Quantitative analysis of protein expression using iTRAQ and mass spectrometry

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Method Article

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Abstract

Introduction

The recent introduction of isobaric peptide tags for relative and absolute quantification \(iTRAQ) of proteins in different samples was a major breakthrough in quantitative proteomics. The iTRAQ method is based on the differential covalent labelling of peptides from proteolytic digests with one of four iTRAQ reagents resulting in the incorporation of 144.1 Da to peptide N-termini and lysine residues. Peptides with different tags are indistinguishable by mass but can be differentiated by collision-induced dissociation \(CID) \(normally applied during MS peptide sequencing) through release of a reporter ion, each of which has a different mass \(114.1, 115.1, 116.1 or 117.1 Da). The analysis of the intensity of reporter ions allows the simultaneous sequencing and quantification of labelled peptides

Reagents

Sample preparation and iTRAQ labelling _Sample homogenisation buffer_ 50mM ammonium bicarbonate pH 8 0.1 % SDS Protease inhibitors \(Sigma) _Acetone_ Chilled to -20 ˚C **Strong cationic exchange HPLC buffers** _Buffer A_ 10mM KH_2PO_4 25 % ACN pH 3.0 with H_3PO_4 MQ H_2O to reach 1000 ml _Buffer B_ 10 mM 1 M KH_2PO_4 25 % ACN pH 3.0 with H_3PO_4 500 mM KCl MQ H_2O to reach 1000 ml _Conditioning buffer_ 0.2 M NaH_2PO_4 0.3 M Na acetate MQ H_2O to reach 500 ml **Reversed phase HPLC buffers** _Buffer A_ 5 % ACN 0.1 %TFA MQ H_2O to reach 1000 ml _Buffer B_ 80 % ACN 0.1 %TFA MQ H_2O to reach 1000 ml _MALDI matrix solution_ 3 mg/ml Alpha-cyano-4-hydroxycinnamic acid 70 % ACN 0.1 % TFA Neurotensin for internal calibration of MALDI-MS/MS **SepPak solutions for sample clean up** 100 % MeOH. _Elution soluion_ 80 % ACN 0.1 %TFA _Washing solution_ 0.1 % TFA

Procedure

Sample preparation 1. Remove amygdala or brain region of interest. 2. Homogenise in 200 µl amonium bicarbonate pH 8, 0.1 % SDS, protease inhibitors, by 10 up and down strokes of a 27G syringe. 3. Sonicate for 2 min. 4. Centrifuge at 13,000 rpm to pellet insoluble material. 5. Determine protein concentration using Bradford Regent. 6. Aliquot 100 µg of each sample. **iTRAQ labeling and digestion** _Clean up a sample by acetone precipitation_ 1. Chill acetone to -20 °C and the sample tube containing the sample to 4 °C. 2. Add six volumes of cold acetone to the cold sample tube. 3. Invert the tube three times. 4. Incubate the tube at –20 °C until precipitate forms \(~1 hr). 5. Decant the acetone. Air dry. _Reduction and cysteine blocking_ 1. To each sample tube containing 100 µg of acetone precipitated protein add 20 µl Dissolution Buffer. 2. Add 1 µl of the Denaturant in the kit and vortex to mix. 3. To each sample tube, add 2 µl Reducing Reagent. 4. Vortex to mix, then spin. 5. Resuspend pellet by vortexing and sonication 6. Incubate the tubes at 60 °C for 1 hr. 7. Spin to bring the sample to the bottom of the tube. 8. To each tube, add 1 µl Cysteine Blocking Reagent. 9. Vortex to mix, then spin. 10. Incubate the tubes at room temperature for 10 min. _Trypsin digestion of sample_ 1. Reconstitute 40 µg of trypsin with 20 µl

dissolution buffer. 2. Vortex to mix, then spin. 3. To each sample tube, add 10 µl \(8 µg) of the trypsin solution \(1:13 enzyme:substrate). 4. Vortex to mix, then spin. 5. Incubate the tubes at 37 °C overnight \(12 to 16 hr). 6. Spin to bring the sample digest to the bottom of the tube. **NB:** The volume of the sample digest must be less than 50 µl. If the volume of the sample digest is greater than 50 µl, lyophilise and then reconstitute with 30 µl Dissolution Buffer. _Labelling the Protein Digests with the iTRAQ Reagents_ 1. Allow each vial of iTRAQ Reagent required to reach room temperature. 2. Spin to bring the solution to the bottom of the tube. 3. Add 70µl of ethanol to each room-temperature iTRAQ Reagent vial. 4. Vortex each vial to mix, then spin. 5. Transfer the contents of one iTRAQ Reagent vial to one sample tube. 6. Vortex each tube to mix, then spin. 7. Incubate the tubes at room temperature for 1 hr. 8. Spin at 13,200 rpm at RT for 15 min. 9. Combine all supernatants. 10. Lyophilise to remove ethanol in speedvac but not to dryness. **Strong Cationic Exchange \(SCX) HPLC** _Sample Preparation_ 1. Add 200 µl of SCX Solvent A. 2. Adjust pH to 3 with acetic acid and add ACN to give final ACN concentration of 25%. 3. Spin at 13,200 rpm at RT for 15min. 4. Put the supernatant into injection vial. _HPLC setup and conditioning of SCX 2.1 mm Poly-S column_ 1. Set flow rate to 300 µl/min. 2. Run 100 % methanol for 30 min. 3. Run 100 % H_2O for 20 min. 4. Run 100 % conditioning buffer for 60 min. 5. Run 100 % H_2O for 15 min. 6. Run 100% buffer B for 15 min. 7. Equilibrate by running 100 % buffer A overnight at 50 µl/min or until UV chromatogram is stable. _SCX fractionation_ 1. Run standard peptides to check column integrity and elution times. 2. Load and inject sample. 3. Fractionate and elute peptides according to **Figure 2**. **Processing of SCX fractions** 1. Lyophilise all fractions to remove ACN. 2. Pool samples based on the SCX elution chromatogram into ~4-6 fractions. 3. Add 0.1% TFA and check pH is ~3. 3. Clean-up samples using sep-pak columns with binding capacity of ~100 µg. 4. Wet the cartridge with 1 ml 100 % MeOH. 5. Wet the cartridge with 1 ml 80 % ACN, 0.1% TFA. 6. Equilibrate with 2 x 1 ml 0.1% TFA. 7. Load the sample. 8. Wash with 2 x 1 ml 0.1 % TFA. 9. Repeat steps 7 and 8 once with the flowthrough. 10. Elute with 500 µl 80 % ACN, 0.1 % TFA. 11. Speedvac dry. **Probot spotting of samples on MALDI plates** _Prepare your Sample_ 1. Dissolve sample in Buffer A and transfer to injection vial _HPLC setup and preparation of of RP 75 µm x 150 mm separation column_ 1. Set flow rate to 0.3 µl/min. 2. Run 100 % buffer A for 30 min. 3. Run a cytochrome c standard to check column integrity. _RP-HPLC fractionation and sample spotting_ 1. Prepare fresh MALDI matrix solution. 2. Fractionate and elute peptides according to **Figure 2**. 3. Initiate spotting at ~35 min when first peptide peaks elute. 3. Mix column effluent directly with MALDI matrix solution. 4. Automatically deposit fractions every 10 s onto the MALDI target plate using a Probot micro-fraction collector. For each HPLC run, a total of 416 spots can be spotted. **ABI 4800 MALDI MS/MS analysis and database searching** Analyse MALDI plates on ABI 4800 MS/MS. Use ProteinPilot software for peptide identifications, and for the analysis of iTRAQ reporter ions for quantitation.

Timing

2-3 days

Critical Steps

Protein determination Solubilisation of sample after acetone precipitation Check the pH of all solutions

References

1. Munton RP, Tweedie-Cullen RY, Livingstone-Zatchej M, Weinandy, F, Waidelich M, Longo D, Gehrig P, Potthast F, Rutishauser D, Gerrits B, Panse C, Schlapbach R, Mansuy IM. Qualitative and Quantitative Analyses of Protein Phosphorylation in Naive and Stimulated Mouse Synaptosomal Preparations. Mol Cell Proteomics, **2007**; 6: 283-93. 2. Tweedie-Cullen RY, Livingstone-Zatchej M, Wollscheid B, Mansuy IM. Neuroproteomics and the detection of regulatory phosphosites. Cur. Proteomics, **2007**; 4: 209-22.

Figures

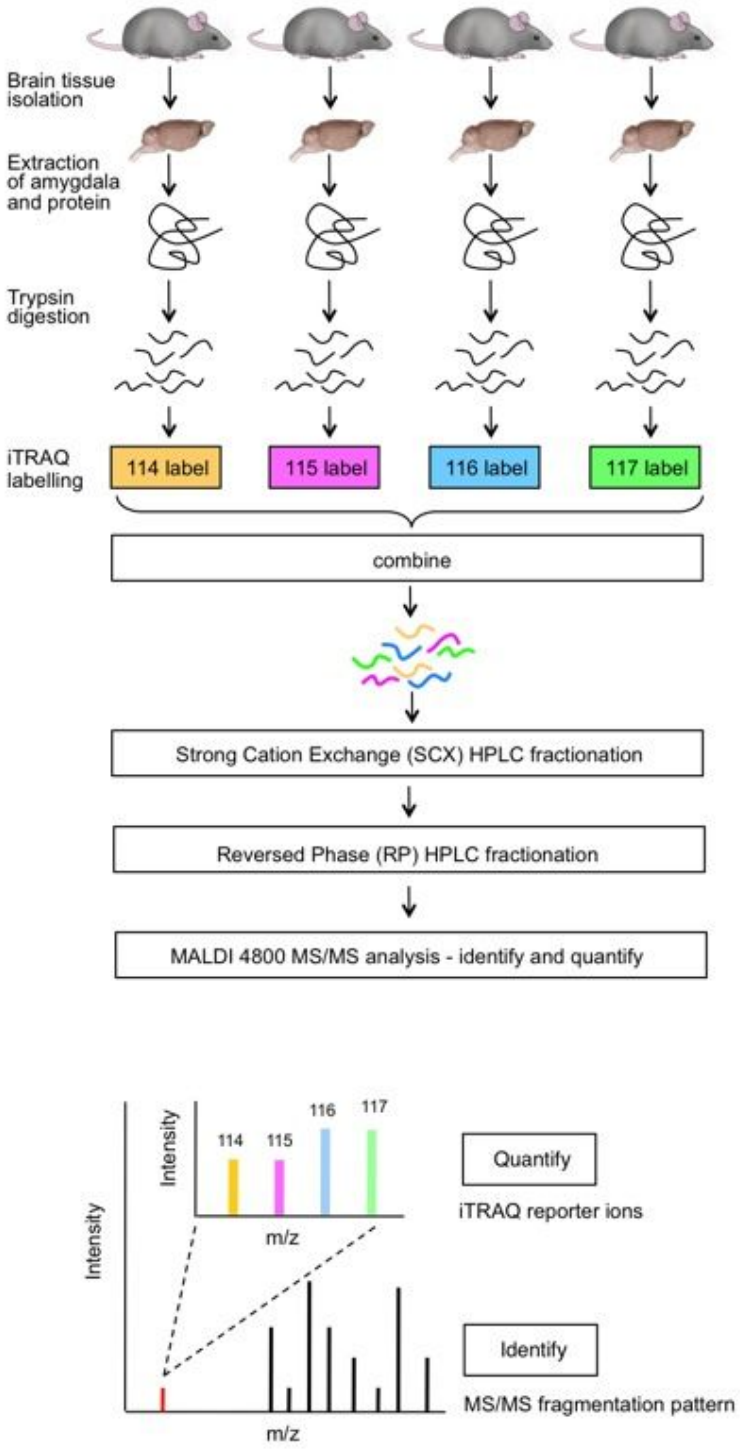

Figure 1

Workflow for the labelling and analysis of samples iTRAQ allows the relative quantification of peptides and proteins. Peptide identification and quantification takes place in the MS/MS scan. Quantification is possible by comparing the peak intensities of the iTRAQ reporter ions.

Strong cation exchange HPLC gradient

Time (min)	% Solvent B
0 - 10	0
10 - 35	0 - 30
35 - 45	30 - 60
45 - 50	60 -100
60	100
60 - 90	0

Reversed phase HPLC gradient

Time (min)	% Solvent B
0 - 10	0
10 - 105	0 - 50
105 - 115	50 - 100
115 - 124	100
124 - 125	100 - 0
125 - 150	0

Figure 2

RP- and SCX-HPLC gradients Two-dimensional liquid chromatography is used to reduce the proteomic sample complexity prior to tandem mass spectrometry analysis.