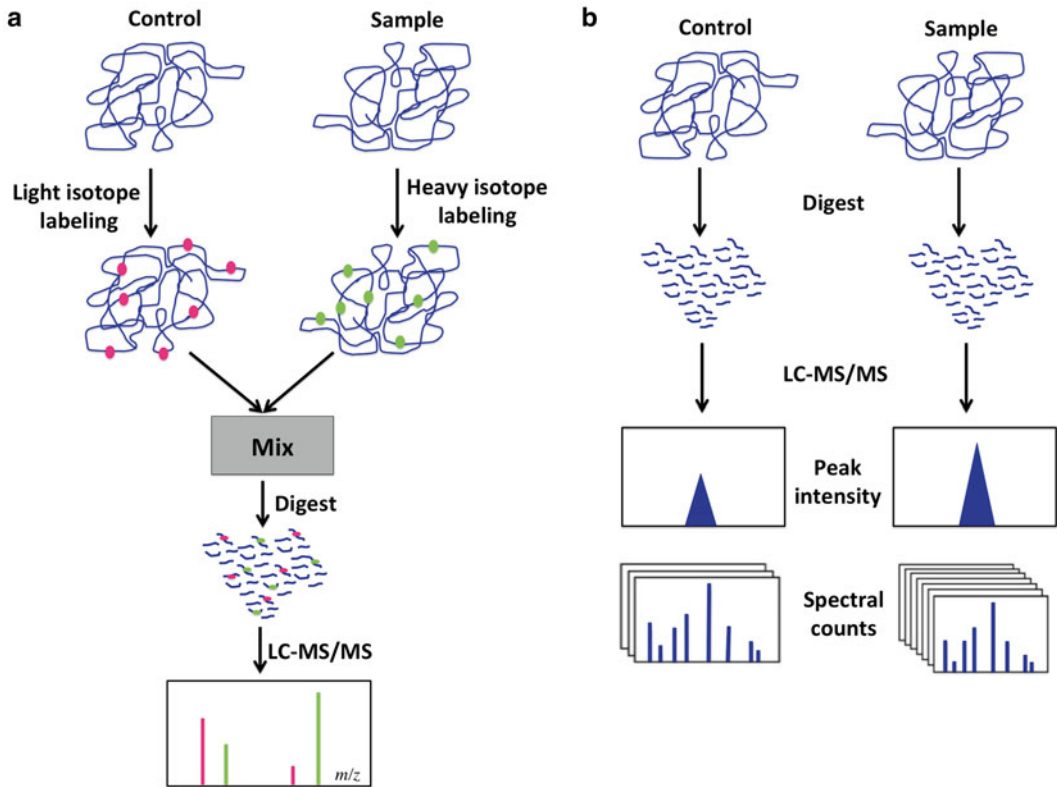


Fig. 2 General quantitative mass spectrometry workflows in differential protein expression analysis. **(a)** Label-based quantitative mass spectrometry and **(b)** label-free quantitative mass spectrometry

peptide digestion and isotope labeling simultaneously, thereby generating peptides that are a few Da heavier. ^{18}O labeling is commonly used and generates peptides 2 or 4 Da heavier due to the exchange of 1 or 2 oxygen atoms with ^{18}O at the carboxyl end of the peptide [3, 8]. However, a limitation with this method is that it has incomplete labeling efficiencies and the rates of labeling for different peptides will vary [1].

Relative quantitation by the label-free approach involves the samples being analyzed separately and the spectra of the peptides are compared for similar masses and retention times (Fig. 2b). In label-free quantitation, two approaches can be used: (1) peak intensities or area under the curve [9] and (2) spectral counting [10]. Measurement of peak area involves calculating and comparing the mean intensity of peak areas for all peptides from each protein in the biological sample [11]. Quantitating proteins based on area under the curve involves measuring ion abundance at a specific retention time for the given peptides [12]. Several factors must be considered when undertaking this approach, such as peak reproducibility and accurate peak detection that may be affected by

biological and technical variations. A limitation associated with this approach is determining a balance between survey scans and fragment scans in order to achieve both protein quantitation and identification [12]. In contrast, spectral counting is based on the number of MS/MS spectra generated from a protein. The more abundant the protein is in the biological sample, the more peptides will be selected for fragmentation [12]. Spectral counting is based on three measures which include observing the number of peptide-to-spectrum match, the number of distinct peptides identified, and the sequence coverage for a select protein in an LC-MS/MS run should correlate with protein quantity [1]. Spectral counting is limited by the differences in peptides' physiochemical properties that can result in variation and bias in MS measurements [12]. However, a comparison of both approaches shows that spectral counting is more reproducible and has a larger dynamic range [13]. As compared to labeling techniques, label-free techniques are less expensive, they can be applied to an array of biological samples, and they have a high analytical depth and dynamic range, which is advantageous when comparing samples with large number of protein changes [4].

Data analysis for label-free quantitation begins with protein identification using database searching via search engines such as MASCOT, SEQUEST, or X!TANDEM [12]. Protein identification is then followed by protein quantitation. Statistical analysis is important in quantitative proteomics experiments to differentiate protein abundance between samples. There are several commercially available software commonly used for label-free quantitation, including Scaffold [14], ProteoIQ, and Elucidator. Here, we describe the methods by which proteins are isolated and identified from cochlear sensory epithelia tissues at different ages and quantitatively differentiated using label-free mass spectrometry with spectral counting.

2 Materials

2.1 Protein Sample Preparation

1. Phosphate Buffered Saline: Prepare 1 L of 1× solution by adding 144 mg of potassium phosphate monobasic, 795 mg of sodium phosphate dibasic heptahydrate, and 9 g of NaCl to 900 mL of water. Bring volume to ~1 L with water (*see Note 1*) then adjust pH to 7.4 with HCl.
2. Protease inhibitor stock solutions: Mix 50 mg/mL of Pefabloc® SC 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF; Fluka St. Louis, MO) in water and store in 100 µL aliquots, 10 mg/mL of leupeptin in water and store in 10 µL aliquots, 10 mg/mL of aprotinin in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 8.0, and store

in 20 μL aliquots, 100 $\mu\text{g}/\text{mL}$ of pepstatin A in 95 % ethanol and 1 mM microcystin-LR (Calbiochem/EMD Biosciences, San Diego, CA) in DMSO and store both in 5 μL aliquots.

3. Lysis buffer stock solutions: 1 M Tris-HCl, pH 8.0 (UltraPure™, Invitrogen, Carlsbad, CA) and 0.5 M ethylenediaminetetraacetic acid (EDTA) UltraPure™ solution (Invitrogen) pH 8.0.
4. Lysis buffer working solution: Use 100 mM Tris-HCl, pH 8.0, 120 mM NaCl, 50 mM NaF, and 5 mM EDTA. Prepare 100 mL solution by adding 5 mL of 1 M Tris-HCl, pH 8.0, 701 mg of NaCl, 210 mg of NaF, and 1 mL of 0.5 M EDTA. Bring volume to 100 mL with water and store at 4 °C.
5. Complete lysis buffer working solution: Use 100 mM Tris-HCl, pH 8.0, 120 mM NaCl, 50 mM NaF, 5 mM EDTA, 500 $\mu\text{g}/\text{mL}$ AEBSF, 10 $\mu\text{g}/\text{mL}$ leupeptin, 100 $\mu\text{g}/\text{mL}$ pepstatin A, 2 $\mu\text{g}/\text{mL}$ aprotinin, and 1 mg/mL microcystin-LR. Prepare a 1 mL volume immediately before use by mixing the following quantities of stock protease and phosphatase inhibitors: 10 μL of AEBSF, 1 μL of leupeptin, 1 μL of pepstatin A, 2 μL of aprotinin, and 0.5 μL of microcystin-LR.
6. Solubilization Buffer: Prepared from complete lysis buffer with 4 % (w/v) sodium dodecyl sulfate (SDS). Other detergents can be used in the solubilization buffer, however careful consideration should be given that the detergent is compatible with the FASP digestion procedure (*see Note 2*).
7. Sonic dismembrator (Model 100; Thermo Fisher).

2.2 Protein Digestion

All solutions are prepared fresh and immediately prior to use.

1. FASP Protein Digestion Kit™ (Expedeon, San Diego, CA): 50 mM $(\text{NH}_4)\text{HCO}_3$, 100 mM Tris-HCl solution, pH 8.5, 8 M urea, iodoacetamide, 0.5 M NaCl solution, and 30 kDa spin filter.
2. Urea Solution: Prepare 8 M urea solution by dissolving one tube of urea from the FASP kit with 1 mL of 100 mM Tris-HCl.
3. Iodoacetamide Solution: Prepare 10 \times iodoacetamide solution by dissolving 100 μL of 8 M urea solution to one tube of iodoacetamide from the FASP kit.
4. Digestion Solution: Prepare 0.1 $\mu\text{g}/\mu\text{L}$ of endoproteinase LysC solution by dissolving 5 μg LysC in 50 μL of 50 mM ammonium bicarbonate solution provided with the FASP kit. Prepare 0.4 $\mu\text{g}/\mu\text{L}$ of trypsin solution by dissolving 20 μg trypsin in 50 μL of 50 mM $(\text{NH}_4)\text{HCO}_3$ solution provided with the FASP kit.
5. Formic acid (*see Note 3*).

6. Benchtop centrifuge (e.g., Microcentrifuge® model 5417R, Eppendorf).
7. NanoDrop spectrophotometer (Model ND 1000; Thermo Fisher Scientific).

2.3 Desalting Peptides Using C₁₈ Spin Columns

1. C₁₈ MacroSpin columns (The Nest Group, Southboro, MA).
2. Acetonitrile, mass spectrometry grade.
3. 0.1 % Formic Acid: 100 µL formic acid in 100 mL of ddH₂O.
4. 90 % Acetonitrile: 90 mL of acetonitrile and 10 mL of ddH₂O.
5. Vacuum concentrator (e.g., SpeedVac® model SPD121P, Thermo Fisher).

2.4 Protein Fractionation Using Ion Exchange Chromatography

1. 200 × 2.1 mm, 5 µm SCX column (Polysulfoethyl A, The Nest Group).
2. Solvent A: 5 mM ammonium formate, pH 3.0 in 25 % acetonitrile and 75 % ddH₂O.
3. Solvent B: 500 mM ammonium formate, pH 6.0 in 25 % acetonitrile and 75 % ddH₂O.
4. 500 µg of Cytochrome C Solution: 100 µL of 1 mg/mL of Cytochrome C in 25 mM NH₄HCO₃. Reduce for 60 min at RT with 5 µL of 200 mM dithiothreitol, followed by alkylation for 60 min with 20 µL of 200 mM iodoacetamide in the dark. Add 20 µL of 20 mM dithiothreitol to consume the remaining alkylating agent. Add 1:100 ratio of trypsin and heat at 37 °C O/N. The following day, add 10 µL formic acid to stop the reaction and dry the cytochrome C digest in a speedvac.
5. High-performance liquid chromatography system (*see Note 4*) and Photodiode array.
6. Fraction collector.

2.5 Nano LC-MS/MS

1. 100 µm × 25 mm sample trap (New Objective, Woburn, MA).
2. 75 µm × 10 cm C₁₈ column (New Objective, Woburn, MA).
3. Solvent A: 95 % ddH₂O and 5 % acetonitrile containing 0.1 % formic acid.
4. Solvent B: 80 % acetonitrile and 20 % ddH₂O containing 0.1 % formic acid.
5. High-resolution LTQ Orbitrap (Thermo Fisher Scientific, MA, USA) mass spectrometer.

2.6 Bioinformatics Tools for Protein ID and Statistical Analyses

1. MASCOT Search Engine (Matrix Science, London, UK).
2. Scaffold (Version 4.3.2, Proteome Software, Portland, OR).
3. Statistica software (Version 12, StatSoft, Inc.).

3 Methods

3.1 Protein Sample Preparation (See Note 5)

Experiments using animal tissue should be approved by the University's Institutional Animal Care and Use Committee as set forth under the guidelines of the National Institutes of Health.

1. Dissect cochleae from post-natal 3-, 14- and 30-day-old (P3, P14, and P30) CBA/J mice under sterile conditions and place them in aliquots of 16 cochlea per tube and store at -80°C .
2. On the day of the experiment, wash tissue with 500 μL of 1 \times phosphate buffered saline (PBS). Centrifuge for 3 min at 1000 $\times g$, and remove the supernatant. Repeat 3 \times .
3. Sonicate tissue for 30 s on ice in 100 μL of complete lysis buffer using a sonic dismembrator. Cool lysate on ice for 1 min between each sonication. Sonicate a total of 3 \times .
4. Centrifuge the extract at 750 $\times g$ at 4°C for 2 min and remove the supernatant to a new microtube. Extract the pellet in 50 μL of complete lysis buffer by sonicating for 30 s on ice. Centrifuge the extract at 750 $\times g$ at 4°C for 2 min. Combine both lysates and centrifuge at 28,600 $\times g$ at 4°C for 60 min. Remove the supernatant to a new microtube and add 20 μL of solubilization buffer to the pellet. Vortex for 1 min and incubate for 60 min at 4°C .
5. Incubate the sample on ice for 30 min, then heat at 95°C for 5 min. Follow with centrifugation at 16,000 $\times g$ at 4°C for 15 min. Collect the supernatant and transfer to a new tube.
6. Extract the pellet in complete lysis buffer by sonicating 1 \times for 30 s on ice.
7. Combine the lysate and previous supernatant and centrifuge at 20,800 $\times g$ at 4°C for 60 min and retain the supernatant for digestion (*see* Note 6).

3.2 Protein Digestion

1. Add a 30 μL aliquot (≤ 400 μg) of cochlear protein extract, containing 4 % SDS, 100 mM Tris-HCl, pH 7.6 and 0.1 M dithiothreitol directly to a 30 K spin filter and mix with 200 μL of 8 M urea in Tris-HCl. Centrifuge at 14,000 $\times g$ for 15 min.
2. Dilute the concentrate with 200 μL of 8 M urea solution and centrifuge at 14,000 $\times g$ for 15 min.
3. Add 10 μL of 10 \times iodoacetamide in 8 M urea solution to the concentrate in the filter and vortex for 1 min. Incubate the spin filter for 20 min at RT in the dark followed by centrifugation at 14,000 $\times g$ for 10 min.
4. Add 100 μL of 8 M urea solution to the concentrate on the filter unit and centrifuge at 14,000 $\times g$ for 15 min. Repeat this

- step 2×. Add 100 μL of 50 mM ammonium bicarbonate solution to the filter unit and centrifuge at $14,000\times g$ for 10 min. Repeat this step 2×.
5. Add 0.1 $\mu\text{g}/\mu\text{L}$ of LysC in a 1:50 (w/w) enzyme-to-protein ratio and incubate O/N at 30 °C.
 6. Following incubation, add 40 μL of 50 mM ammonium bicarbonate solution to the filter unit and centrifuge at $14,000\times g$ for 10 min. Repeat this step 1×.
 7. Add 50 μL of 0.5 M NaCl solution to the spin filter and centrifuge at $14,000\times g$ for 10 min. Transfer the filtrate containing the LysC peptides to a fresh microtube and acidify with formic acid to 1.0 %.
 8. Wash the filter unit with 40 μL of 8 M urea, and then wash 2× with 40 μL 18 M Ω water.
 9. Wash the filter unit 3× with 100 μL of 50 mM ammonium bicarbonate solution. After the final wash add 0.4 $\mu\text{g}/\mu\text{L}$ of trypsin in 1:100 (w/w) enzyme-to-protein ratio and incubate O/N at 37 °C.
 10. Elute tryptic peptides from the second digest by adding 40 μL of 50 mM ABC solution to the filter unit and centrifuge at $14,000\times g$ for 10 min. Repeat this step 1×.
 11. Add 50 μL of 0.5 M NaCl solution to the filter unit and centrifuge at $14,000\times g$ for 10 min. Transfer the filtrate containing the tryptic peptides to a fresh microtube and acidify with formic acid to 1.0 %.

3.3 Desalting Peptides Using C_{18} Spin Columns (see Note 7)

1. Activate a C_{18} spin column by adding 500 μL of acetonitrile and centrifuge at $1100\times g$ for 1 min. Discard the flow-through after centrifugation.
2. Equilibrate the column with 500 μL of 0.1 % formic acid and centrifuge at $1100\times g$ for 1 min. Discard the flow-through and repeat this step 1×.
3. Add up to 500 μL of the LysC peptide digest to the column and centrifuge at $1100\times g$ for 1 min. If the sample volume is greater than 500 μL then repeat this step.
4. Wash the column with 500 μL of 0.1 % formic acid and centrifuge at $1100\times g$ for 1 min. Discard the flow-through. Repeat this step 1×.
5. Add 250 μL of a 90:10 acetonitrile-to-water ratio to the column and centrifuge at $1100\times g$ for 1 min. Collect the eluent containing the desalted peptides and transfer to a fresh microtube. Repeat this step 1×.
6. Dry the desalted peptide sample in a vacuum centrifuge and avoid letting the sample dry completely.

7. Repeat this procedure to desalt the tryptic peptide digest.
8. The peptide samples were quantified based on absorbance at 280 nm using a NanoDrop spectrophotometer.

3.4 Protein Fractionation Using Ion Exchange Chromatography

1. Inject 100 μg of Cytochrome C digest onto the SCX column to verify column separation.
2. Inject 50–100 μg of LysC peptide digest onto a SCX column to separate peptides.
3. Use a gradient of 2–40 % solvent B over 50 min with a flow rate of 250 $\mu\text{L}/\text{min}$.
4. Monitor the peptide fractions at 280 nm and collect the fractions in 2 min intervals using a fraction collector.
5. Repeat the procedure for the tryptic peptide digest.
6. Dry fractions in a vacuum concentrator and store at $-80\text{ }^{\circ}\text{C}$ until ready to use for nano LC-MS/MS analysis (*see Note 8*).

3.5 Nano LC-MS/MS

1. Reconstitute dried LysC and tryptic peptide digests fractions in 20 μL of 0.1 % formic acid and sonicate for 15 min.
2. Centrifuge samples at $20,000\times g$ for 10 min and remove the top 95 % of sample to a new sample vial.
3. Inject 5 μL of each peptide fraction onto a sample trap to remove salts and contaminants and separate peptides on a C_{18} column.
4. Use a gradient of 2–40 % solvent B over 100 min with a flow rate of 200 nL/min.
5. Collect ten tandem mass spectra for each MS scan on the LTQ Orbitrap.

3.6 Identification of Proteins (See Note 9)

1. Proteins are identified by peptide mass fingerprinting using the MASCOT search engine with the UniProt protein database (*see Note 10*).
2. The following search parameters can be used in MASCOT: *Mus musculus* for taxonomy, set the parent and fragment ion maximum precursors to ± 8 ppm and ± 1.2 Da, respectively (these parameters are instrument-specific). A fixed modification of carbamidomethyl of cysteine, variable modifications of oxidation of methionine and protein N-terminal acetylation (these are selected based on the reduction and alkylation performed during the digestion procedure), select the enzyme used to digest the proteins, and set a limit of two missed cleavages (*see Note 11*).
3. The peptide and protein identifications can be validated using Scaffold software. Load the MASCOT .dat files for the P3 LysC fractions, then create a new experiment and load the .dat

files for the P3 tryptic fractions. Repeat this for the other P3 biological replicates and give the sample category the name P3 so that all the replicates will be grouped as P3. Perform the same process for P14 and P30. Hence, all three sample categories, P3, P14, and P30 will be contained in one Scaffold file.

4. Scaffold parameters can be set to 95 and 99 % probability for peptide and protein, respectively, and contain a minimum of two identified peptides.
5. Remove decoys and contaminants such as keratin from the list of proteins.
6. Validate peptide identity assigned to spectra for protein identification by observing signal-to-noise ratio, verifying that high intensity peaks are labeled, and the fragment peaks line up with the amino acid assignment (*see Note 12*).

3.7 Normalizing Spectral Counts

Scaffold provides spectral counts for each protein and normalizes the spectral counts between samples, which allows for comparison of protein abundance between samples, whether a protein is up- or down-regulated. If the total amount of protein varies between samples, normalization can be used to create the total amount of all proteins in each sample to be about the same.

1. In Scaffold, select the option Quantitative Value, which allows you to view the normalized spectral counts (*see Note 13*).
2. The normalized spectral count data can then be used to calculate a ratio of the average spectral counts or fold change obtained for each age group, P3/P14, P3/P30, and P14/P30. Proteins with a ratio of average spectral counts that are twofold or greater are considered significant (*see Note 14*).

3.8 Analysis of Differentially Expressed Proteins (See Note 15)

Statistica software can be used for the statistical analysis. Spectral counts correlate with protein abundance. Therefore, the mean normalized spectral counts can be used to determine differential abundance between the age groups.

1. Once the data sets are comparable after normalization, changes can now be determined between the age groups (*see Note 16*).
2. To find differentially abundant proteins, export the mean normalized spectral counts from Scaffold into an excel spreadsheet and import the data into the statistical software, Statistica (*see Note 17*).
3. Analyze the data using a One-way Analysis of Variance (ANOVA) followed by a *post-hoc* test. For example you can use the Bonferonni test. Select “Age Group” as the Category Predictor *aka* Effect and the mean normalized spectral counts as the Dependent Variables (*see Note 18*).

4. Statistica will report p -values that are drawn from pairwise comparison between age groups, Sums of Squares (SS), Mean Square (MS), MS between groups divided by MS within groups (F -value), tests of all age groups together using ANOVA (P), and Degrees of Freedom (df). Proteins between age groups can be considered significantly different when $p \leq 0.05$.
5. Statistica provides statistical values, including p -values comparing all age groups. For example, values are given for P3 vs. P14, P3 vs. P30, and P14 vs. P30. The results can be exported to an excel spreadsheet.
6. Using the ratio of the average spectral counts, as described in Section 3.7, and the p -values calculated from Statistica, the differentially expressed proteins can be sorted in an excel spreadsheet to determine which proteins are up- and down-regulated as well as exclusively expressed.

4 Notes

1. All solutions should be prepared using Milli-Q water (minimum of 18 M Ω cm) and chemicals used are at minimum Analytical Reagent Grade quality.
2. SDS is a detergent with relatively small micelles and high critical micelle concentration (CMC), and, therefore, easily passes through the FASP filtering membrane and is depleted from the protein lysate. In contrast, detergents with large micelles and low CMCs are not easily removed from the FASP filtering membrane which could result in inhibition of the protease(s) used for digestion as well as ion suppression in mass spectrometry. This outcome will result in no data [15].
3. FORMIC ACID can be used in place of the recommended trifluoroacetic acid (CF₃CO₂H) to acidify the digested peptides and stop the digestion.
4. If the SCX column begins to exhibit signs of increased back pressure over extended use, the column should be flushed with 20 column volumes of high salt buffer, followed by 20 column volumes of 40 % methanol, then 20 column volumes of water, followed by column equilibration.
5. Prior to protein quantitation the proteins have to be identified, hence it is important to select a proteomics approach that will reduce the proteome dynamic range. Shotgun proteomics, which involves digesting proteins and separating peptides prior to tandem mass spectrometry is preferred, because we can combine different separation methods prior to mass spectrometry to enrich protein samples, detect different classes of proteins, and identify low abundant proteins.

6. Biological replicates should be performed in order to eliminate random variations. Performing replicates allows random variations to be averaged out and consistent signals to be confirmed.
7. Peptide desalting is important to remove salt and other contaminants that interfere with mass spectrometry analysis.
8. To facilitate the removal of excess salt, 500 μL of 5 % FORMIC ACID in 50:50 ACETONITRILE:H₂O can be added to the partially dried SCX collected fractions. These fractions are then dried again in a vacuum centrifuge. It is important to not dry the samples completely to avoid losing peptide identification during LC-MS/MS.
9. A challenge with using spectral counts is that zero counts can be given for a protein when absent in one sample, but it may be detected in another, which makes it impossible to calculate a fold change. In addition, when comparing samples, there may be peptides that will not be fragmented and detected due to low abundance and low ionization efficiency. Hence, optimizing peptide fractionation and chromatographic separation is critical to peptide identification.
10. It is important to search the data against a decoy database that contains reversed, shuffled, and random protein sequences to reduce the number of false positives.
11. MASCOT search engine uses mass spectrometry data for protein identification. A commonly used approach is peptide mapping, which refers to the identification of proteins using data from intact peptide masses. For peptide mass fingerprinting, MASCOT requires the input of specific parameters. The taxonomy filter allows selection of the species studied, which limits the number of matches in the results. The modification filter allows selection of fixed modifications, which assumes that in every instance a particular residue has been modified and variable modification, which tests each potential site with and without the modification. Variable modification should be used carefully because it can increase the chance of random matches. The parent and fragment ion maximum precursors allow for input of the error window on experimental peptide mass values and the error window for MS/MS fragment ion mass values, respectively. If the parent ion maximum precursor is set too tight this can result in the loss of valid peptide identifications and if set too loose this can result in an increase of false-positive identifications. The missed cleavage filter allows for missed cleavages during protein digestion. If not certain whether the protein digestion was perfect, a selection of 1 or 2 missed cleavage sites may be selected. The selection of a larger number of missed cleavage sites can result in an increased number of random peptide matches.

12. The signal-to-noise (S/N) ratio should be at least 3:1 to be considered significant.
13. To obtain normalized Quantitative Values between samples in Scaffold, the sum of the unweighted spectral counts (the total number of spectra associated with a protein as well as those shared with other proteins) for each sample is scaled by determining a sample specific scaling factor. The scaling factor used for each sample is then applied to all proteins in that sample. The scaling factor is determined by dividing the sum of spectral counts by the average spectral counts across all biological samples.
14. Protein variation between samples can occur for numerous reasons, including differences in protein abundances, variation in sample preparation, sample digestion, protein extraction procedure, change in chromatography, or the MS/MS acquisition. Hence, a normalization process is required to take into account these changes and properly identify significant changes in protein abundance between sample groups.
15. When performing a quantitation study it is important to consider whether the proteins of interest are known or unknown. If the proteins are known they can be targeted. In such a case, we would not use label-free quantitation, but another approach such as Selected Reaction Monitoring (SRM). However, if the proteins of interest are unknown, we can observe differential expression using a label-based or label-free approach.
16. Fold change values should be considered along with p -values ≤ 0.05 using ANOVA. The significance of the fold change can vary depending on the average spectral counts being compared. For example, a fold change of 2 is more significant between average spectral counts of 54 and 27 as compared to between 2 and 1.
17. Scaffold has several statistical analysis tools, such as Fold change, t -test, and ANOVA that can be used to identify differential protein abundance between samples. However, Scaffold does not have the ability to perform a *post-hoc* test that confirms where differences occur between sample groups. Therefore, additional statistical analyses have to be performed using different statistical software.
18. Data sets can be analyzed using statistical tests such as a Student's t -test. However, when there are more than two groups to compare ANOVA should be performed.

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