

# A Comprehensive Phosphoproteome Workflow

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## Overview

**Purpose:** Optimize sample preparation, LC/MS methodology, and data analysis workflows to maximize phosphoproteome coverage.

**Methods:** Biological samples from multiple sources were processed to enrich the phosphopeptide content, evaluated using LC/MS, including iterative peptide ion exclusion lists, and annotated using Proteome Discoverer software.

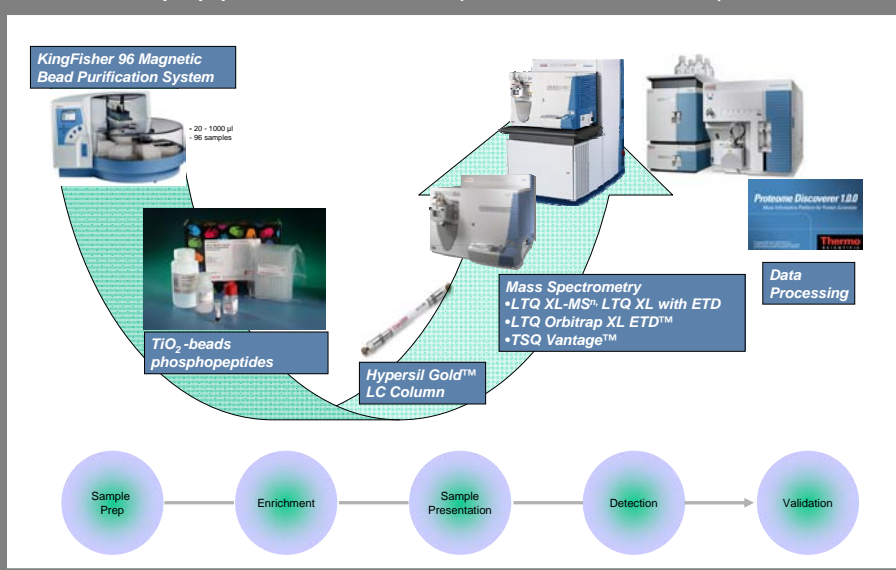
**Results:** Utilization of automated TiO<sub>2</sub> enrichment, iterative LC/MS analysis strategies, and results annotation significantly improves the coverage and information content for the phosphoproteome.

## Introduction

A comprehensive evaluation of a phosphoproteome of whole cell digests or digests of biological fluids via LC/MS requires a rigorous sample preparation methodology, appropriate LC/MS protocols, and intelligent data analysis workflows. Inadequacies in any of these steps lead to reduced numbers of identified phosphopeptides. This study is a comprehensive effort to optimize each of these steps to produce a workflow that produces the maximal phosphoproteome coverage from complex protein mixtures.

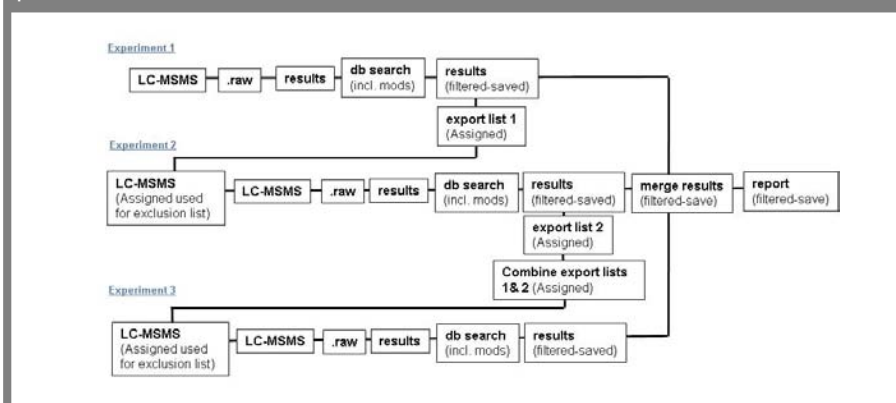
## Methods

FIGURE 1. Phosphopeptide workflow overview (see also Poster P-MON-103)



- Proteins are isolated from whole cell preparations and then proteolytically digested.
- Phosphopeptides are enriched using the Pierce Magnetic TiO<sub>2</sub> Phosphopeptide Enrichment Kit, automated with the Thermo Scientific Kingfisher Magnetic Bead Purification System.
- LC-MS/MS analysis is facilitated with an LTQ FT<sup>TM</sup> MS ultra high resolution mass spectrometer or LTQ XL<sup>TM</sup> with ETD equipped with a Surveyor<sup>TM</sup> HPLC system.
- Results are evaluated and iterative experiments designed with Proteome Discoverer 1.0 software (Figure 2).
- Biological relevance of the results are obtained through the InforSense VM option within the Proteome Discoverer 1.0 package (Figure 5).

FIGURE 2. Flow diagram for iterative LC/MS analysis of protein digests. Peptide identifications are output from Proteome Discoverer 1.0 and imported into XCalibur<sup>TM</sup> as exclusion lists. Data from three iterative LC-MSMS runs are merged. Both Normal (0-2000 m/z) and Extended mass ranges (0-4000 m/z) experiments were performed.



## Results

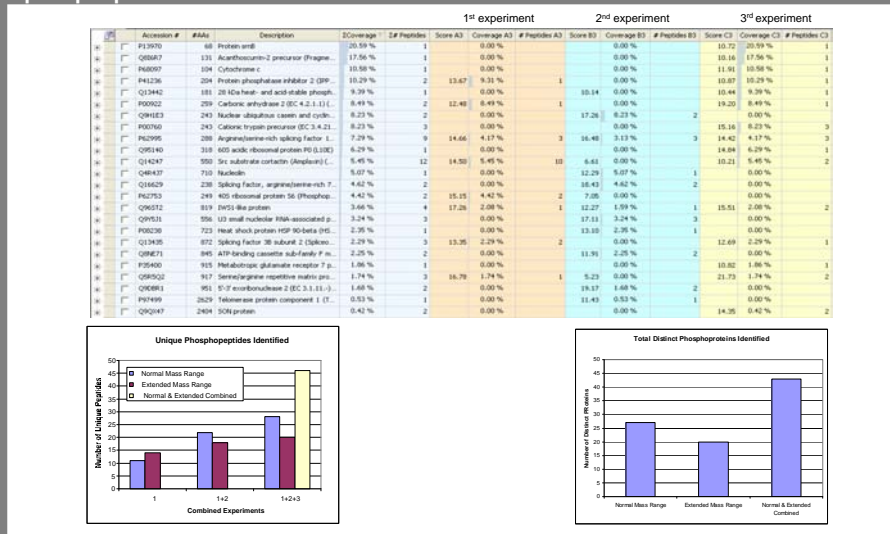
### Automated TiO<sub>2</sub> Phosphopeptide Enrichment

FIGURE 3. Automated enrichment of phosphopeptides from blood lymphocytes using Pierce TiO<sub>2</sub> Magnetic Phosphopeptide Enrichment Kit and KingFisher. Data acquired on an LTQ FT MS ultra high resolution mass spectrometer. Starting material was 2 mg of total lymphocyte tryptic peptide digest. Column A – With Enrichment, Column B – No Enrichment

	A	B
Total number of proteins identified	185	247
Total number of phosphoproteins identified	160	1
Total number of peptides identified	2347	2457
Total number of phosphopeptides identified	2009	7
Total number of unique phosphopeptides	181	1
Relative enrichment for phosphopeptides (%)	86	0.3

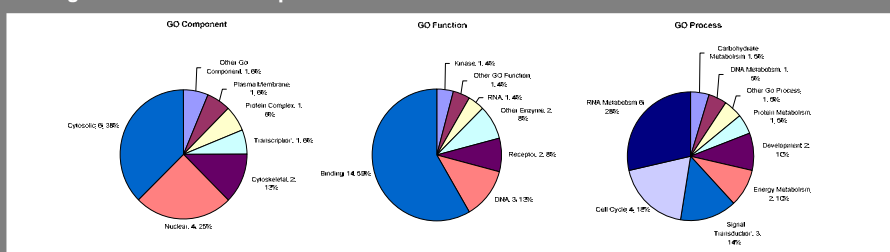
- Automated enrichment strategies simplify and increases the robustness of sample preparation methodologies.
- Automated TiO<sub>2</sub> enrichment significantly enhances the numbers of phosphopeptide identifications.
- The total numbers of peptides in both preparations are similar, but the majority of peptides in the enriched sample are phosphopeptides.
- Iterative LC/MS Analysis Strategy

FIGURE 4. ZCore analysis of ETD data generated from a dilute TiO<sub>2</sub>-phosphopeptide enriched protein digest obtained from a Human cancer cell line. Data acquired on a LTQ-XL with ETD using the iterative LC-MSMS strategy and analyzed with the Proteome Discoverer software package. Top: combined data from the normal mass range experiments; Bottom: distribution of unique phosphopeptides and distinct phosphoproteins.



- Iterative LC-MSMS strategies increase the numbers of identified peptides and proteins.
- Use of consecutive exclusion lists reduces the numbers of repeated identifications, resulting in an increase in the number of unique identifications.
- Combining data from normal and extended mass range experiments significantly increases the numbers of identified peptides and proteins.
- Comprehensive Results Annotation

FIGURE 5. Gene Ontology Annotation results from the iterative LC-MSMS experiments through the InforSense VM option in Proteome Discoverer



- Automated data annotation significantly reduces the time necessary to fully evaluate the biological import of LC-MS/MS data.
- Retrieval of posttranslational modifications (data not shown) and GO category distributions aid in the design of follow-up experiments and optimization of sample preparation methodologies.

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