Quantitative analysis of protein expression using iTRAQ and mass spectrometry

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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.
4. Clean-up samples using sep-pak columns with binding capacity of ~100 µg.
5. Wet the cartridge with 1 ml 100 % MeOH.

6. Wet the cartridge with 1 ml 80 % ACN, 0.1% TFA.
7. Equilibrate with 2 x 1 ml 0.1% TFA.
8. Load the sample.
9. Wash with 2 x 1 ml 0.1 % TFA.
10. Repeat steps 7 and 8 once with the flowthrough.
11. Elute with 500 µl 80 % ACN, 0.1 % TFA.
12. 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.
4. Mix column effluent directly with MALDI matrix solution.
5. 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

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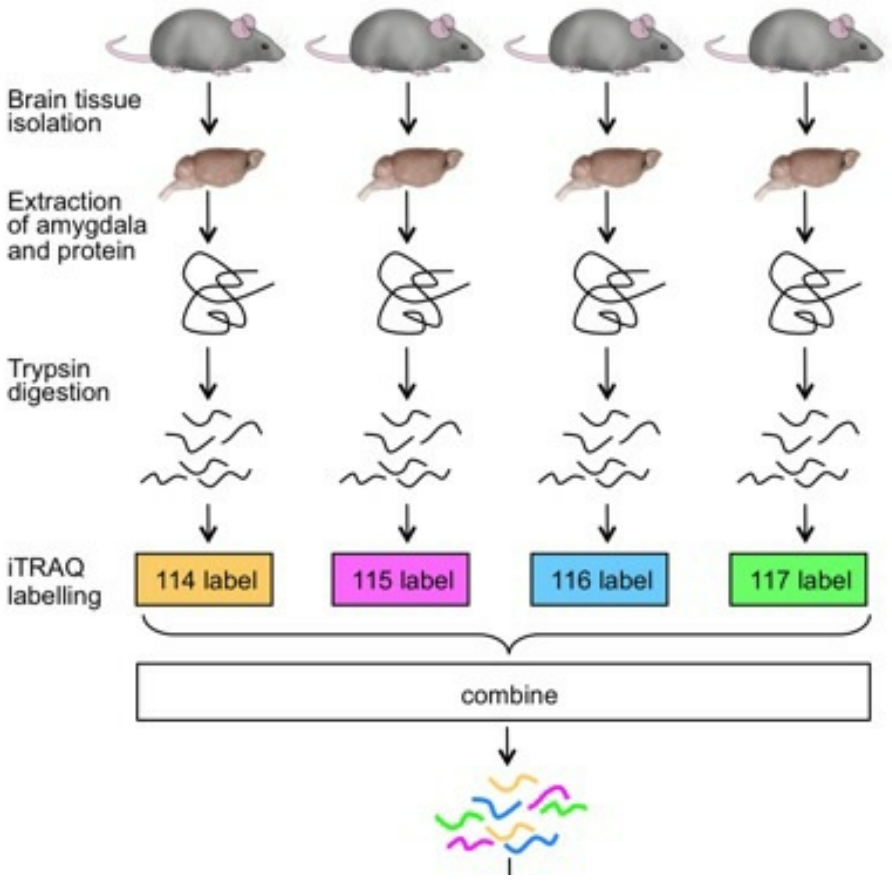
Figures



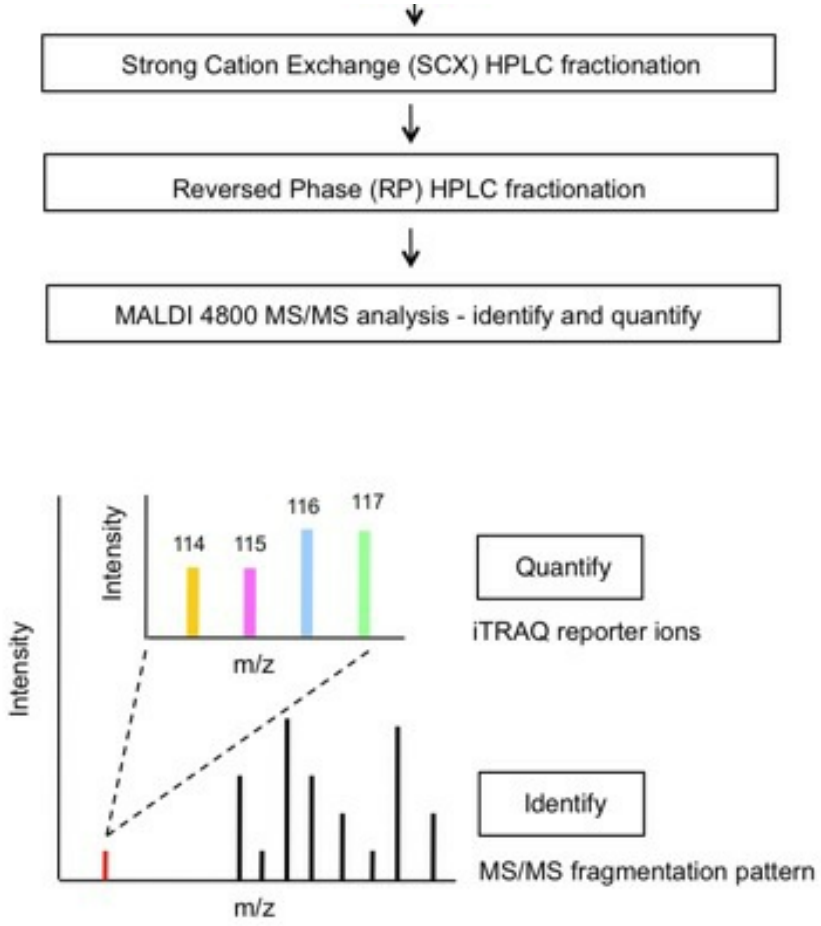


Figure 1

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.

Control of the establishment of aversive memory by calcineurin and Zif268

by Baumgärtel K, Genoux D, Welzl H, +5
Nature Neuroscience (26 March, 2008)