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Identification of utilized phosphorylation sites in tagged proteins

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Method Article

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Abstract

Introduction

Examination of utilized phosphorylation sites in an expressed, tagged protein requires a robust method that allows for maximum sequence coverage and identification of even low stoichiometry sites. The following protocol uses two to four enzymes to achieve high sequence coverage. Each of these digestions is analyzed un-enriched \(C18) and enriched by TiO chromatography. By using this strategy and the high dynamic range and mass accuracy of the LTQFT hybrid linear ion trap – FTICR, sites modified at least down to the 0.1% level can be determined.

Reagents

10 mM DTT 50 mM iodoacetamide trypsin \(reductively alkylated) alternate enzymes: Asp-N, Lys-C, Glu-C, Arg-C and Chymotrypsin glacial acetic acid acetonitrile 0.5% NH4OH/40% acetonitrile 20% acetonitrile/2% formic acid 80%acetonitrile/2% formic acid 2% formic acid 200 mM ammonium bicarbonate 1% acetic acid 0.1M acetic acid

Equipment

Analytical columns fabricated in-house by packing 7.5 cm Jupiter 10 µm C18 packing material \(Phenomenex, Torrance, CA) into a 25 cm length of 360x75 µm fused silica \(Polymicro Technologies, Phoenix, AZ) behind a bottleneck with integrated tip of ~5µm. TiO2 columns fabricated from 15 cm 360x150 µm fused silica with a kasil frit in one end, packed with 2 cm of Titansphere packing material \(GL Sciences Inc., CA). LTQ-FT hybrid instrument from Thermo Electron Corporation \(Waltham, MA).

Procedure

Digestion: 1. Receive samples in the lab as immunoprecipitates in a PBS buffer \(~50pmol). 2. Reduce protein with the addition of 5 µL of 10 mM DTT for 30 minutes at room temperature. 3. Alkylate protein with the addition of 5 µL of 50 mM iodoacetamide for 30 minutes at room temperature. 4. Add trypsin \(or alternative enzyme) at a ratio of 1:20 and allow to react at room temperature overnight \(as second aliquot of enzyme used over next night). 5. Quench the digestion by adding 5% \(v:v) glacial acetic acid. **Analytical LC and phosphopeptide enrichment**: 1. Load sample directly onto analytical columns for the C18 analytical runs \(3%, duplicate runs). 2. To begin phosphopeptide enrichment, wash TiO_2 column with 20 µL 0.5% NH4OH/40% acetonitrile, pH 10.5. 3. Equilibrate column with 20 µL 20% acetonitrile/2% formic acid. 4. Reconstitute sample \(previously dried down) in 20% acetonitrile/2% formic acid. 5. Load sample onto TiO_2 column and wash with 20 µL 80%acetonitrile/2% formic acid. 6. Rinse with 5 µL 2% formic acid. 7. Elute bound phosphopeptides from TiO_2 column with 50 µL 200 mM ammonium bicarbonate, pH 9.0, directly into a C18 analytical column. 8. Rinse C18 column with 1% acetic acid before analysis. **Relevant LC/MS parameters**: The gradient elution was 0-90%

acetonitrile/0.1M acetic acid over two hours. Data was collected in a top 10 mode, meaning that one FT scan \(100K resolution) was taken followed by 10 MS/MS fragmentation spectra of the top intensity ions collected in the linear ion trap. Isolation width for the MS/MS was 3 m/z, and the collision energy was set to 35. After MS/MS fragmentation was done on a particular parent ion, the m/z was placed on an exclusion list to enable greater dynamic range and prevent repeat analysis of the same peptide. The repeat count for the exclusion list was 1, the repeat duration was 30 seconds, and the exclusion duration was 120 seconds. The electrospray voltage was set to 2.5 kV, and the capillary temperature was 230 degrees. The flow rate was ~500 nL/min. **Manual validation of phosphorylation sites**: 1. Perform a Sequest search against a limited database containing only the protein of interest. 2. The initial searches should use a parent mass tolerance of 8ppm and fragment tolerance of 1Da. 3. Perform an additional search using a parent tolerance of 2.1Da to identify potential spectra where the monoisotopic parent mass was incorrectly calculated by the software. 4. Manually verify all potential phosphopeptides receiving an Xcorr above 1.8 in either the analytical or phosphopeptide enrichment runs, both for accuracy of identification as well as accuracy of the site of modification. 5. Where ambiguities cannot be clearly resolved, denote the site as such.

Timing

Approximately 2 hours for titanium enrichment. Mass spectrometry data collection is 2 hours per run.

Critical Steps

Ti column flow rate should be ~1uL/min for each step so flow rate has to be adjusted using gas pressure.

Anticipated Results

The number of phosphorylation sites can vary from a low of 1-2 to a high of 50+ and will vary with cell line, growth conditions and inhibitors used.

References

Canti _et al_., _Anal. Chem_., 2007, **79**:4666-4673.

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