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iTRAQ-based Shotgun Neuroproteomics

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Abstract

Shotgun proteomics involves the analysis of peptides obtained by enzymatic digestions of proteins and subsequent identification via tandem mass spectrometry. This approach is an effective method for studying global protein expression in neuronal systems. The method described here is a quantitative shotgun neuroproteomics method using amine-specific isobaric tags for a relative and absolute quantitation (iTRAQ)-based workflow. We will provide the technical details for sample preparation, 2-dimensional liquid chromatography, tandem mass spectrometry, database search and statistical analysis to identify differentially expressed proteins. We will use a recent study on a rat model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE) to illustrate the successful application of this method.

Keywords

Shotgun proteomics; liquid chromatography; tandem mass spectrometry; iTRAQ; EAE; multiple sclerosis; post-translational modification

1. Introduction

Shotgun proteomics refers to the direct analysis of complex peptide mixtures to rapidly generate a global expression profile of the proteins within complex systems¹. It is an effective tool for the identification and quantification of a large number of proteins by a workflow sequentially involving 2-dimensional (2D) liquid chromatography (LC), tandem mass spectrometry (MS/MS) and protein database-searching algorithms. In recent years, it has received increasing attentions for neuroproteomics analysis, and has uncovered key proteins involved in the neuronal dysfunction and an inflammatory response associated with neurodegenerative disorders^{2, 3}. The shotgun approaches, based on 2D-LC-MS/MS, have advantages over 2D gel-based proteomics technologies in terms of both detection sensitivity and proteome coverage⁴. Quantification of differentially expressed proteins using the shotgun methods can be divided into either labeled or label-free approaches. Several chemical labeling technologies have emerged recently, including ¹⁸O-labeling⁵, stable isotope-labeled amino acids in culture (SILAC)⁶, iTRAQ^{7, 8} and isotope-coded affinity tag (ICAT)⁹ methods. Label-free quantification approaches include LC/MS quantification of

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MS/MS precursors and spectral counting¹⁰. Both approaches have their strengths and limitations. Chemical labeling allows better quantification precision, but suffers from the potential for incomplete peptide labeling or unintended side reactions. Label-free methods are simpler, but quantification outcomes are less precise than labeled methods.

iTRAQ technology provides a means to quantify up to eight different samples simultaneously. In our experimental design, we used four labeling reagents consists of an N-methylpiperazine quantification group, a carbonyl stable-isotope mass balance group and a hydroxyl succinimide ester group that reacts with the primary amines on peptide N-termini and lysine side chains¹¹. Peptide quantification is based on the relative abundance of the four quantification reporter ions (m/z 114, 115, 116 and 117) generated through MS/MS fragmentation of iTRAQ-labeled peptide mixtures. Since iTRAQ reagents are efficient at labeling nearly all peptides¹¹, this method is more effective at providing higher protein identification sequence coverage than the ICAT method. In addition, the peptide b- and y-series ions derived from all four samples are combined in MS/MS spectra, resulting in better ion statistics for more accurate peptide identifications¹².

We will present here an iTRAQ-based shotgun neuroproteomics method that we've used to study EAE, an animal model of multiple sclerosis, with inflammatory and demyelinating symptoms of the central nervous system. Although the pathological manifestations of EAE are well defined, the molecular mechanisms underlying neural deficits remain elusive. In order to discover novel therapeutic targets for multiple sclerosis, we conducted a proteomics study of EAE animals (see Fig. 1). Proteins extracted from four rat lumbar spinal cords were first digested with trypsin. Four iTRAQ reagents were utilized to label two control and two EAE samples. Equal amounts of the labeled peptides were combined and separated using strong cation exchange LC (SCXLC), and subsequent reversed-phase LC (RPLC). The resulting peptide fractions were spotted onto matrix-assisted laser desorption/ionization (MALDI) plates and analyzed on a tandem mass spectrometer. Bioinformatic analysis of the MS/MS spectra enabled us to identify 41 differentially expressed proteins in EAE rat spinal cords³.

2. Materials

2.1. Protein Extraction

1. Dissect lumbar spinal cords from two adjuvant-treated controls and two EAE rats. Rinse well to remove blood completely.
2. Lysis buffer: 25 mM triethylammonium bicarbonate (TEAB), 20 mM Na₂CO₃ and 0.1% (v/v) of protease inhibitor cocktail (Sigma, St Louis, MO), pH 10.0 (see Note 1).

¹The lysis buffer described here is only for extracting soluble proteins. In order to extract more hydrophobic proteins, an alternative lysis buffer containing SDS, NP40, Triton-100, Tween-20 and urea may be used. For example, 10 mM HEPES (pH 8.0), 150 mM TEAB, 0.02% sodiumazide, 0.1% SDS, 1% NP40, 0.5% deoxycholic acid, 0.2 mM PMSF, 2 µg/mL aprotinin, 2 µg/mL leupeptin, 2 µg/mL pepstatin, and 50 mM NaF may be used. Since high concentrations of detergents will affect trypsin digestion, the lysate should be diluted with H₂O to reduce the detergents concentrations prior to trypsin digestion. The final concentrations of SDS should be 0.05%, NP-40 0.1%, Triton X-100 0.1%, Tween 20 0.1%, CHAPS 0.1%, Urea 1M.

3. An ultrasonic homogenizer with a microtip that fits a typical 1.5 ml eppendorf tube (e.g., Omni Ruptor 250 ultrasonic homogenizer with a 5/32" solid titanium microtip, Omni international, Marittha, GA).
4. A Bradford protein assay kit (e.g., from Bio-Rad, Hercules, CA).
5. A microplate spectrophotometer (e.g., Spectra MAX 190 from Molecular Devices, Sunnyvale, CA).

2.2. iTRAQ Labeling

1. Dissolution buffer: 0.5 M TEAB, pH 8.5.
2. Denaturing reagent: 2 % SDS (w/v).
3. Reducing reagent: 50 mM tris-(2-carboxyethyl) phosphine (TCEP).
4. Cysteine blocking reagent: 200 mM methyl methanethiosulfonate (MMTS).
5. HPLC grade ethanol and water.
6. Mass spectrometry grade modified trypsin (e.g., from Promega, Madison, WI).
7. iTRAQ™ Reagents: 114, 115, 116 and 117 (Applied Biosystems Inc. (ABI), Forster City, CA).
8. Vacuum concentrator (e.g., the Eppendorf 5301, Westbury, NY).

2.3. Liquid Chromatography Systems

2.3.1. Strong Cation Exchange liquid chromatograph (SCXLC)

1. Mobile phase A: 10 mM KH₂PO₄ and 20% acetonitrile (ACN), adjust to pH 3.0 with 85% H₃PO₄.
2. Mobile phase B: 600 mM KCl, 10 mM KH₂PO₄ and 20% ACN, adjust to pH 3.0 with 85% H₃PO₄.
3. BioCAD Sprint™ perfusion chromatography system (ABI).
4. Column: Polysulfoethyl-A column (4.6 × 200 mm, 5 μm, 300 Å, Poly LC Inc., Columbia, MD).
5. pH paper.

2.3.2. Peptide Desalting

1. PepClean C₁₈ spin columns (Pierce, Rockford, IL).
2. Loading solution: 5% ACN containing 0.5% trifluoroacetic acid (TFA).
3. Activation solution: 50% ACN containing 0.5% TFA.
4. Elution solvent: 70% ACN.

2.3.3. Reversed-phase Liquid Chromatography (RPLC)

1. Solvent A: 5% ACN containing 0.1% TFA.

2. Solvent B: 95% ACN containing 0.1% TFA.
3. MALDI matrix solution: 7 mg/mL α -cyano-4-hydroxycinnamic acid (Sigma) in 60% ACN, 5 mM ammonium monobasic phosphate and internal calibrants (50 fmol/mL each of (Glu¹)-fibrinopeptide B (GFP, m/z 1570.677, Sigma) and adrenocorticotrophic hormone 18–39 (ACTH 18–39, m/z 2465.199, Sigma)).
4. LC-Packings Ultimate Chromatography System equipped with a Probot MALDI spotting device (Dionex, Sunnyvale, CA).
5. C₁₈ PepMap 0.3 × 5 mm, 5 μ m, 100 Å, trapping column (Dionex).
6. C₁₈ PepMap 0.1 × 150 mm, 3 μ m, 100 Å, capillary column (Dionex).

2.4. Mass Spectrometry

1. 4700 Proteomics Analyzer (ABI).
2. MALDI plates (ABI).
3. Mass Standards Kit containing 6 peptides mixture (ABI, cat no. 4333604).

2.5. Data Analysis Software

1. 4000 Series Explorer (ABI).
2. GPS Data Explorer v3.5 (ABI).
3. Mascot Search Engine v1.9 (Matrix Science Ltd. London, UK).

3. Methods

3.1. Protein Extraction

In order to obtain reproducible quantification results, it is important to prepare all four neuronal samples under identical conditions (see Note 2). In addition, the protein amounts among the four samples prior to trypsin digestion must be made equal.

1. For each sample, place ~15 mg of spinal cord tissue into a 1.5 mL Eppendorf tube on ice. Add 300 μ L of the lysis buffer into each tube.
2. Disrupt the tissues on ice via ultrasonication (20 s sonication followed by a 30 s pulse). Repeat five times (see Note 3).
3. Pellet the insoluble materials by centrifugation for 15 min at 20,000 g at 4 °C in a bench-top centrifuge.
4. Transfer the supernatants into a fresh 1.5 mL Eppendorf tube kept on ice.

²In the EAE model, eight-week old female Lewis rats were immunized with myelin basic protein emulsified in Complete Freund's Adjuvant (CFA), or CFA/vehicle. The animals exhibiting hind limb paralysis were euthanized by exposure to CO₂. The spinal cords were dissected out, meninges were carefully removed and the tissues were thoroughly rinsed with saline to remove blood. The lumbar spinal cord was immediately frozen on dry ice and stored at –80 °C until use.

³In most cases, ultrasonication for 5 times should be enough to break the cells and extract the proteins. However, if the cells do not break completely, ultrasonication can be repeated more times until the proteins can be thoroughly extracted.

5. Measure the protein concentrations for all four samples using the Bradford assay with bovine serum albumin diluted in the lysis buffer as standards (see Note 4).
6. Adjust the protein concentration of each sample to the same level with the Lysis Buffer.

3. 2. Trypsin Digestion and iTRAQ Labeling

1. Pipette 90 µg of protein from the four samples into four separate tubes (see Note 5). Add 1 µL of the denaturing reagent into each tube.
2. To each sample, add 2 µL of reducing reagent and vortex. Bring down the contents with a brief centrifugation. Incubate the sample tubes at 60 °C for 1 h. Spin briefly to settle the liquid to the bottom of each tube.
3. To each sample, carefully add 1 µL of the cysteine blocking reagent. Mix by vortexing and centrifuge briefly to collect the solutions to the bottoms of the tube. Incubate at room temperature for 10 min.
4. Reconstitute two vials of trypsin (20 µg/vial) with 25 µL each of HPLC grade water. Vortex briefly.
5. To each sample tube, add 10 µL of the trypsin solution, vortex and centrifuge briefly to collect the solution to the bottom of the tube. Incubate at 37 °C for 12 to 16 h. Spin briefly to bring the sample solution to the bottom of the tubes (see Note 6).
6. Bring the iTRAQ reagents to room temperature. Add 70 µL of ethanol into each reagent vial, cap the vial and vortex vigorously, then centrifuge briefly to settle the iTRAQ reagents to the bottoms of the vials (see Note 7).
7. Transfer the entire content of one iTRAQ reagent vial into each of the four sample tubes, and vortex to mix thoroughly. Spin briefly to collect the liquid to the bottom of the tubes. Peptides derived from the two control samples are labeled with iTRAQ Reagents 114 and 115 whereas peptides obtained from the two EAE samples are labeled with iTRAQ Reagents 116 and 117. Incubate the reaction vials at room temperature for 1 h.
8. Carefully combine the entire contents of all four iTRAQ labeled samples into one tube, mix thoroughly by vortexing, then centrifuge briefly (see Note 8).

⁴To obtain accurate iTRAQ quantitative results, it is very important to adjust the protein concentration to be equal among all four samples. If the lysis buffer contains detergents, the Bradford assay will not be suitable for accurate protein concentration estimation. A BCA protein assay may be used instead.

⁵Based on iTRAQ instructions from ABI, each protein sample should be between 5 to 100 µg for each iTRAQ labeling reaction. To ensure maximum labeling efficiency, sample volumes should be less than 50 µL each. If the sample volume is larger than 50 µL, a speedvac can be used to reduce the sample volume before iTRAQ labeling.

⁶It is important to check the protein digestion efficiency before iTRAQ labeling. Take 1 µL of each digested sample and desalt it using a C18 ZipTip (Millipore, Billerica, MA). Mix the eluted peptides with the MALDI matrix solution in a 1:1 ratio and spot them onto a MALDI plate. Acquire MS spectra to check if the peptide ion signals are comparable.

⁷To maximize labeling efficiency, the concentration of organic reagents (ethanol and iTRAQ reagents) in iTRAQ labeling reactions should be larger than 60% (v/v).

3. 3. SCXLC

The combined peptide mixture will be first separated by SCXLC on a polysulfoethyl-A column to first remove the excess iTRAQ reagents and then fractionate the peptides.

1. In order to remove both TEAB and the organic solvent from the sample, dry the combined sample completely in a vacuum concentrator (see Note 9).
2. Reconstitute the peptides by adding 500 μ L of SCX mobile phase A and confirm the pH value using pH paper. If the pH is not between 2.5 and 3.0, adjust it by adding additional SCX mobile phase A or drops of 1 M phosphoric acid.
3. Before SCX separation, centrifuge the sample at 20,000 g for 10 min to pellet any particulates.
4. Equilibrate the column with mobile phase A, then inject the iTRAQ labeled peptides onto the SCX column through a 500 μ L sample loading loop.
5. Peptides will be eluted with a 60 min linear gradient from 100% mobile phase A to 100% mobile phase B at a flow rate of 1.0 mL/min. Collect 2-min fractions into Eppendorf tubes.
6. Dry all the SCX fractions completely in a vacuum concentrator for subsequent desalting.

3.4. Peptides Desalting using C₁₈ Spin Columns

1. Resuspend the peptides in each fraction in 150 μ L of the loading solution (see Note 10).
2. Add 200 μ L of the activation solution into a C₁₈ spin column and centrifuge at 1,500 g for 1 min to clean the spin column. Repeat this step once.
3. Equilibrate the spin column with 200 μ L of the loading solution and then centrifuge the column at 1,500 g for 1 min. Repeat this step 3 times.
4. Separately for each SCX fraction, load 150 μ L of the peptides dissolved in the loading solution onto a spin column and centrifuge at 1,000 g for 1 min to remove the unbound materials. Reload the flow through materials onto the spin column and repeat this step twice (see Note 10).

⁸In cases of the lysis buffer containing high concentrations of detergents or organic solvents, protein concentration measurements and sample pipetting accuracy may be affected by the lysis buffer. Additional adjustments in peptide levels may be needed to ensure even peptide mixing in order to obtain reliable protein quantification results. For example, prior to combining all four iTRAQ-labeled samples, a small aliquot (i.e. 1/20) of each iTRAQ labeled sample can be combined into one tube. The excess iTRAQ reagents are removed by an SCX cartridge column (ABI) followed by a C₁₈ spin column for desalting (Pierce, cat no. 89870). Dry the eluted peptides in a vacuum concentrator and reconstitute the peptides in RPLC solvent A. Follow steps 3.5. to 3.8., the peptides can be separated by RPLC and spotted onto a MALDI plate. MS/MS spectra of the peptides are acquired and data are analyzed using the GPS server (ABI). Calculate the population reporter ion peak area (RPA) median of each quantification reporter ion (114, 115, 116 and 117). Assuming most of proteins do not change among the four samples, the relative RPA medians of the four quantification reporter ions should be close to 1:1:1:1. If one or more samples varies from the others by greater than 10%, the volume(s) of that sample(s) needs to be adjusted accordingly to ensure an equal peptide mixture.

⁹To remove all of the TEAB, reconstitute the combined iTRAQ labeled samples in 100 μ L of HPLC grade water and dry the sample in a vacuum concentrator. Repeat this step twice to ensure all the TEAB is evaporated.

¹⁰For high salt fractions, 150 μ L of the loading solution may not be enough to resuspend the salts and peptides. More loading solution should be added until all the contents are solubilized. The entire content of the tube should be loaded onto the spin column for desalting.

5. Wash the bound peptides with 200 μ L of the loading solution and centrifuge at 1,500 g for 1 min to remove salts. Repeat this step 3 times.
6. Elute the peptides using 100 μ L of the elution solvent via centrifugation at 1,500 g for 1 min and repeat twice. Collect all three eluents into the same Eppendorf tube.
7. Dry the peptides in a vacuum concentrator before RPLC (see Note 11).

3.5. Reversed-phase Liquid Chromatography

1. Reconstitute the samples in 8 μ L of solvent A, vortex vigorously, then centrifuge at 10,000 g for 2 mins. Transfer the samples into the bottom of auto-sampler vials and place them in cooled autosampler wells.
2. Equilibrate the RPLC column with 5% solvent B for at least 15 min prior to sample injection.
3. Each fraction (6.4 μ L) will be loaded onto a C₁₈ trapping column using a 'microliter pickup' method at a flow rate of 20 μ L/min. The bound peptides are subsequently resolved on a C₁₈ capillary PepMap column at a flow rate of 200 nL/min with the following gradient:

Time (min)	Solvent A	Solvent B
0	95	5
4	92	8
34	82	18
57	62	38
64	5	95
69	5	95
70	95	5
85	95	5

4. The eluted peptides are mixed with the MALDI matrix in a 1:1 ratio through a 30 nL mixing tee, and spotted onto MALDI plates in an 18 $\times$ 18 spot array format (total 324 spots) using the Probot, which collects a spot every 12 s. Repeat the RPLC steps for each SCX fraction.

3.6. Mass Spectrometry

1. Load the sample plates into the plate loader of a MALDI tandem mass spectrometer, in our case an ABI 4700 Proteomics Analyzer.
2. Tune the instrument (e.g., deflectors X1, Y1, X2, Y2) using a mass standard mixture (e.g., kit from ABI) for optimal MS/MS sensitivity. For our instrument, we

¹¹Approximately 30 SCX fractions are collected. In order to reduce the number of subsequent RPLC samples, some fractions can be combined based on the peptide complexity in each fraction. Take 1 μ L from each fraction after desalting, mix it with the MALDI matrix in a 1:1 ratio and spot them onto the MALDI plate. Acquire MS spectra, and evaluate MS ion complexity in the spectra. SCX fractions whose MS spectra contain less than 100 peaks (S/N $\geq$ 5) between m/z 1,000 to 2,000 may be combined prior to RPLC separations.

also optimize both the metastable ion suppressor and the timed-ion-selector (TIS) for the most precise precursor ion selection at the maximal resolution of 200, corresponding to ± 5 Da at a m/z of 1,000. Instrument MS/MS sensitivity should be evaluated daily to ensure optimal iTRAQ quantification outcome.

3. Update the MS calibration file using the masses of the peptides in the mass standard mixture. Update the MS/MS calibration file using GFP MS/MS ion spectra (internal calibrant).
4. Create a new spot set (an Oracle database interface that contains both sample and MS method information on the of RPLC eluents spotted on to a MALDI plate, a spread sheet unique to the ABI 4000 Series Explorer software), load and align the iTRAQ sample plates.
5. iTRAQ labeled peptides from each MALDI spot are analyzed in a data-dependent fashion. All positive ion MS/MS data will be acquired using a method optimized with a 1 kV collision energy.
6. Set up the acquisition, processing and job-wide interpretation methods for both MS and MS/MS analysis. In the MS acquisition method, set the mass range to m/z 850–3,000; the focus mass is set at m/z 1,900; MS spectra are acquired with 2,500 laser shots at a laser intensity of 3,300. In the MS processing method, GFP (m/z 1570.677) and ACTH 18–39 (m/z 2465.199) masses are used for internal calibration. In the interpretation method, MS ions that meet the precursor selection criteria (200 ppm spot-to-spot precursor exclusion, S/N ratio 100 and a maximum of ten most abundant precursors per spot) are selected for subsequent MS/MS analysis, starting from the weakest to the strongest ion. In the MS/MS acquisition method, spectra are acquired with 3,000 laser shots at a laser intensity of 3,850. The spectra are smoothed with a Savitsky-Golay algorithm (FWHM = 9, polynomial order = 4) (see Note 12). An MS/MS spectrum example is shown in Fig. 2.

3.7. Database Search

1. Peptide identification is performed by searching the MS/MS spectra against rodent sequences in the SwissProt protein database (see Note 13) using a local Mascot search engine (v1.9) on a GPS server (v3.5, ABI).
2. The following parameters are used for the search: trypsin digestion with a maximum of one missed cleavage; precursor mass tolerance set at 50 ppm; MS/MS mass tolerance set at 0.3 Da; iTRAQ-labeled N-termini and lysines as well as

¹²Different MS instruments have different optimal MS and MS/MS methods for the analysis of iTRAQ labeled peptides. In the 4700 Proteomic Analyzer, we update both MS and MS/MS calibration files daily and tune the deflector parameters weekly to obtain the maximum sensitivity and mass accuracy.

¹³It is important to use the latest version of the protein database to ensure comprehensive peptide identification. Swissprot, IPI, NCBI protein database or EST (6 frame translation into protein sequences) can be used, with increasing number of entries and database size. Generally speaking, using bigger databases will likely increase one's chance to match a spectrum to a peptide sequence. However, it will also increase the odds for random matching. We chose the Swissprot database for our study, because of its high protein sequence accuracy and low redundancy.

MMTS-labeled cysteines are set as fixed modifications; oxidized methionines and iTRAQ-labeled tyrosines are set as variable modifications (see Note 14).

3. In the GPS Result Browser, choose MS/MS Summary Tab and copy both the isotope carry-over corrected iTRAQ reporter ion peak areas (RPAs) of m/z 114 to 117 and the corresponding Mascot peptide identification matching results to a Microsoft Excel datasheet (see Note 15).
4. Only peptides identified with a confidence interval (C.I.) value $\geq 95\%$ are used for protein identification and quantification. Mascot will assign a peptide to a corresponding protein entry with the highest score (usually the protein with the most number of peptides matched) if more than one entry share identical peptide sequence. To reduce the probability of a false identification, we will only quantify proteins containing at least two matched peptides.
5. To estimate the false positive rate (FPR) for protein identification, all spectra are searched against a decoy SwissProt database with all protein sequences reversed using the same database searching criteria outlined above. The FPR is computed as¹³:

$$\text{FPR} = 2 \times (N_{\text{decoy}}) / (N_{\text{decoy}} + N_{\text{forward}})$$

N_{decoy} : Number of proteins identified using the decoy database

N_{forward} : Number of proteins identified using the regular database

6. If FPR is higher than the generally accepted 5%, increase your peptide identification C.I. value cutoff from 95% to 97% or higher for subsequent expression analysis until the calculated FPR value is less than 5%.

3.8. Bioinformatics Analysis to Determine Differentially Expressed Proteins

1. Minor adjustments to the raw RPA values need to be performed prior to protein quantification analysis. For the peptides with RPA values of 0, assign a nominal signal count of 100 to facilitate later mathematical analysis (see Note 16).
2. For each sample, the peptide population median RPA (M_{114} , M_{115} , M_{116} , M_{117} for control 1, control 2, EAE1 and EAE2 samples, respectively) is calculated in Microsoft Excel.
3. Assuming a comparable overall protein concentration in each sample, individual peptide RPA values are normalized by dividing the raw RPA value with corresponding normalization factors (F_{norm}), calculated using the following formulas (see Note 17).

¹⁴Using too many variable modifications can significantly lower the spectra matching confidence interval (C.I.) values due to increased random matches. Only include modifications relevant to the experiment. iTRAQ labeling is usually very efficient¹¹; however, it may be prudent to evaluate iTRAQ labeling efficiency for each experiment. To that end, change the fixed iTRAQ modifications into variable modifications and repeat the GPS search. For all the peptide matches with C. I. $\geq 95\%$, the number of iTRAQ-labeled N-termini (N_{ti}) and lysines (N_{ki}) are compared with the total number of peptide N-termini (N_{tt}) and lysines (N_{kt}). The iTRAQ labeling efficiency is estimated as: $100\% \times (N_{\text{ti}} + N_{\text{ki}}) / (N_{\text{tt}} + N_{\text{kt}})$.

¹⁵Database search and data display can take hours depending on your computer hardware and the scale of your iTRAQ experiment.

¹⁶The denominators for peptide expression ratio computation cannot be zero. Therefore, use a nominal signal count 100 to represent the background noise level.

$$F_{\text{norm114}} = M_{114} / M_{114} \quad (\text{Control 1})$$

$$F_{\text{norm115}} = M_{115} / M_{114} \quad (\text{Control 2})$$

$$F_{\text{norm116}} = M_{116} / M_{114} \quad (\text{EAE 1})$$

$$F_{\text{norm117}} = M_{117} / M_{114} \quad (\text{EAE 2})$$

4. A protein expression change is derived from all of its corresponding peptide changes. For each peptide, the mean of the four iTRAQ RPA values ($\overline{RPA}$) is computed in Microsoft Excel. Since low ion signals tend to produce larger quantification inaccuracies, only peptides with $\overline{RPA}$ values larger than 5,000 counts will be included for protein expression analysis¹¹.
5. In order to ensure that different peptides belonging to the same protein contribute equally towards the statistical determination of differentially expressed proteins, regardless of their peptide ionization efficiency, relative RPA values are computed using Microsoft Excel. For each peptide, all of its relative RPA values are calculated as the ratios of normalized individual RPA values divided by the RPA value from the 114-labeled sample. Such ratios are then transformed into $\log_2$ values (R_{114} , R_{115} , R_{116} and R_{117} , see Note 18). In addition, the relative RPAs between EAE1 (m/z 116) and EAE2 (m/z 117) are also computed and transformed into $\log_2$ values for the later evaluation of within-group protein variation between the two EAE samples (R_{76}). In cases of multiple MS/MS spectra matched to the same peptide sequence, the peptide ratio is calculated and weighted based on the relative proportion of the $\overline{RPA}$ values of each spectrum.
6. Protein $\log_2$ ratios (P_{114} , P_{115} , P_{116} , P_{117} and P_{76} (EAE2/EAE1)) are computed as the mean of R values (R_{114} , R_{115} , R_{116} , R_{117} and R_{76} , respectively) of all its corresponding peptides.
7. The relative protein expression between EAE and the control samples (calculated as the pooled protein $\log_2$ ratios) are computed based on the following equation:

$$P_i = \frac{(P_{116i} + P_{117i})}{2} - \frac{(P_{114i} + P_{115i})}{2}$$

¹⁷The assumption of using the median values to normalize RPAs is that four iTRAQ-labeled peptide populations should produce a similar RPA signal distribution profile. In case the RPA signal distribution profiles are dissimilar, LOWESS or other advanced normalization routines can also be used¹⁴.

¹⁸A log transform is used to produce a symmetrical peptide log ratio distribution and provide a means for an accurate estimation of the standard deviations for peptide and protein ratios¹⁵. More importantly, this procedure will ensure that both an increase and decrease in peptide expression levels contribute equally towards the determination of differentially expressed proteins. We choose 2-based log transformation, because it enables concise interpretations of protein fold changes. For example, a difference of one in this scale corresponds to a two-fold change.

P_i : the pooled protein log₂ ratio of the i th protein.

($i = 1, 2, 3, \dots N$, where N is the total number of identified proteins).

8. To identify differentially expressed proteins, both p -values in Student's t -tests and the pooled standard deviation of within-group protein variation (S_p , see Note 19) need to be considered (see Note 20). The p -values are generated by comparing each protein log₂ ratio in the control group (P_{114} and P_{115}) to those in the EAE group (P_{116} and P_{117}) using Microsoft Excel. S_p is computed using the following formula:

$$S_p = \sqrt{\frac{S_C^2 + S_E^2}{2}}$$

S_p : pooled standard deviation of within-group protein log₂ ratios; S_C : standard deviation of all protein log₂ ratios between the two control samples, which is calculated as the standard deviation of P_{115} with Microsoft Excel; S_E : standard deviation of all protein log₂ ratios between the two EAEs, which is calculated as the standard deviation of P_{76} with Microsoft Excel (see step 6). Differentially expressed proteins must meet two criteria: 1) p -values < 0.05 and 2) P_i values are larger than $1.65S_p$ or smaller than $-1.65S_p$ (corresponding to the top 5% increased and the bottom 5% decrease proteins) (see Notes 21 and 22).

9. Anti-log₂ of P_i values are calculated to produce the exact protein fold change values.

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¹⁹ S_p is a pooled standard deviation to estimate the degree of the protein expression changes caused by random protein-to-protein variations within the control or EAE groups.

²⁰Student's t -test is utilized here to account for the relative protein expression value between groups for each protein. Limited by the availability of only 4 versions of iTRAQ reagents at the time of our experiments, the t -test can be underpowered. However, recent availability of 8 versions of the iTRAQ reagents should substantially improve the t -test statistical power.

²¹Using the methods described here, we acquired 13,834 MS/MS spectra. 2,488 unique peptides from 510 proteins were identified with an estimated false identification rate 3.1%. Forty-one proteins were found differentially expressed in EAE³. To further identify changes in peptides with post translational modifications (PTMs), additional PTMs can be set as variable modifications for repeated database PTM searches. The peptides identified have to meet the following criteria: 1) a C.I. value > 80 ; 2) the PTM peptide matching score is higher than the score when the same spectrum is searched without considering this PTM and 3) must pass a manual spectral inspections.¹⁶

²²It is very important to verify the iTRAQ quantification results using Western blotting, immunohistochemistry, or other biological methods for validation purposes.

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Fig. 1. iTRAQ-based shotgun neuroproteomics workflow

Proteins extracted from the rat lumbar spinal cords are first digested into peptides by trypsin. Two control samples are labeled with the iTRAQ reagents 114 and 115, and two EAE samples are labeled with the iTRAQ reagents 116 and 117. The labeled peptides are combined and separated using SCXLC and RPLC and analyzed on a tandem mass spectrometer. Database search and bioinformatics procedures are used for protein identification and quantification.

Fig. 2. MS/MS spectrum of a lysozyme C peptide

Lysozyme C is found elevated in EAE samples. (A) The peak areas of iTRAQ quantification ions, m/z 114 through 117 are used to determine the relative abundance of the peptide. (B) Peptide sequence is deduced from the MS/MS spectrum, based on the continuous series of N-terminal (b series) and C-terminal (y series) ions.