Absolute quantitation of *in vitro* expressed plant membrane proteins by targeted proteomics (MRM) for the determination of kinetic parameters

Carsten Rautengarten[1], Berit Ebert[1] and Joshua L. Heazlewood[1,2]*

[1]School of BioSciences, The University of Melbourne, Victoria 3010, Australia
[3]Joint BioEnergy Institute, Lawrence Berkeley National Laboratory, Berkeley, CA 94702, USA

***Corresponding Author**
Joshua L. Heazlewood
School of BioSciences
The University of Melbourne
Victoria 3010, Australia
Tel: +61 3 8344 2677
Email: jheazlewood@unimelb.edu.au

Running Head: MRM quantitation of plant membrane proteins

Summary

The purification of a functional soluble protein from biological or *in vitro* expression systems can be problematic, the enrichment of a functional membrane protein for biochemical analyses can be a serious technical challenge. Recently, we have been characterising plant endomembrane nucleotide sugar transporters using a yeast expression system. However rather than enriching these *in vitro* expressed proteins to homogeneity, we have been conducting biochemical characterization of these transport proteins in yeast microsomal fractions. While this approach has enabled us to estimate a variety of kinetic parameters, the accurate determination of turnover number of an enzyme-substrate complex (k_{cat}) requires that the catalytic site concentration (amount of protein) in the total reaction volume is known. As a result, we have been employing targeted proteomics (multiple reaction monitoring) with peptide standards and a triple quadrupole mass spectrometer to estimate the absolute amount of protein in a mixed protein microsomal fraction. The following method details the steps required to define the absolute quantitation of an *in vitro* expressed membrane protein to define complete kinetic parameters.

Key Words

membrane proteins, multiple reaction monitoring, enzyme kinetics

1. Introduction

The development of the Michaelis–Menten model over 100 years ago [1,2], in which the rate of the enzyme is related to the substrate concentration, now represents one of the most widely used equations for the examination of enzyme kinetics. While the original derivation of the equation was based on a number of assumptions, some of which were subsequently addressed [3], the equation is still incredibly useful in understanding an enzymes function as it provides a kinetic description of its activity. Significantly, both the Michaelis constant (K_m) providing a measure of substrate affinity and the maximal rate or maximum velocity of the enzyme (V_{max}) can be empirically derived by measuring catalytic rates at various substrate concentrations. However, while the determination of both K_m and V_{max} do not require any knowledge of the enzyme concentration, this information is necessary to derive the turnover number (k_{cat}) of the enzyme. In general, the turnover number (k_{cat}) and specificity constant (k_{cat}/K_m) are now regarded as the principal steady state kinetic parameters necessary to adequately describe an enzyme [4].

The determination of an enzymes kinetic parameters is commonly accomplished after the enrichment and purification of the enzyme to near homogeneity. This enrichment process was traditionally accomplished directly from complex biological material [5], but is now more commonly achieved after *in vitro* expression in a heterologous system such as *Escherichia coli* [6]. However, the purification and enrichment of membrane proteins to near homogeneity using the above approaches can be extremely challenging. While there are documented successes using such approaches with membrane proteins [7], these likely hide the numerous failures. We recently faced similar problems when attempting to biochemically characterize members of the Nucleotide Sugar Transporter family from Arabidopsis [8,9]. Although we had developed a sensitive assay [10] using microsomal fractions from *Saccharomyces cerevisiae* (yeast) expressing our transporters of interest, the nature of these membrane proteins (8 to 10 predicted transmembrane domains), incentivised us to develop an alternative approach over further enrichment and purification. The determination of both K_m and V_{max} was possible, however an estimation on the amount of protein in this mixed microsomal fraction was required to determine k_{cat}. As a result, we developed an MRM-based assay for the absolute quantitation of our expressed transporter in yeast microsomal preparations using protein specific synthetic peptides and triple quadrupole mass

spectrometry. This approach has now been successfully applied on numerous occasions [8,9] to determine the concentration of a protein and to determine complete kinetic parameters. The method details the in vitro expression of a membrane protein in yeast, the enrichment of a mixed membrane lysate contacting the membrane protein of interest and the absolute quantitation of the expressed protein by triple quadrupole mass spectrometry (MRM) and peptide standards for the estimation of k_{cat}.

The accurate characterisation of enzymes will be a vital aspect in our capacity to develop next generation agricultural crops. Future bioengineering programs will rely on large-scale systems biology approaches to develop models that predict engineered outcomes and thus the accuracy of these models will directly rely on the quality of data and the contributions of well-characterized parts [11,12].

2. Materials

Prepare solutions using ultrapure water (18 MΩ cm at 25°C) and analytical grade reagents. Utilize LC-MS grade reagents for solutions and buffers used in conjunction with mass spectrometry. Prepare all reagents at room temperature, unless otherwise indicated, reagents can be stored for several months and room temperature.

2.1. *In vitro* expression in *Saccharomyces cerevisiae*

1. *Saccharomyces cerevisiae* (yeast) strain INVSc1 (*see* **Note 1**).
2. Yeast inducible expression vector, such as pYES-DEST52 (*see* **Note 2**).
3. Yeast transformation kit, such as, S.c. EasyComp Transformation Kit (Thermo Fisher Scientific) (*see* **Note 3**).
4. Agar
5. Yeast Nitrogen Base without amino acids (*see* **Note 4**).
6. Yeast Synthetic Drop-out Medium, lacking the appropriate selectable marker, such as uracil (*see* **Note 5**).
7. 50 mL tubes.
8. 250 mL baffled flask (*see* **Note 6**).
9. Bench top centrifuge with rotor for 50 mL tubes capable of 3,000 x *g*.
10. 1000 mL baffled flask (*see* **Note 6**).
11. Preparative centrifuge for 250 mL tubes and capable of 3,000 x *g*.
12. 20 % dextrose (filter sterilized).
13. 20 % galactose (filter sterilized) (*see* **Note 7**).
14. 1 M sodium azide solution (*see* **Note 8**).

2.2. Isolation of yeast microsomes

1. Yeast Resuspension Buffer: 50 mM potassium phosphate, pH 7.1, 1.4 M sorbitol, 10 mM NaN_3 and 40 mM 2-mercaptoethanol.
2. Lyticase from *Arthrobacter luteus*.
3. Yeast Lysis Buffer: 0.8 M sorbitol, 10 mM triethanolamine / acetic acid pH 7.2, 1 mM EDTA
4. Protease inhibitors, such as, SigmaFAST™ Protease Inhibitor Cocktail Tablet (Sigma-Aldrich)
5. 100 mM phenylmethanesulfonyl fluoride (PMSF)

6. Microsome Resuspension Buffer: 10 mM N-(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine (Tricine-KOH) pH 7.5, 50 mM potassium D-gluconate, 20 % glycerol (*see* **Note 9**).
7. Bench top centrifuge with rotor for 15 mL and 50 mL tubes capable of up to 8,000 x *g*.
8. Ultracentrifuge with rotor for 30 mL tubes and capable of 100,000 × *g* for pelleting microsomes.
9. glass beads acid-washed
10. Colorimetric protein quantification assay.

2.3. Analysis of microsomes by immunoblotting

1. Mini electrophoresis chamber for protein separation.
2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) precast gels compatible with the electrophoresis chamber (*see* **Note 10**).
3. Electrophoresis Buffer: 248 mM Tris-base, 1.92 M glycine, 1 % (w/v) SDS.
4. Sample Buffer: 40 mM Tris-HCl (pH 6.8), 5 % (w/v) SDS, 0.4 % (w/v) bromophenol blue, 1 % (v/v) 2-mercaptoethanol, 1 X protease inhibitor, 5 mM PMSF, 8 M urea, 0.1 mM EDTA.
5. 2-mercaptoethanol solution.
6. SDS-PAGE protein markers.
7. Immunoblot apparatus.
8. Western Transfer Buffer: 25 mM Tris-base, 192 mM glycine, 10 % (v/v) methanol.
9. 100 % (v/v) methanol.
10. Filter paper for immunoblotting.
11. Nitrocellulose membrane.
12. TBST Buffer: 50 mM Tris-HCl (pH 7.6), 150 mM NaCl and 0.1 % (v/v) Tween-20.
13. Skim milk powder.
14. Protein tag antibody, such as, Anti-V5 antibody (Invitrogen) (*see* **Note 11**).
15. Secondary antibody, Anti-Mouse- or Anti-Rabbit- IgG Peroxidase (*see* **Note 12**).
16. Chemiluminescent substrates for horseradish peroxidase.
17. Chemiluminescent imaging system.

2.4. Determination of peptides for MRM quantitation

1. Tandem mass spectrometer (MS/MS) with liquid chromatography (LC) delivery system capable of data dependent acquisition (DDA) / independent data acquisition (IDA) (*see* **Note 13**).
2. Reverse phase column for peptide separation, such as a C_{18} HPLC column.
3. Digestion Buffer: 1 M urea and 10 mM tris(hydroxymethyl)aminomethane (Tris-HCl), pH 8.5 solution (*see* **Note 14**).
4. 1 M dithiothreitol (DTT) (*see* **Note 15**).
5. 1 M iodoacetamide (IAA). (*see* **Note 16**).
6. Sequencing grade trypsin.
7. Solid phase extraction (SPE) for peptides, such as Micro SpinColumns with C_{18} (Harvard Apparatus, MA, USA).
8. SPE Buffer 1: 80% acetonitrile (v/v) with 0.1% trifluoroacetic acid (v/v).
9. SPE Buffer 2: 2% acetonitrile (v/v) with 0.1% trifluoroacetic acid (v/v).
10. MS Buffer A: 2 % acetonitrile, 0.1 % formic acid.
11. SpeedVac concentrator.
12. Access to search engine to identify proteins from data generated by tandem mass spectrometry (*see* **Note 17**).

2.5. Quantitation of induced membrane protein by MRM

1. Triple quadrupole mass spectrometry (MS) system coupled to a liquid chromatography (LC) delivery system capable of MRM mode (*see* **Note 13**).
2. Synthetic peptide standards > 95 % purity (*see* **Note 18**).
3. Infusion Buffer: 50 % acetonitrile and 0.1 % formic acid
4. Syringe pump
5. Gastight Hamilton syringe 500 µL
6. MS Buffer A: 2 % acetonitrile, 0.1 % formic acid.
7. Reverse phase column for peptide separation, such as a C_{18} HPLC column.
8. Software for MRM method development, profiling and quantitation, such as Skyline (https://skyline.gs.washington.edu/labkey/project/home/software/Skyline/begin.view) (*see* **Note 19**).

3. Methods

3.1. Yeast transformation and protein induction

1. Transform the expression vector containing the sequence encoding your gene of interest (*see* **Note 20**) into *Saccharomyces cerevisiae* (yeast) using a commercial yeast transformation kit (*see* **Note 21**).
2. Make dropout media using Yeast Synthetic Drop-out Medium and Yeast Nitrogen Base according to the product description. For plates add agar (20 g / L). After autoclaving, add dextrose to a final concentration of 2 %.
3. Plate transformed yeast onto agar plates and incubate for 3 to 4 days at 30 °C.
4. Pick a colony and inoculate a 50 mL starter culture (dropout media, *see* **Note 5**) in a 250 mL flask.
5. Incubate with shaking for approximately 24 hours at 30 °C.
6. Transfer culture to a 50 mL tube and centrifuge at 2,500 x *g* for 5 min at room temperature.
7. Take yeast pellet and inoculate 200 mL of dropout media, replacing the 2 % (w/v) dextrose with 2 % (w/v) galactose, in a 1000 mL flask (*see* **Note 22**).
8. Incubate with shaking overnight at 30 °C (*see* **Note 23**).
9. Harvest yeast cells from the induced culture by centrifugation at 2,500 x g for 5 min at room temperature.
10. Wash the pellet with 30 mL of 10 mM sodium azide (*see* **Note 8**) and repeat centrifugation.
11. Decant supernatant and freeze pellet at -80 °C.

3.2. Enrichment of microsomes from yeast cultures

1. Weigh the yeast pellet to estimate the amount of cells (fresh weight), should be around 2.5 g.
2. Resuspend the yeast pellet in 10 mL of Yeast Resuspension Buffer.
3. Add the appropriate amount of Lyticase to the yeast solution (*see* **Note 24**).
4. Incubate at 37 °C for 1 h to generate spheroplasts (*see* **Note 25**).
5. Harvest spheroplasts by centrifugation at 2,500 x *g* for 5 min.
6. Wash the spheroplasts pellet with Yeast Lysis Buffer.
7. Resuspend the washed spheroplasts in 5 mL Yeast Lysis Buffer containing protease inhibitor cocktail and 1 mM PMSF.

8. Add 5 ml acid-washed glass beads and disrupt spheroplasts by vortexing for 1 min, place on ice for 1 min, repeat twice.
9. Centrifuge spheroplast lysate at 3,000 x *g* for 5 min, keep supernatant on ice.
10. Resuspend the un-ruptured spheroplast pellet in 5 mL Yeast Lysis Buffer containing protease inhibitor cocktail and 1 mM PMSF and repeat the vortexing step.
11. Collect supernatant by centrifuging at 3,000 x *g* for 5 min; repeat this step two more times to produce a 20 mL lysate.
12. A microsomal fraction is obtained by sequential centrifugation.
13. Centrifuge the 20 mL lysate at 8,000 x *g* for 10 min and take supernatant.
14. Centrifuge the supernatant at 100,000 x *g* for 75 min and discard supernatant.
15. Resuspend the microsomal pellet in Microsome Resuspension Buffer (*see* **Note 9**).
16. Estimate the protein concentration in the microsomal fraction using a protein quantification assay and then store fractions at -80°C.

3.3. Assessment of protein induction by immunoblotting

1. To ensure the protein has been induced and is present in the microsomal fraction, immunoblotting can be undertaken using a protein specific antibody or with an antibody against a tag, such as the V5-tag or His-tag (Fig. 1A).
2. If using a mini-gel system, around 2 µg (~0.5 µL) of protein is suitable. Add Sample Buffer (~5 µL) then incubate at 65°C for 15 min.
3. Load samples and protein standards onto a preassembled precast gel (*see* **Note 26**).
4. Separate the protein samples on the gel at 200 V for 45 to 60 min or until the dye front reaches the bottom of the gel.
5. Remove the gel from the plates and place into Western Transfer Buffer.
6. Cut nitrocellulose membrane to the size of the gel and briefly rinse in water, then place in Western Transfer Buffer (*see* **Note 27**).
7. Cut two pieces of filter paper to the size of the gel and soak in Western Transfer Buffer.
8. Assemble transfer stack according to the manufactures instructions, normally in the following order from the cathode, blotting pad (2), filter paper, gel, nitrocellulose membrane, filter paper and blotting pad (2).
9. Place transfer stack into the Western apparatus, assemble, add Western Transfer Buffer and connect to electrophoretic power source.

10. Transfer at the recommended current for 1 hour (*see* **Note 28**).
11. Disassemble transfer stack, remove membrane and place in TBST Buffer.
12. Incubate membrane with gentle shaking in TBST Buffer with 5 % skim milk powder for 1 hour at room temperature.
13. Dilute primary antibody in TBST Buffer with 1 % skim milk powder (*see* **Note 29**).
14. Incubate membrane with primary antibody diluted in TBST Buffer with 1 % skim milk powder with gentle shaking at room temperature for 1 hour (*see* **Note 30**).
15. Wash membrane with TBST Buffer for 10 min with shaking. Repeat wash in TBST Buffer two more times.
16. Dilute HRP conjugated secondary antibody in TBST Buffer with 1 % skim milk powder (usually around 10,000 fold dilution) and incubate with washed membrane with gentle shaking at room temperature for 1 hour (*see* **Note 31**).
17. Wash membrane with TBST Buffer for 10 min with shaking. Repeat wash in TBST Buffer two more times.
18. Prepare the HRP substrate (about 1 mL) by mixing the two reagents (*see* **Note 32**).
19. Drain excess liquid from the membrane and add HRP substrate and incubate for 1 minute.
20. Image using a chemiluminescence imager.

3.4. Determining optimal peptides for quantification by MRM

1. Resuspend 50 µg of microsomal protein in about 100 µL Digestion Buffer.
2. Add DTT to the diluted protein extract to a final concentration of 25 mM and incubate 30 min (room temperature).
3. Add IAA to a final concentration of 50 mM and incubate 30 min (room temperature) in the dark.
4. Add trypsin at a 1:10 trypsin:protein ratio (*see* **Note 33**) and incubate overnight (37°C) (*see* **Note 34**).
5. Remove urea and concentrate samples with a Micro SpinColumn (25 to 75 µL capacity). Initially hydrate the C_{18} matrix with ultrapure water (75 µL) for 10 min and centrifuge (1000 x *g*, 2 min) as per manufacturer's instructions.
6. Wash the SpinColumn with the 50 µL SPE Buffer 1 and centrifuge (1000 x g, 2 min) and prime twice with 50 µL SPE Buffer 2, centrifuging (1000 x g, 2 min) after each step.

7. Add digested peptides in urea solution to the SpinColumn and centrifuge (1000 x g, 2 min), wash twice with 50 µL SPE Buffer 2, centrifuging (1000 x g, 2 min) after each step.
8. Finally elute into a new tube with 25 to 50 µL SPE Buffer 1 by centrifuging 1000 x g for 2 min. Concentrate and remove acetonitrile with a SpeedVac concentrator until 1 to 5 µL of peptide solution remains in the tube.
9. Analyse about 1 µg (approximately 0.1 µL) of the trypsin digested microsomal preparation by nanoflow liquid chromatography tandem mass spectrometry (LC-MS/MS) using an automated data dependent acquisition method optimized for the analysis of complex protein samples (*see* **Note 35**) (Fig. 1B).
10. Data produced after LC-MS/MS analysis can be interrogated with search algorithms, such as the software package Mascot (Matrix Science), to identify peptides from the induced membrane protein within the microsomal fraction suitable for MRM (*see* **Note 36**).
11. Select unique peptides specific for the expressed protein based on precursor (optimally 500 to 700 m/z), charge state (optimally $[M+ 2H]^{+2}$) and peptide match score, as it provides an indication of a MS compatibility factor.
12. Obtain synthesize selected peptides (2 to 3) from a commercial supplier specific for the induced membrane protein to use as standards for MRM.

3.5. Estimating abundance of induced membrane protein by MRM

3.5.1 Develop the MRM method and assess standard peptides

1. Dilute synthesized peptides standards to around 1 pmol / µL with Infusion Buffer. Then using the syringe pump and the Hamilton syringe, infuse the mixture into the triple quadrupole mass spectrometer at about 50 µL / min (or a similar flow rate to be used for MRM analysis) and monitor peptide using a Q1 scan mode (*see* **Note 37**).
2. Optimize conditions for the peptides, for example deculstering potential, gas flow rate, gas temperature (*see* **Note 38**) to maximize their signal-to-noise ratio.
3. Optimize the fragmentation conditions for each peptide in turn using Product Ion mode (MS2) or equivalent. Take note of the value used for the collision energy (CE) that resulted in the absence of the precursor ion (peptide) and also produced product

ions with optimal signal-to-noise ratio. Take note of 2 or 3 product ions corresponding to valid *y*- or *b*-series ions for each peptide.

4. Create MRM method using the experimentally optimized values for each transition including Q1 mass (precursor ion), Q3 masses for each precursor ion (product ions), Collision Energy (CE) and a time (msec) for each transition (*see* **Note 39**) (Fig. 2).
5. Create a simple LC elution method (30 to 50 min) with a 10 to 20 min acetonitrile gradient (2 % acetonitrile to 80 % acetonitrile).
6. Dilute synthesized peptides in MS Buffer A, load about 5 pmol and analyse using the MRM and LC methods outlined above.
7. View and assess the subsequent results using the MS software provided with the mass spectrometer, making note of the elution window for each peptide, elution times, and signal intensities for different transitions (product ions) from the same peptide. At this stage, transitions (product ions) resulting in poor signals could be eliminated from the method (retain at least two), peptides could be eliminated if they are not retained on the column, elute as a split peak or elution occurs over a wide window (*see* **Note 40**).
8. Add the peptide sequences to the Skyline software package, then select the precursor mass using "pick children" with the corresponding charge state as observed in the mass spectrometer (e.g. ++ or +++ or ++++). Select the product ions using the "pick children" option for the appropriate charge state empirically determined and analysed above (e.g. *y*6, *b*3). Save the target list as a Skyline file (Fig. 3A).
9. Import the raw MS data file from the 5 pmol standard peptide analysis into Skyline and check to ensure that all peptides and transitions have imported correctly by selecting each peptide and transition and comparing them to the results observed on the MS software provided with the mass spectrometer (Fig. 3B).

3.5.2 Absolute quantification of the induced protein in microsomal membrane fraction

1. Utilize approximately 10 µg (1 µL) of the trypsin digested microsomal preparation (see Section 3.4) in MS Buffer A and analyse using the method which was developed and validated with the standard peptides (see section 3.5.1).
2. View and assess the results using the MS software provided with the mass spectrometer. Check the elution times and the signal intensities for different transitions and compare to the results from the 5 pmol peptide standard analysis.

3. If the peptides are clearly detectable in the microsomal preparation, a rough estimation of induced protein content can be made by comparing the intensity from the 5 pmol standard sample to the microsomal sample.
4. Based on comparisons between the signals from the 5 pmol standard peptide and the 10 µg microsomal preparation, estimate a range of concentrations required to generate a standard curve from the peptide standards. Based on numerous examples, we have found the following standard curve seems to cover most variations in expression when conducting MRM analysis at flow rates around 400 µL / min: 0.1 pmol, 0.25 pmol, 0.5 fmol, 1 pmol, 5 pmol, 20 pmol.
5. Create a sample sequence with the MRM method that analyses the range of concentrations for the standard curve (step 4 above), with the digested 10 µg microsomal preparation interspersed in the sequence (Fig. 1C). The objective is to generate a minimum of three replicates for each concentration of the standard peptide solution and a similar number for the microsomal preparation (10 µg each).
6. Open the previously saved peptide target list in Skyline and import the raw MS data files for the replicates of the microsomal preparation and the standard curve (Fig. 3C).
7. Inspect each peptide from these samples to ensure that the correct peaks have been assigned. Compare the retention time to that of the synthetic standard peptides. The correct peak can be manually selected in Skyline using the mouse. The retention times for all samples and all peptides can be easily checked using View > Retention Times > Replicate Comparison option. Also check that each fragment transition is present and that each one has a similar proportion as the fragment transitions found for the synthetic standard peptides.
8. Export the results (File > Export > Report) as a .csv (a report) file with data corresponding to precursor 'Total Area' (*see* **Note 41**).
9. Open exported data in Microsoft Excel (or similar) and generate a standard curve using data from the synthetic standard peptides e.g. amount loaded (pmol) against the precursor 'Total Area' values for each peptide.
10. Using the standard curve and the 'Total Area' values, estimate the average amount (pmol) of peptide in the microsomal fractions. This value corresponds to the number of moles of the expressed membrane protein. Estimate the amount (g) of protein in the total analysed microsomal fraction (10 µg) using the calculated molecular weight of

the expressed fusion protein. Then estimate the percentage of expressed protein in the microsomal fraction (*see* **Note 42**).

11. Estimate the amount of expressed protein used in the assay and in combination with the previously determined value for V_{max} to calculate k_{cat}.

4. Notes

1. The yeast strain, INVSc1, (Thermo Fisher Scientific) is a fast-growing strain and ideal for protein expression. INVSc1 genotype: MATa his3D1 leu2 trp1-289 ura3-52 MAT his3D1 leu2 trp1-289 ura3-52
2. Any suitable yeast expression vector should suffice. The pYES-DEST52 expression vector is galactose inducible with a uracil (URA3) selectable marker for yeast growth and with a C-terminal V5 epitope and 6xHis tags. The vector is also a Gateway destination vector with antibiotic resistance (ampicillin) for initial cloning in *Escherichia coli*. We have used this vector successfully to express over 50 plant, fungi and mammalian membrane proteins in yeast.
3. Transformation of yeast can be readily undertaken without a commercial kit, however these systems are a cost effective and efficient means to accomplish a successful transformation, especially if being undertaken for the first time.
4. Contains nitrogen source, vitamins, trace elements and salts for optimal yeast growth and is used in conjunction with the yeast drop-out media. The ratio required for yeast media will depend on the supplier.
5. Contains amino acids and other nutrients (e.g. adenine, inositol and etc.) absent from the Yeast Nitrogen Base. The pYES-DEST52 vector contains the auxotrophic selection marker URA3 (orotidine 5'-phosphate decarboxylase) which converts orotidine monophosphate (OMP) to uridine monophosphate (UMP). Depending on the yeast expression vector, the appropriate drop-out media is required.
6. Baffled flasks create a turbulent flow and increase gas exchange at the surface of the liquid to increase oxygen intake.
7. The pYES-DEST52 vector contains the promoter sequence of the GAL1 gene upstream of the *att*R region for induction of the gene of interest. The appropriate induction method is dependent on the yeast expression vector utilized.
8. The addition of sodium azide (often referred to as STOP buffer) will inhibit further activity and arrest metabolism. Take adequate precautions as sodium azide is a potent inhibitor of mitochondrial respiration.
9. The type of buffer and volume used will greatly depend on the downstream enzyme assay that will be conducted.

10. A 12 % polyacrylamide gel is a good compromise for the generic separation of proteins. If purchasing precast polyacrylamide gels, consider a gradient gel employing 8 to 16 % acrylamide.

11. The selection of the primary antibody will be determined by the protein tag (if any) in the expression vector.

12. The type of secondary antibody will depend on the origin of the primary antibody, but is commonly either Anti-Mouse (usually monoclonal antibodies) or Anti-Rabbit (usually polyclonal antibodies).

13. In recent times, access to tandem mass spectrometers has become relatively cost-effective with a variety of service-based facilities at many institutions.

14. Urea can be unstable and degrade in solution when exposed to elevated temperatures (> 25°C) or over time. Degradation products such as isocyanic acid can react with the amino terminus of proteins and sidechains of lysine and arginine residues. Consequently, it is recommended that urea solutions be made fresh as required.

15. A stock solution of DTT can be stored in aliquots at -20°C.

16. A stock solution of IAA can be stored in aliquots at -20°C. IAA is used to alkylate thiol group on cysteine residues after reduction with DTT. It is virtually impossible to detect cysteine containing peptides unless controlled alkylation is undertaken. IAA is light and heat sensitive and should be stored in the dark.

17. Tandem mass spectrometers designed for proteomic workflows will usually ship with software for the interrogation of tandem spectra for the identification of proteins and it is likely that any MS service facility will have a variety of packages. However there are a number of open source software packages available as well as a range of third party products that can be accessed online. The algorithms are generally similar in their approach, although we use Mascot (a third party software package from Matrix Science) as it provides quality results when high confidence cut-offs are employed. Access to the software is free if submitting less than 1200 spectra in a single submission (http://www.matrixscience.com/).

18. The selection of the synthetic peptides to use as standards for MRM-based quantitation will depend on a number of factors after inspecting the results from the LC-MS/MS analysis of the microsomal fraction. If a yeast expression vector with a protein tag has been employed, e.g. the V5 epitope, it is often cost-effective to select a tryptic peptide from this region that can be used in future experiments with different

proteins. If no tag was employed or if no suitable tag derived peptides were identified after shotgun analysis of the microsomal fraction, select protein specific tryptic peptides. The objective is to select MS-compatible tryptic peptides with no miscleavage and no modifications (avoid methionine and cysteine containing peptides). Optimally, 2 to 3 doubly charged $[M+2H]^{2+}$ peptides in the range of 500 to 700 m/z appear to produce optimal results with regard to signal and fragmentation . If no peptides were identified after shotgun analysis of the microsomal fraction, first ensure adequate expression and if necessary optimize protein expression. If there are still no candidates, there are a number of utilities that can predict peptides suitable for MRM from the amino acid sequence, such as, the Arabidopsis Proteotypic Predictor [13].

19. The Skyline software package is a freely-available, open-source application for developing MRM methods and analysing the resultant data. The software can be used to select candidate peptides and fragment ions for an MRM experiment from a protein sequence. It can also be used to optimise the declustering potential and collision energy for a set of peptides. The Skyline website has an extensive collection of tutorials and help pages.

20. In parallel, ensure that an empty vector control is also transformed and resultant microsomes are employed throughout the entire assay.

21. While there are numerous protocols for yeast transformation, we have found that using a commercial kit is cost and time effective.

22. The pYES-DEST52 vector harbours a galactose inducible promoter for induction of the transgene. This step will be dependent on the expression vector being employed.

23. If there are difficulties obtaining a stably expressed protein, check expression by immunoblotting at earlier time points, such as after 6 hours.

24. Depending on the source and batch of Lyticase, the appropriate proportion to add to the solution, based on mg fresh weight of yeast, will need to be calculated.

25. The use of 37 °C is optimal for digestion of yeast cell walls, however the expressed protein may be heat sensitive. Consequently, this step may require assessment depending on protein expression levels. A lower temperature for a longer incubation period can be used.

26. Using a precast gel system is the simplest and most convenient way to analyse multiple fractions. Assemble the apparatus with the precast gel and compatible buffers following the manufacturer's instructions.

27. Ensure that the methanol has been added to commercially sourced transfer buffers.

28. A range of conditions can be used including overnight transfers, however voltage adjustments are required. Check the instructions of the specific transfer apparatus for advice on specific conditions.

29. The dilution of a primary antibody will vary depending on its specificity. A range will be provided by the supplier; however some testing may be required to obtain an optimal signal. A dilution range between 500 and 5000 is common for polyclonal antibodies.

30. To minimize the amount of primary antibody employed, only make enough of this solution so as to cover the membrane and employ a small container.

31. A variety of conjugations are available for the detection of secondary antibodies, however HRP (horse radish peroxidase) is one of the most common as it is a robust enzyme that enables detection by a variety of techniques.

32. When mixed, the HRP substrate is light sensitive. Therefore keep away from light (wrapped) and use immediately.

33. The recommended ratio of trypsin to protein (w/w) is generally 1:20 or 1:50. However this will depend on the type of trypsin being employed. If using modified trypsin (not subject to autolysis), then the recommended ratios can be used, however if unmodified trypsin is used, we recommend using it at higher ratios. The advantage of unmodified trypsin, is that autolytic products (peptides) can act as internal controls for sample handling and mass spectrometry.

34. An overnight digestion is likely excessive, however the timing usually suits the standard sample processing workflow.

35. The analysis of 1 µg of a complex peptide lysate by nano-LC-MS/MS should result in the high-confidence identification of between 1000 and 2000 proteins for species with well curated genomes. In this instance, up to 1000 yeast proteins are likely to be detected using this approach.

36. To ensure that only high-confidence proteins and peptides are identified from the fragmentation spectra, employ recommended protein and peptide cut-offs to ensure spectral match probabilities are < 0.05 or false discovery rates are < 1 %. If using

Mascot, an ions score value is provided for peptide matches and needs to be entered manually in the "Ions score or expect cut-off" box. Using such an approach, only high-confidence peptides matches are used to define the resultant identified proteins. This means that even proteins identified by a single peptide can be regarded as high-confidence matches, although a replicate experiment will be required to independently identify and confirm its existence in the sample.

37. As indicated above, the Skyline software package can be used to create the MRM method and to optimize collision energy and fragment ions (File > Export > Method) after entering peptide information into the software.

38. Check that you have selected the most abundant charge state for the peptide, often peptides > 1,600 Da will be found in two charge states $[M+2H]^{+2}$ and/or $[M+3H]^{+3}$.

39. Newer instruments can employ quite short dwell times to monitor hundreds of MRM transitions, however given that only a handful of peptides will be analysed, dwell times of 50 to 100 msec can be readily employed.

40. The peptide elution window will depend on a number of factors. Using a traditional setup employing nanoflow or capflow LC delivery rates, peaks widths of 1 to 2 minute would be expected. However, it is now more common to conduct MRM-type analyses using Ultra Performance Liquid Chromatography (UPLC) and depending on the flowrate employed, peptide elution windows can be less than 10 seconds.

41. Skyline has a collection of report templates, however none provide 'Total Area' i.e. the area of all transitions of a given peptide. Simply edit a report template e.g. Peptide RT Results, and add 'Total Area' to the report. The 'Total Area' option is found under Proteins > Precursors > Precursor Results.

42. For proteins exhibiting good expression in yeast, after MRM quantitation, we have calculated that the expressed protein comprises around 1 % of total microsomal protein.

Acknowledgments

This work was supported by the U. S. Department of Energy, Office of Science, Office of Biological and Environmental Research, through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the U. S. Department of Energy. JLH is supported by an ARC Future Fellowship [FT130101165].

5. References

1. Michaelis L, Menten ML (1913) The kenetics of the inversion effect. Biochemische Zeitschrift 49:333-369. doi:
2. Henri V (1903) Lois générales de l'action des diastases. Hermann, Paris
3. Briggs GE, Haldane JBS (1925) A note on the kinetics of enzyme action. Biochemical Journal 19:338-339. doi:
4. Johnson KA, Goody RS (2011) The original Michaelis constant: Translation of the 1913 Michaelis-Menten paper. Biochemistry 50:8264-8269. doi: 10.1021/bi201284u
5. Matern U, Grisebach H (1977) UDP-Apiose-UDP-Xylose Synthase - Subunit composition and binding studies. European Journal of Biochemistry 74:303-312. doi: DOI 10.1111/j.1432-1033.1977.tb11394.x
6. Rautengarten C, Ebert B, Ouellet M et al. (2012) Arabidopsis deficient in cutin ferulate encodes a transferase required for feruloylation of omega-hydroxy fatty acids in cutin polyester. Plant Physiology 158:654-665. doi: 10.1104/pp.111.187187
7. Lim L, Linka M, Mullin KA et al. (2010) The carbon and energy sources of the non-photosynthetic plastid in the malaria parasite. FEBS Letters 584:549-554. doi: 10.1016/j.febslet.2009.11.097
8. Ebert B, Rautengarten C, Guo X et al. (2015) Identification and characterization of a Golgi localized UDP-xylose transporter family from Arabidopsis. The Plant Cell 27:1218-1227. doi: 10.1105/tpc.114.133827
9. Rautengarten C, Ebert B, Moreno I et al. (2014) The Golgi localized bifunctional UDP-rhamnose/UDP-galactose transporter family of Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America 111:11563-11568. doi: 10.1073/pnas.1406073111
10. Ito J, Herter T, Baidoo EE et al. (2014) Analysis of plant nucleotide sugars by hydrophilic interaction liquid chromatography and tandem mass spectrometry. Analytical Biochemistry 448:14-22. doi: 10.1016/j.ab.2013.11.026
11. Aderem A (2005) Systems biology: Its practice and challenges. Cell 121:511-513. doi: 10.1016/j.cell.2005.04.020
12. Arkin AP (2013) A wise consistency: engineering biology for conformity, reliability, predictability. Current Opinion in Chemical Biology 17:893-901. doi: 10.1016/j.cbpa.2013.09.012

13. Taylor NL, Fenske R, Castleden I et al. (2014) Selected reaction monitoring to determine protein abundance in Arabidopsis using the Arabidopsis proteotypic predictor. Plant Physiol 164:525-536. doi: 10.1104/pp.113.225524

Figure Captions

Fig. 1. Workflow outlining the basic procedure for quantification of expressed membrane proteins in yeast.

(A) Immunoblot analysing the expression of an induced membrane protein in yeast microsomal fractions using the Anti-V5-tag antibody (see Section 3.3)

(B) Shotgun LC-MS/MS analysis of yeast microsomal preparations containing the induced membrane protein to identify unique peptides for MRM (see Section 3.4).

(C) Analysis of yeast microsomal preparations containing the induced membrane protein using MRM with a triple quadrupole mass spectrometer (see Section 2.5.2).

Fig. 2. Basic parameters to be included in the MS method necessary to conduct MRM analyses.

The 'Q1 Mass' corresponds to the observed *m/z* of the peptide. The 'Q3 Mass' represents the *m/z* of dominant product ions. The 'Time' represents the time spent collecting data on each transition. The 'CE' indicates the collision energy. Both the dominant product ions and the collision energy can be obtained empirically by initially infusing the synthesized peptide.

Fig. 3. Overview of the main features of Skyline.

(A) An example peptide entry in Skyline with the charge state for the peptide selected (++) and the three dominant product ions [y10], [y9] and [y5] shown. The green circles indicate that all three transitions were detected in the sample.

(B) MRM raw data for a synthesized peptide (FYLPDIFPGLNK) imported into Skyline. Note the differences in transition intensity, with [y9] and [y5] producing more intense signals under these condition compared to the [y10] fragment ion.

(C) MRM data from a complex lysate with a protein containing the tryptic peptide (FYLPDIFPGLNK). Data from complex biological samples result in background and reduced signal. Note that the retention time matches the one of the standard peptide and the profile of transitions also corresponds, albeit the signal for the [y10] fragment ion is low.

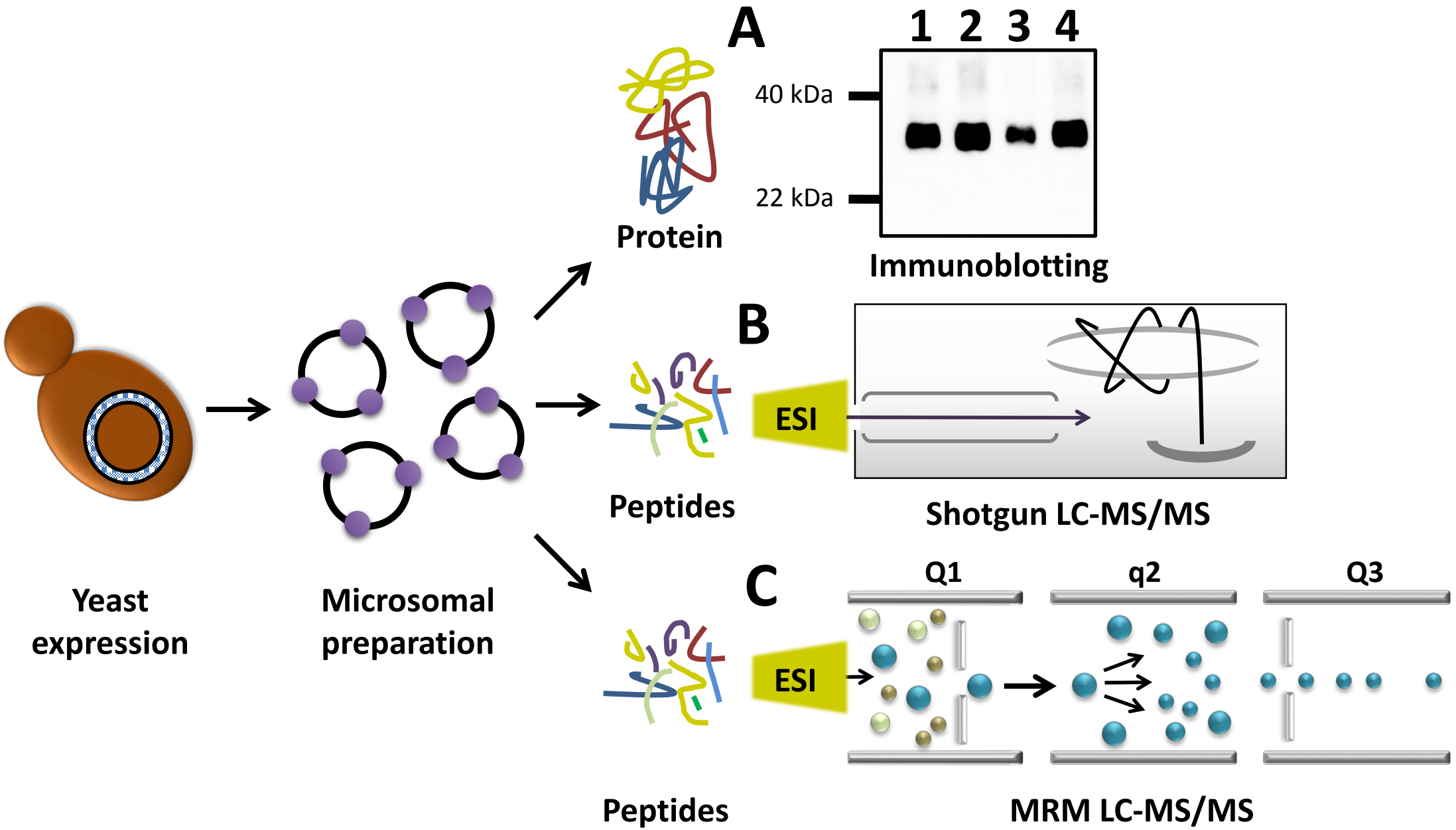
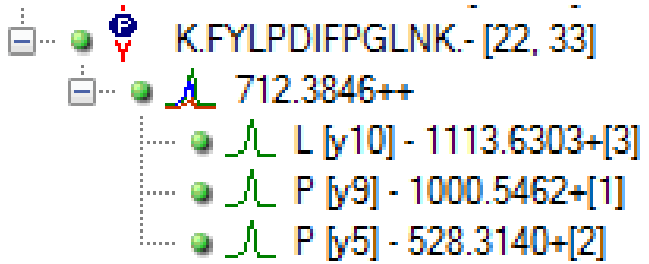

A
1 2 3 4
40 kDa
22 kDa
Protein
Immunoblotting
B
ESI
Peptides
Shotgun LC-MS/MS
C
Q1
q2
Q3
ESI
Peptides
MRM LC-MS/MS
Yeast expression
Microsomal preparation

Figure 1

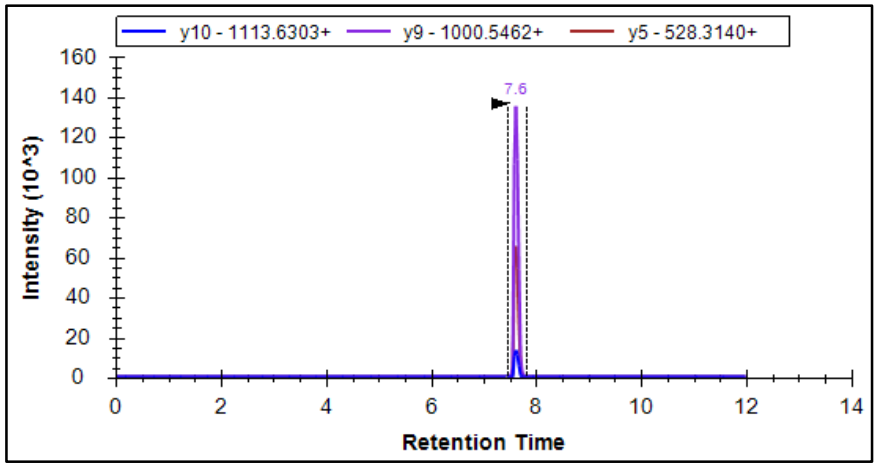
	Q1 Mass (Da)	Q3 Mass (Da)	Time (msec)	CE (volts)
1	712.400	1113.600	100.0	42
2	712.400	1000.500	100.0	42
3	712.400	528.300	100.0	42

Figure 2

A


K.FYLPDIFPGLNK.- [22, 33]
712.3846++
L [y10] - 1113.6303+[3]
P [y9] - 1000.5462+[1]
P [y5] - 528.3140+[2]

B


y10 - 1113.6303+
y9 - 1000.5462+
y5 - 528.3140+
Intensity (10^3)
Retention Time
7.6

C

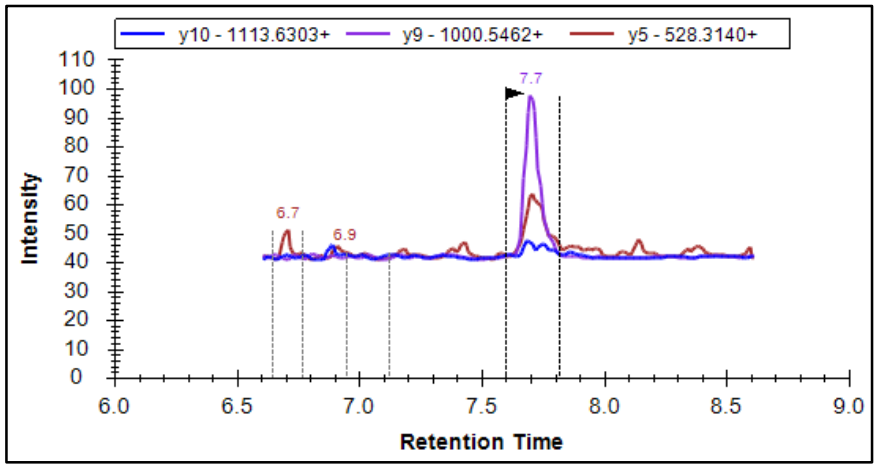

y10 - 1113.6303+
y9 - 1000.5462+
y5 - 528.3140+
Intensity
Retention Time
7.7
6.7
6.9

Figure 3

Author/s:
Rautengarten, C; Ebert, B; Heazlewood, JL

Title:
Absolute Quantitation of In Vitro Expressed Plant Membrane Proteins by Targeted Proteomics (MRM) for the Determination of Kinetic Parameters.

Date:
2018

Citation:
Rautengarten, C., Ebert, B. & Heazlewood, J. L. (2018). Absolute Quantitation of In Vitro Expressed Plant Membrane Proteins by Targeted Proteomics (MRM) for the Determination of Kinetic Parameters.. Mock, H-P (Ed.). Matros, A (Ed.). Witzel, K (Ed.). Plant Membrane Proteomics: Methods and Protocols, (1), 1696, pp.217-234. Humana Press.

Persistent Link:
http://hdl.handle.net/11343/235807

File Description:
Accepted version