An Accelerated Phosphoproteomics Workflow

Reversible phosphorylation plays a key role in a wide range of cellular processes. Given the extent to which antibodies have trouble differentiating small differences in proteins, mass spectrometry is uniquely suited to the identification and quantification of phosphorylation. Recent advances in phosphopeptide isolation techniques and high resolution mass spectrometry have enabled the characterization of dynamic protein phosphorylation networks.

Despite recent successes in phosphoproteomics, the overall workflow remains time consuming when coupled with traditional digestion protocols. Presented here is an accelerated phosphoproteomics workflow via combinations of Polymer-based Metal-ion Affinity Capture (PolyMAC, Tymora Analytical Operations) and SMART Digest or Soluble SMART Digest technologies (Thermo Fisher Scientific).

Materials and Methods

Burkitt's Lymphoma DG75 human B cells (ATCC) were cultured in RPMI-1640 media supplemented with 10% heat-inactivated FBS, 1% sodium pyruvate, 0.5% streptomycin/penicillin, and 0.05% 2-mercaptoethanol. The cells were washed once with PBS, collected, and frozen at −80 °C. The sample containing 8×10^7 cells was lysed in 1 mL of lysis buffer consisting of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1x phosphatase inhibitor cocktail (Sigma) for 20 min on ice. The cell debris was cleared by centrifugation at 16,100 × *g* for 10 min. The supernatant containing soluble proteins was collected. A BCA assay was carried out showing ~8mg/mL protein concentration. The lysate was further split into 4 samples - 230uL each (each sample ~2mg).

Sample 1 - Control Overnight Digestion:

a. To one 230uL lysate sample, prepared as described above, dry urea was added to a final concentration of 8M urea. Following the addition of 12uL of 200mM DTT the sample was incubated for 30 minutes at 37°C.

b. After reduction, alkylation was performed using 24uL of 300mM IAA. The sample was then reacted in the dark for 50 minutes.

c. Following alkylation, the sample was diluted 10 fold. 20ug of sequencing grade trypsin was added and the sample digested overnight at 37°C.

d. The digested sample was acidified and desalted using a 500mg Sep-Pak column, aliquoted into 200ug aliquots, dried and stored at -80°C.

Sample 2 - SMART Digest Kit (immobilized trypsin):

a. To one 230uL lysate sample, prepared as described above, 690uL of SMART Digest buffer and 60uL of SMART Digest beads were added to the sample which was then digested at 70°C for 60 minutes at 1400 rpm.

b. Following digestion, the sample was reduced using 28uL of 200mM DTT reacted for 30 minutes at 37°C.

c. After reduction, the sample was alkylated using 56uL of 300mM IAA and reacted in the dark for 50 minutes.

d. Following reduction and alkylation, the sample was acidified and desalted using a 500mg Sep-Pak column, aliquoted into 200ug aliquots, dried and stored at -80°C.

Sample 3 – Soluble SMART Digest kit (in-solution trypsin):

a. To one 230uL lysate sample, prepared as described above, 690uL of SMART Digest buffer and 10uL of Soluble SMART Digest solution was added to the sample which was then digested at 70°C for 60 minutes.

b. Following digestion, the sample was reduced using 28uL of 200mM DTT reacted for 30 minutes at 37°C.

c. After reduction, the sample was alkylated using 56uL of 300mM IAA and reacted in the dark for 50 minutes.

d. Following reduction and alkylation, the sample was acidified and desalted using a 500mg Sep-Pak column, aliquoted into 200ug aliquots, dried and stored at -80°C.

Sample 4 – Soluble SMART Digest kit (in-solution trypsin) – 3x trypsin used:

e. To one 230uL lysate sample, prepared as described above, 690uL of SMART Digest buffer and 30uL of Soluble SMART Digest solution was added to the sample which was then digested at 70°C for 60 minutes.

f. Following digestion, the sample was reduced using 28uL of 200mM DTT reacted for 30 minutes at 37°C.

g. After reduction, the sample was alkylated using 56uL of 300mM IAA and reacted in the dark for 50 minutes.

h. Following reduction and alkylation, the sample was acidified and desalted using a 500mg Sep-Pak column, then aliquoted into 200ug aliquots, dried and stored at -80°C.

100ug of each sample were enriched using a PolyMAC kit per manufacturer's instructions. The phospho-enriched peptides were dried en vacuo.

Following phosphopeptide enrichment, peptide samples were re-dissolved in 8 µL of 0.25% formic acid in 3% acetonitrile and injected into a Thermo EASY nLC system. The separation was performed using a C18 capillary column packed in-house with 3 µm C18 Magic beads resin (Michrom; 75 µm i.d. and 40 cm of bed length). The mobile phase buffer consisted of 0.1% formic acid in ultra-pure water with the eluting buffer of 0.1% formic acid in acetonitrile, run over a shallow linear gradient over 60 min (2% to 30% acetonitrile) with a flow rate of 0.3 µl/min. The electrospray ionization emitter tip

was generated on the prepacked column with a laser puller (Model P-2000, Sutter Instrument Co.). The HPLC system was coupled online with an LTQ Orbitrap Velos hybrid mass spectrometer equipped with ETD (Thermo Fisher, San Jose, CA, USA). The mass spectrometer was set to operate in the data-dependent CID mode in which a full-scan MS was followed by 15 MS/MS scans of the most abundant ions. Charge state of +1 and unassigned charge states were excluded. The mass exclusion time was 90s.

Data analysis was performed with SEQUEST algorithm using Proteome Discoverer software V2.1 (ThermoFisher Scientific, San Jose, CA, USA). RAW files were searched against *Homo sapiens* database with no redundant entries. Peptide mass tolerance was set at 10 ppm, and MS/MS tolerance was set at 0.6 Da. Search criteria included a static modification of cysteine residues of +57.0214 Da, variable modifications of +15.9949 Da to include potential oxidation of methionines, and a modification of +79.996 Da on serines, threonines and tyrosines for identification of phosphorylation. Searches were performed with full tryptic digestion and allowed a maximum of two missed cleavages on the peptides analyzed from the sequence database. The parameters for FDR were set for 1% for each analysis. Proteome Discoverer software generated a reverse "decoy" database from the chosen database, and any peptides passing the initial filtering parameters that were derived from this decoy database are defined as false positive identifications. The minimum cross-correlation factor (Xcorr) filter was then re-adjusted for each individual charge state separately in order to optimally meet the predetermined target FDR of 1% based on the number of random false-positive matches from the reversed "decoy" database. Thus, each dataset had its own passing parameters

Results

Digestion Conditions	# of Peptides Identified	# of Phosphopeptides Identified
Sequencing Grade Trypsin, Overnight	5794	5493
SMART Digest, 1 hour	6607	6004
Soluble SMART Digest, 1 hour	5721	5235
3x Soluble SMART Digest, 1 hour	6098	5756

Discussion

The combination of PolyMAC phosphopeptide enrichment and SMART digestion generated results in <3 hours which were almost identical to those obtained using an overnight method. The SMART digests performed optimally with the NP40 lysed cells. The overnight digestion required pretreatment with 8M urea in order to denature the samples. This step was not required with the SMART Digest protocol. In fact, the number of peptides identified decreased significantly when SMART Digest was used in combination with urea (data not shown). This observation is in agreement with previous studies demonstrating the negative impact of chaotropes on digestion at elevated temperature (1). The two experiments performed using varying amounts of Soluble SMART Digest performed similarly. However, experiments performed using 15uL of Soluble SMART Digest trypsin per 1mg of protein performed slightly better than 5uL. It is possible that either a further titering of the enzyme amount (e.g. to 10 uL of trypsin per mg of protein) or a slight increase in the digestion time would produce similar results.

1. Selecting Buffers to Remove Uncertainty in Tryptic Digestion