Proteome-wide quantitative phosphoproteomic analysis of *Trypanosoma brucei* insect and mammalian lifecycle stages

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Running title:

T. brucei quantitative phosphoproteomics

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Abstract

Mass spectrometry based proteomics allows the identification and quantification of protein and phosphorylation site abundance on a proteome wide scale. Here we describe the metabolic labelling of cultured *Trypanosoma brucei* cells in either the bloodstream or procyclic lifecycle stage using stable isotope labelling of amino acids in cell culture (SILAC), and the production of samples suitable for analysis by liquid chromatography tandem mass spectrometry. The protocols require little specialist equipment, and they typically enable quantification of over 4,500 proteins and 9,000 phosphorylation sites.

Key words *Trypanosoma brucei,* SILAC, High pH reverse phase, proteomics, phosphoproteomics

1 Introduction

The identification and quantification of phosphorylation site dynamics is a crucial component in the elucidation of signaling pathways, and may also improve drug discovery efforts through improved candidate selection and detailed mode-of-action studies [1]. Recent advances in the field of mass spectrometry based phosphoproteomics have resulted in the routine identification and quantification of thousands of *in vitro* phosphorylation sites, generating evidence of dynamic phosphorylation in an unbiased, system-wide approach [2]. The increasing availability of commercial analytical services has allowed the technique to be applied outside of specialist mass spectrometry laboratories.

Global phosphoproteomic experiments involve digesting proteins into peptides (typically with trypsin), enrichment of phosphopeptides, and fractionation before analysis by liquid chromatography- mass spectrometry (Figure 1). The peptides and phosphopeptides are identified

and quantified from the mass spectra and mapped to the intact protein by automated database searching [3]. It is essential to enrich for phosphopeptides prior to analysis due to the low stoichiometry of phosphorylation and the poor ionization of phosphopeptides in the mass spectrometer. Measurement of both peptide and phosphopeptide abundance is important to distinguish whether changes occur at the level of protein or through a change in phosphorylation site occupancy. Fractionation is required to reduce the sample complexity and ensure that low abundance peptides and phosphopeptides can be detected, and results in improved depth of coverage. Although quantitation can be achieved in several ways [4], metabolic labelling achieved through stable isotope labelling of amino acids in cell culture (SILAC) provides high accuracy and reproducibility. SILAC has been applied to both the mammalian and insect form of *Trypanosoma brucei* [5-7], and to the insect forms of *Trypanosoma cruzi* [8] and *Leishmania* species [9]. If direct labelling of samples is unfeasible then spike-in SILAC may be used, where a heavy isotope labelled standard may be used to compare *ex vivo* or clinical samples [10].

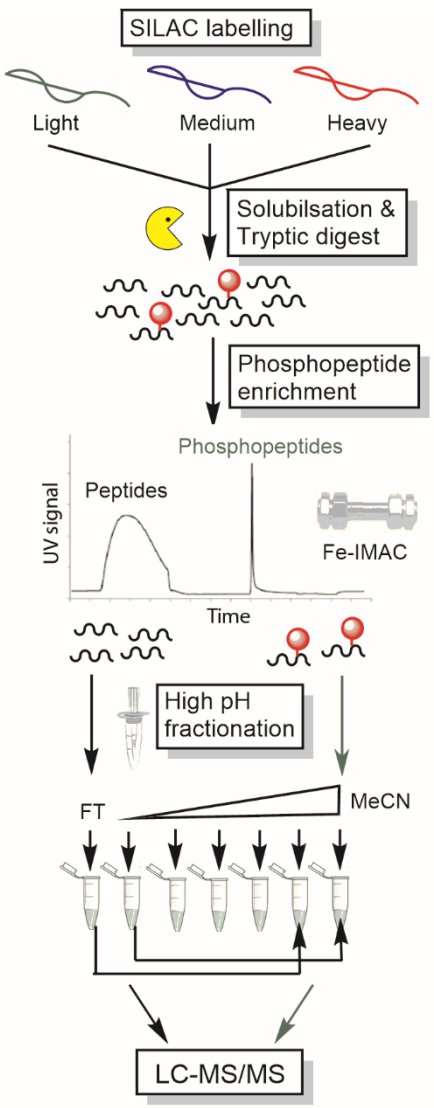

Figure 1. Quantitative global proteomic and phosphoproteomic analysis of *T. brucei*. SILAC labelled cells are treated according to the experimental regimen, combined, and the proteins solubilized and subjected to tryptic digest. Phosphopeptides are enriched by Fe-IMAC, and recovered peptides and phosphopeptides processed in parallel using high pH fractionation to reduce sample complexity prior to analysis by liquid chromatography – tandem mass spectrometry.

2 Materials

For best results, all chemicals must be of the highest grade available, and ideally solvents should be of LC-MS grade. Where solutions must be made freshly, this is indicated in the text.

2.1 SILAC labelling of cultures and cell lysis

1. SILAC HMI-11T [6] (*see* **Note 1**): To 450 ml Iscove's Modified Dulbecco's Medium (IMDM) without L-arginine and L-lysine (Pierce) add the following ingredients in order with stirring: 55 mg Pyruvic acid (Na salt), 19.5 mg Thymidine, 14 mg Bathocuproinedisulphonic acid, 2.5 ml Hypoxanthine solution (1.36 g in 100 ml of 0.1 M NaOH, warm to dissolve, store at -20 ^{0}C), 50 ml dialysed fetal calf serum (*see* **Note 2**), 5 ml 100× L-alanyl-L-glutamine. Adjust to pH 7.3 – 7.4 as required, filter sterilise and incubate at 37 ^{0}C for >24 h prior to use to check for absence of microbial growth.

2. SILAC SDM-79 [5] (*see* **Note 3**): To 425 ml SDM-79 without L-arginine and L-lysine (custom synthesis) add the following ingredients in order with stirring: 375 µL Haemin stock (10 mg/ml in 0.1 M NaOH), 1 g Sodium bicarbonate, 75 ml dialysed heat inactivated fetal calf serum (*see* **Note 2**), 5 ml 100× L-alanyl-L-glutamine. Adjust to pH 7.3 – 7.4 as required, filter sterilise and incubate at 37 ^{0}C for >24 h prior to use to check for absence of microbial growth.

3. SILAC medium that has been stability approved must be supplemented with either normal isotopic concentration of L-arginine and L-lysine (R0K0, light label), $^{13}C_6$ L-arginine and $^{2}H_4$ L-lysine (R6K4, medium label), or $^{13}C_6{}^{15}N_4$ L-arginine and $^{13}C_6{}^{15}N_2$ L-lysine (R10K8, heavy label) under sterile conditions. The heavy, medium and light labels are used at 30% of standard concentrations as given in Table 1 (*see* **Note 4**).

Table 1. Quantities of stable isotope labelled L-arginine and L-lysine

Label (100mg/ml, -20 ^{0}C)	**Mw**	**HMI-11**	**SDM-79**
L-Arg.HCl (R0)	210.6	25.3 mg/L	64.5 mg/L
L-Arg.HCl (R6-$^{13}C_6$)	216.6	25.8 mg/L	66.3 mg/L
L-Arg.HCl (R10-$^{13}C_6$,$^{15}N_4$)	220.6	26.2 mg/L	67.5 mg/L
L-Lys.HCl (K0)	182.6	43.8 mg/L	21.6 mg/L
L-Lys.2HCl (K4-$^{2}H_4$)	223.1	53.5 mg/L	26.7 mg/L
L-Lys.2HCl (K8-$^{13}C_4$,$^{15}N_4$)	227.1	54.5 mg/L	27.3 mg/L

4. Culture flasks (25 cm^2 and 150 cm^2 surface area), non-treated.

5. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4.

6. Cell lysis buffer: 0.1 µM Tosyl phenylalanyl chloromethyl ketone (TLCK), 1 µg/ml leupeptin, 1x phosphatase inhibitor cocktail II (Calbiochem), 1 mM phenyl methyl sulfonyl fluoride (PMSF), 1 mM Benzamidine. Prepare fresh just prior to use; stocks of TLCK and leupeptin can be prepared and stored at -20 ^{0}C, but Benzamidine and PMSF (in dry isopropanol) must be freshly prepared each time.

2.2 Protein solubilisation and tryptic digest

1. Solubilisation buffer: 8% sodium dodecylsulfate (SDS), 200 mM Tris pH 8.5, 200 mM dithiothreitol (DDT). Add DTT immediately before use.

2. Centrifugal filter devices: Vivaspin 20, 30 kDa molecular weight cut-off (Sartorius)

3. UA buffer: 8 M urea, 0.1 M Tris pH 8.5, prepared fresh on the day.

4. Iodoacetamide solution (IAA): 50 mM iodoacetamide in UA buffer, prepared immediately before use.

5. Ammonium bicarbonate (ABC): 50 mM ammonium bicarbonate, prepared fresh on the day.

6. Trypsin stock solution: MS grade Trypsin (Promega) at a concentration of 1 µg/µl in 50 mM acetic acid, store at -80°C for up to a month.

7. Optional: SDS-PAGE gels, sample loading buffer, running buffer and rapid Coomassie stain.

2.3 Desalting using a C18 column

1. Methanol

2. C18 columns: Discovery DSC-18, 3 ml tubes (Supelco) or Micro Spin Columns (Harvard Apparatus)

3. C18 wash buffer: 0.1% trifluoroacetic acid (TFA)

4. C18 elution buffer: 0.1% TFA, 60% acetonitrile

2.4 Fe-immobilised metal ion affinity chromatography (Fe-IMAC)

All solutions should be filtered through a 0.2 µM membrane and de-gassed before use

1. HPLC column: ProPac, IMAC-10, BioLC Guard, 4 × 50 mm (Thermo Scientific).

2. HPLC or FPLC capable of maintaining a flow rate of 1-200 µl/min, injecting 100 µl samples and UV monitoring at 280 nm, and collecting fractions every 2 min.

3. 20 mM formic acid (FA).

4. Iron charging solution: 25 mM $FeCl_3$ in 100 mM acetic acid.

5. 50 mM EDTA.

6. IMAC loading solvent: 30% acetonitrile, 0.07% TFA.

7. IMAC elution solvent: 30% acetonitrile, 0.5% NH_4OH.

8. LoBind protein 1.5 ml microcentrifuge tubes (Eppendorf).

2.5 High pH fractionation

1. High pH Reversed-Phase Peptide Fractionation Kit (Pierce)

2. Elution buffers: 2%, 3%, 4%, 6%, 10% and 50% acetonitrile in 5 mM NH_4OH. 1 ml of each is sufficient per peptide/phosphopeptide sample pair.

3. LoBind protein 1.5 ml microcentrifuge tubes (Eppendorf).

2.6 Liquid Chromatography tandem mass spectrometry (LC-MS/MS)

1. LC-MS/MS system: Ultimate U3000 Nano LC System (Dionex) coupled to an LTQ Orbitrap Velos Pro mass spectrometer (Thermo Scientific) equipped with a Proxeon nanospray ion source (*see* **Note 5**).

2. LC columns: 1 × 5 mm C_{18} trap column and a 75 µm × 15 cm reverse phase C_{18} nanocolumn.

2.7 Data analysis

1. Proteomic processing software: MaxQuant [11], freeware available from the Max Plank Institute of Biochemistry.

2. Statistical software: Perseus [12], freeware available at from the Max Plank Institute of Biochemistry.

3 Methods

3.1 SILAC labelling of cultures and cell lysis

1. Culture bloodstream form (Bsf) in SILAC-HMI11-T at 37 ^{0}C, 5% CO_2, 100% humidity or procyclic form (Pcf) in SILAC-SDM-79 at 28 ^{0}C. Grow for more than seven cell divisions in the appropriate label before harvest of log-linear culture (*see* **Note 6**). *Optional – apply treatment as dictated by the experimental regimen* (*see* **Note 7**).

2. Harvest cells by centrifugation at 1,000 × g for 10 min at 4 ^{0}C.

3. Remove the supernatant and resuspend the cell pellet in PBS.

4. Transfer cells to microfuge tube and harvest at full speed for 15 sec.

5. Remove the supernatant, lyse cells at 1 × 10^9 cells/ml in freshly prepared ice-cold lysis buffer and incubate at room temperature for 5 min (*see* **Note 8**).

6. Divide into suitable aliquots and store at -80°C.

3.2 Protein solubilisation and digestion

1. Thaw cell lysates and combine according to the experimental design (*i.e.* equal number of light and heavy cells, or light, medium and heavy cells) (*see* **Note 9**).

2. Add an equal volume of 2× solubilisation buffer.

3. Vortex for 30 sec, boil at 95°C for 3 min, vortex again for 30 sec, and sonicate for 3 min in a sonic bath. Repeat these steps if sample still viscous (*see* **Note 10**).

4. Spin at 16,000 × g for 10 min and discard the pellet. *Optional - Save a small sample of the supernatant for SDS-PAGE analysis (start sample).*

5. Dilute the remaining supernatant 1:6.6 with UA buffer and mix well by pipetting.

6. Transfer to a Vivaspin 6 column (30 kDa MWCO) and spin at 4,000 × g for 60 min at RT or until the volume is ≤ 0.5 ml (*see* **Note 11**).

7. Add 6 ml UA and spin at 4,000 × g for 60 min at RT until volume is ≤ 0.5 ml.

8. Add 3 ml IAA solution and mix for 1 min.

9. Incubate 20 min at RT in the dark without mixing. *Optional - Take a small sample for SDS-PAGE analysis (after IAA sample).*

10. Spin at 4,000 × g for 45 min at RT until volume is ≤ 0.3 ml.

11. Add 3 ml UA and spin at 4,000 × g for 45 min at RT until volume is ≤ 0.3 ml. Repeat twice.

12. Add 1.5 ml ABC and spin at 4,000 × g for 30 min at RT until volume is about 0.3 ml. Repeat twice (*see* **Note 12**). *Optional - Save a small sample before tryptic digest for SDS-PAGE analysis (before trypsin sample).*

13. Add 600 μL ABC + 1:50 approximate ratio trypsin to protein.

14. Seal the filter tube with parafilm and incubate for more than 18 h at 37°C in an oven. *Optional - Following overnight digestion, take a small sample for SDS-PAGE analysis (after trypsin sample). Run all four protein samples on an SDS-PAGE gel and stain the gel with Coomassie to confirm that the amount of trypsin used is sufficient (see* **Note 13***).*

15. If sample is completely digested, dilute it to a final volume of 3 ml with ABC buffer and remove from the spin column (*see* **Note 14**).

16. Add TFA to a final concentration of 0.1% and proceed with desalting.

3.3 Desalting using a C18 cartridge

1. Wash a 3 ml C18 column with 3 ml of 100% methanol (*see* **Note 15**). Repeat twice.

2. Wash column with 3 ml of C18 elution buffer. Repeat twice.

3. Wash column with 3 ml of C18 wash buffer. Repeat twice.

4. Apply sample to column.

5. Wash column with 3 ml of C18 wash buffer. Repeat twice.

6. Elute sample with 3 ml of C18 elution buffer.

7. Add 2 ml of milliQ water and lyophilise overnight (*see* **Note 16**).

3.4 Fe-IMAC to separate peptides from phosphopeptides

1. To charge the column with iron, first rinse with 3 column volumes (CVs) of FA at 100 µl/min, charge the column with 3 CVs of Fe charging solution at 100 µl/min, then flush out unbound Iron with at least 20 CVs of FA at 100 µl/min (*see* **Note 17**).

2. Remove column from system and store. Flush the HPLC lines (without a column) at 200 µl/min with ≥20 ml ddH_2O at 200 µl/min, then ≥20 ml 50 mM EDTA at 200 µl/min and finally with ≥10 ml ddH_2O at 200 µl/min.

3. Reconnect the column to system. Wash the column at 100 µl/min with 5 ml Fe IMAC elution solvent, then equilibrate with ≥20 ml Fe IMAC loading solvent until the absorbance at 280 nm is stable.

4. Dissolve the lyophilised sample in 100 µl of Fe IMAC loading solvent, spin at 16,000 × g for 1 min to remove insoluble material (*see* **Note 18**). Prepare a 100 µl blank sample (Fe-IMAC loading solvent only) as well.

5. Run the blank followed by the sample on the following gradient:

0-3 min – Fe IMAC loading solvent at 50 µl/min

3-4 min – Fe IMAC loading solvent at 150 µl/min

4-15 min – Fe IMAC loading solvent at 100 µl/min

15-76 min – 0-45% Fe IMAC elution solvent at 100 µl/min

76-77 min – 100% Fe IMAC elution solvent at 100 µl/min

77-82 min – 100% Fe IMAC elution solvent at 100 µl/min

82-85 min – 100-0% Fe IMAC elution solvent at 200 µl/min

85-115 min – Fe IMAC loading solvent at 200 µl/min

6. Monitor the UV absorption at 280nm and collect fractions of the sample every two minutes in protein Lobind tubes (*see* **Note 19**). Peptides that don't bind to the column will generally elute immediately in fraction 2 (after 2-4 min) while phosphopeptides will elute at a concentration of approximately 11% Fe-IMAC elution solvent in fraction 15 (after 28-30 min) (*see* **Note 20**).

7. Lyophilise the appropriate fractions overnight.

8. To strip the Fe IMAC column, flush at 200 µl/min with 30 CVs of 50 mM EDTA, then with ≥10 ml of FA. Recharge and equilibrate (steps 1-3) before next run (*see* **Note 17**).

3.5 High pH fractionation

1. Peptide and phosphopeptide fractions should be processed in parallel, but only 10% of the total peptide fraction should be processed further due to its higher abundance. Re-dissolve lyophilised combined peptide fraction in 1 ml 5 mM NH_4OH and the combined phosphopeptide fraction in 100 µl 5 mM NH_4OH.

2. Wash the spin column with 300 µl acetonitrile (5,000 × g, 2 min), discard the flow through.

3. Wash the spin column with 300 µl 0.1% TFA (5,000 × g, 2 min), discard the flow through.

4. Wash the spin column twice with 300 µl 5 mM NH_4OH (5,000 × g, 2 min), discard the flow through.

5. Apply 100 µl sample to column, collect and re-apply the flow through (3,000 × g, 2 min).

6. Collect flow-through for desalting (using C18 spin tips) before combining with other fractions (*see* **Note 21**).

7. Wash column 3× with 300 µl 5 mM NH_4OH (3,000 × g, 2 min), discard the flow through.

8. Elute with 300 µl of 2, 3, 4, 6, 10 and 50% acetonitrile in 5mM NH_4OH, saving each elution in separate protein Lo-bind tubes (*see* **Note 19**).

9. Concatenate the eluates into five fractions in protein Lo-bind tubes (*see* **Note 19**) using the following scheme: F1 = 2% and 50%, F2 = 3%, F3 = 4%, F4 = 6%, F5 = desalted FT and 10% (*see* **Note 22**). Lyophilise overnight.

3.6 Liquid chromatography tandem mass spectrometry (LC-MS/MS)

1. Resuspend samples in 0.1% formic acid (buffer A), inject onto a 1 × 5 mm C_{18} trap column, wash onto a 75 µm × 15 cm reverse phase PepMap C_{18} nanocolumn (LC Packings, Dionex), and elute with a linear gradient from 5 to 40% buffer B over 65 min into an LTQ Orbitrap Velos Pro mass spectrometer (Thermo Scientific) (*see* **Note 5**).

2. For phosphoproteomic analysis, operate in data dependent mode to perform a survey scan over a range 335 – 1800 m/z in the Orbitrap analyzer (R = 60,000), with each MS scan triggering fifteen MS^2 acquisitions of the fifteen most intense ions using multistage activation on the neutral loss of 98 and 49 Thomsons in the LTQ ion trap [13] (*see* **Note 23**). It is recommended to acquire two technical replicates of each fraction to improve the coverage of the phosphoproteome.

3. For proteomic analysis, operate in data dependent mode with each MS scan triggering fifteen MS^2 acquisitions of the fifteen most intense ions in the LTQ ion trap. Calibrate the Orbitrap mass analyzer on the fly using the lock mass of polydimethylcyclosiloxane at m/z 445.120025.

3.7 Data analysis

1. Process data using MaxQuant [11] to search against a protein sequence database containing *T. brucei brucei* 927 annotated proteins (obtained from TriTrypDB [14] http://www.tritrypdb.org/) supplemented with frequently observed contaminants (*see* **Note 24**).

2. Use the experimental design to appropriately group samples to experimental groups, allowing matching between runs for the concatenated fractions in each experiment and group two technical replicates of the phosphoproteomic samples together (*see* **Note 25**).

3. Set carbamidomethylation of cysteine as a fixed modification and oxidation of methionine, *N*-terminal protein acetylation and *N*-pyroglutamate as variable modifications. For phosphoproteomic data only use phosphorylation of serine, threonine and tyrosine residues as variable modifications. Set the false discovery rates (FDRs) of 0.01 at the levels of peptides, proteins and modification sites. Allow SILAC ratios to be calculated only where at least one peptide could be uniquely mapped to a given protein group and there is a minimum of two SILAC pairs.

4. Import the data into Perseus for visualization and statistical analysis. Filter to remove known contaminants and reverse sequences, and any phosphorylation sites with a < 0.75 localisation probability. Log_2 transform ratio data and perform further analysis as appropriate for the experiment.

4. Notes

1. SILAC HMI-11T is a modification of original HMI11 [15] (HMI9 without serum plus) based on IMDM without L-arginine and L-lysine (Pierce), and substituting 1-Thioglycerol for β-mercaptoethanol and L-alanyl-L-glutamine for L-glutamine to improve stability.

2. Fetal calf serum dialysed against PBS with a 1,000 molecular weight cut-off membrane (1K MWCO) is recommended in preference to 3K or 10K MWCO. It is critical to batch test dialysed FCS for growth with the specific *T. brucei* cell line, as the quality of the serum is critical to successful cell culture and may lead to slower growth rates and lower final cell densities. Often, this is more critical for Bsf cells than Pcf cells. Occasionally poor growth may improve on prolonged passage in dialysed serum, particularly when genetically altered cell lines are used.

3. At the time of writing SILAC SDM-79 is not commercially available, and must be purchased as a custom synthesis made to the original SDM-79 formulation [16] but without L-arginine and L-lysine for SILAC labelling and with sodium bicarbonate and L-glutamine removed to improve the shelf life. The SILAC SDM-79 medium has also been used to SILAC label *Leishmania* [9] and *Trypanosoma cruzi*

[8] for quantitative proteomics, and the sample processing protocols described here are applicable to these species.

4. We have found that reducing the quantity of L-arginine and L-lysine in the media to 30% of the original concentration has no effect on the cell growth or efficiency of SILAC labelling, and substantially reduces the cost of the media.

5. It is not essential to have access to a suitable LC-MS/MS system in-house, as analysis is increasingly available as a commercial service. Alternative instruments with high mass accuracy and resolution will yield similar coverage.

6. Greater than seven cell divisions is required to ensure >98% incorporation of isotopic label. The culture can be expanded while this is ongoing to save media and time.

7. Once labelling is complete, the samples can be treated according to the experimental regimen i.e. addition of external stimulus, induction of RNAi etc. Careful consideration must be given to experimental design to ensure that suitable control experiments are performed (i.e. swapping label in replicates) and that sufficient biological replicates are employed to ensure effects are reproducible.

8. Cells lysis can be verified by microscopy during this incubation step. The final cell lysate is 0.5×10^9 cells/ml or approximately 5 mg/ml total protein content.

9. The total number of cells recommended for a combined proteomic and phosphoproteomic experiment is approximately 5×10^8 combined over the different labels used, allowing quantification of over 4,500 proteins and 9,000 phosphorylation sites. Lower amounts of starting material will yield a reduced coverage of lower abundance proteins and phosphorylation sites. If only proteomic analysis is required, a total of 5×10^7 cells is sufficient.

10. A minimum of two cycles of boiling and sonication is needed to solubilise structural and membrane proteins and shear DNA. Viscosity can be checked by pipetting the sample up and down. It is crucial to completely solubilise the sample and reduce its viscosity at this point, otherwise the filter might become clogged later on and centrifugation times will increase disproportionately.

11. Centrifugation times can vary from run to run and it is therefore recommended to check how fast or slow the current sample is flowing through the filter, thus the times stated are an indication only. The sample must be reduced to ≤ 0.5 ml before dilution to ensure efficient removal of SDS which interferes with downstream MS analysis.

12. The required centrifuge running times increase a lot during the ABC washes. If the flow rate becomes close to zero, dilute the sample in ABC buffer until the concentration of urea is below 1 M, and proceed to tryptic digest according to protocol but with a larger total reaction volume.

13. Analysis of success of the solubilisation and digestion by SDS-PAGE is optional but recommended, as it may identify any problems before the expense of LC-MS/MS analysis. Take care to stop the gel well before the dye front runs out or you will lose your peptide band. Alternatively use a gradient gel, which helps in clearly visualising the digested peptides.

14. While the original FASP procedure [17] allows the digested peptides to elute through the centrifugal filter device, this led to variable results in our hands with peptides being retained within the column and even persisting there following the high salt washes. To avoid this we simply removed the digested peptide solution from the column and proceeded directly with desalting.

15. Washes, loading and elution are by gravity flow, but can be accelerated by applying a gentle pressure to the column (for example by using a customised syringe or a vacuum manifold).

16. Dilution is required to reduce the organic content sufficiently to allow lyophilisation. Aliquot samples out into 1.5 ml tubes for lyophilisation to allow for faster freezing and more efficient drying.

17. A new Fe-IMAC column can be charged directly; strip column first if it has not been used for over a week or in between samples of different origin.

18. The sample usually dissolves completely, but can be sonicated if any insoluble residue is apparent.

19. Protein Lo-bind tubes are essential to avoid loss of peptides and phosphopeptides on untreated plastic surfaces.

20. The phosphopeptide peak usually appears when the pH changes from acidic to basic during the run; this corresponds roughly to 11% of freshly prepared Fe-IMAC elution solution. This might differ depending on the age/storage conditions of the buffers and it is recommended to collect all fractions (every 2 minutes) to avoid losing the important one.

21. Desalting using C18 spin tips follows the same principle as the 3 ml C18 columns with reduced volumes. All washing and elution steps are in 100 µl volumes and centrifugation is at 200 x g for 1 min per step. Final elution is in 3 x 100 µl.

22. Concatenation of the seven high pH fractions reduces the number of sample analysed by MS, and hence reduces the cost, without sacrificing coverage as the combined sample have complementary LC-MS/Ms profiles.

23. In our experience, using collision induced decay (CID) with multistage acquisition (MSA) outperforms the use of High-energy C-trap dissociation (HCD) for the complex samples required for global phosphoproteomic studies.

24. Alternative data processing programmes are available. The computational resources required can be considerable for larger data sets, but Maxquant is scalable and can be run on a standard desktop machine albeit with long analysis times. As a rough guide, processing 120 LC-MS/MS phosphoproteomic data files requires ~600 Gb storage and currently takes approximately 48 h to run on 24 fast CPU cores with ≥2 Gb RAM per core.

25. Instructions for use of MaxQuant are beyond the scope of this protocol, but help and tutorials are available on-line and there are annual residential training courses.

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