

SCX And IMAC Prefractionation on Spin Columns Prior to 2D PAGE Followed by Nano-LC ESI MS and AP MALDI MS in Proteomic Analysis

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Introduction: Reducing the complexity of large proteomes, which may include as many as 10^6 - 10^{12} different proteins, is a formidable challenge in proteomics. Sample prefractionation is a preferred method to improve subsequent analysis by 2D PAGE and mass spectrometry. Prefractionation can be achieved by differential protein extraction, purification of cell organelles, preparative isoelectric focusing, or chromatographic techniques. Many of these procedures are relatively time consuming and have a low throughput. Here we describe the set of methods for the analysis of complex proteome samples comprising rapid fractionation with centrifugal membrane adsorber units prior to 2D electrophoresis with either Cy3 and Cy5 labeling or Pro-Q Diamond and Sypro Ruby staining followed by protein identification by nano-LC ESI IT MS and AP MALDI IT MS.

Methods: Here, we used membrane adsorbers with IEX (SCX) and IMAC ligands in small spin columns placed in centrifuge tubes driven by centrifugal force to fractionate proteins. Wild type and *pcu3* knockout *Schizosaccharomyces pombe* cell lysates were labelled with cyanine dye 5 (Cy5) and cyanine dye 3 (Cy3), respectively, and pooled before being applied to the SCX spin columns. The combined sample was eluted in three fractions of increasing sodium chloride concentration. The three fractions, as well as the total lysate, were analyzed by differential 2D gel electrophoresis (DIGE). In separate experiments, IMAC spin columns charged with Fe^{3+} were utilized prior to 2D gel electrophoresis in order to enrich phosphorylated proteins of fission yeast cell lysates and to study the difference in phosphorylation level of the same strains of *S. pombe*. Spots on the gels corresponding to the proteins of interest were excised, destained, and enzymatically digested. One third of each sample was analyzed by nano-LC ESI IT MS and another third by AP MALDI IT MS. A Surveyor LC pump (ThermoElectron, CA) with a self-packed column 75 μ m i.d. x 15 cm was used for nano-LC experiments. An LCQ Deca XP Plus ESI mass spectrometer (Thermo Electron, CA) was used in all experiments. AP MALDI ion source from MassTech (MD) was used in all MALDI experiments. In data-dependent MS/MS scanning, a full MS scan between 400 and 2000 m/z was followed by five full MS/MS scans for the five most intense ions from the MS scan in all ESI experiments and by three MS/MS scans for AP MALDI experiments. MS/MS data-dependent acquisition, followed by database searching with SEQUEST (for both ESI and AP MALDI) and PeptideMapping (for AP MALDI data only) (BioWorks 3.1, ThermoElectron) allowed protein identification. Fully tryptic peptides were matched with SEQUEST at a delta correlation (ΔCn) of greater than 0.08 and correlation ($Xcorr$) greater than 1.9, 2.2 and 3.5 for charged states of +1, +2 and +3, respectively. The search was performed against the whole NCBI non-indexed *S. pombe* protein fasta database.

Results and Discussion: Prefractionation on IEX spin columns led to acidic and basic protein fractions, which resulted in a better resolution in 2D PAGE in comparison to the original protein lysate. In one example, the original cell lysate provided a complex gel with 354 protein spots. The three eluted fractions led to three gels with 495, 365, and 431 spots each, amplifying the detection of low abundance proteins and making for a much simpler analysis. Both the amount of protein eluted from the column (relative to the amount loaded) as well as the reduced complexity

of the gel images are consistent. Differential 2D gel electrophoresis (DIGE) of fractionated lysates revealed a number of differentially expressed proteins in genetically diverse *S. pombe* cell cultures (Fig. 1). Fractionation on IMAC columns led to noticeable enrichment of phosphorylated proteins which resulted in better rate of identification for those proteins. Regularly, approximately 80% of the gel spots gave at least one protein ID found out by SEQUEST database search using nano-LC ESI and 50-60% of the spots using AP MALDI MS/MS. The use of Peptide Mapping software (Thermo Electron, CA) helped to improve the protein identification rate by AP MALDI IT MS. Thus, AP MALDI peptide mapping resulted in 70-80% identifications identical to IDs gotten by nano-LS ESI MS. Also, AP MALDI MS fingerprinting gave significant overlap with the identification results by nano-LC ESI MS/MS. Some spots (~10-20%) observed after phosphospecific Pro-Q Diamond staining enabled identification of phosphorylated peptides followed by MS analysis. Future coupling of AP MALDI ion source to more sensitive and fast mass spectrometers is planned. Results of the analysis of differentially expressed and post-translationally modified proteins of genetically diverse *S. pombe* cell cultures seemed to be biologically rational. Further proteomic experiments are planned to be used in studies of functional mechanisms regulating cullin ubiquitin ligase assembly and substrate-specific functions in fission yeast.

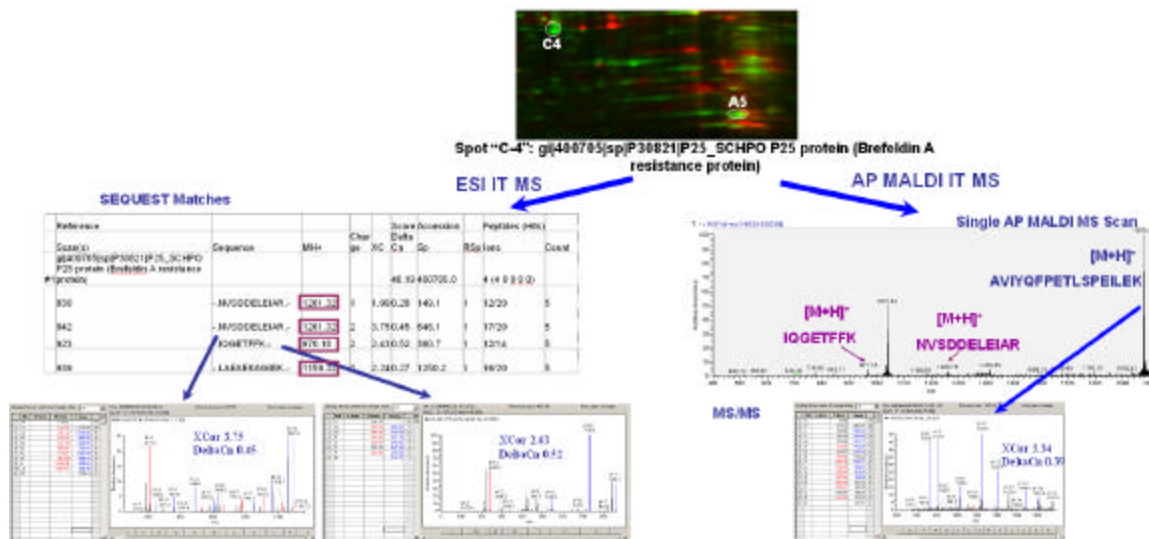


Figure 1: An example of protein identification analysis by using SCX prefractionation followed by DIGE, nano-LC ESI IT MS/MS and AP MALDI IT MS/MS for in-gel digested up-regulated protein P25