Automated Identification and Sequencing of Post-Translational Modifications Using PTM Discovery on the 4000 Q TRAP® LC/MS/MS System

**Purpose**
This application note describes an automated workflow to identify post-translational modifications (PTMs) as well as determine the site of modification. The 4000 Q TRAP® system—along with Pro ID and BioAnalyst™ software—provides a high sensitivity, single-run solution for PTM analysis.

**Overview**
Protein discovery research most commonly involves the identification and quantification of proteins that are relevant to a given biological state. Crucial to this is the characterization of the post-translational modifications on the proteins of interest. PTMs are covalent modification or processing events that change the properties of a protein through proteolytic cleavage or by addition of a chemical moiety to one or more amino acid residues in a protein sequence. These modifications can affect a variety of protein properties including its three-dimensional structure, turnover, activity state, and interactions with other proteins.

The direct combination of high sensitivity triple quadrupole and ion trapping capabilities in the 4000 Q TRAP® hybrid triple quadrupole-linear ion trap LC/MS/MS system uniquely allows for automated investigation of low level post-translational modifications in biological samples, including phosphorylation and glycosylation. Integrated, advanced software identifies the PTMs, determines the protein of origin for each modified peptide, and identifies the site(s) of modification on the peptide. The PTM discovery method described here provides automated, single-run identification and characterization of modified proteins.

**Key Features**
- Automated, single-run identification of PTMs
- Identification and sequencing of low level modified peptides in complex mixtures
- High sensitivity and specificity allows screening for specific modification groups of interest
- Advanced BioAnalyst™ software with Pro ID application identifies the protein, peptide sequence and site of PTM and compares to theoretical ions

**Experimental Conditions**
Samples were tryptic digests of proteins analyzed using nanoflow chromatography coupled to a 4000 Q TRAP hybrid triple quadrupole-linear ion trap LC/MS/MS system equipped with a NanoSpray™ source and NanoSpray interface. For the selective identification of phosphorylation sites, the 4000 Q TRAP system was set to detect the characteristic PO$_3$$^-$ fragment ion using a true precursor ion scan of a triple quadrupole at either m/z 216 for Tyr phosphorylation or m/z 79 in negative ion mode for Ser, Thr or Tyr phosphorylation during LC/MS/MS.

**Results and Discussion**
For the PTM discovery experiments, a sensitive and specific triple quadrupole scan—either a precursor ion (PI), neutral loss (NL), or multiple reaction monitoring (MRM) scan—is combined with linear ion trap enhanced resolution (ER) and enhanced product ion (EPI) scans to obtain the sequence and site of the PTMs on the modified peptides. The method is automated by employing Information Dependent Acquisition (IDA) to seamlessly link the scan functions and gain the most information from any given run.

For example, in the workflow diagram...
shown in Figure 1, examples of different types of survey scans that could be used include:

- PI scan at (+) 216.0 for phosphotyrosine
- PI scan at (+) 204.1 for N-acetylhexosamine
- PI scan at (+) 126 for methylated lysines
- PI scan at (-) 80 for any sulfated residue
- PI scan at (-) 79.0 for any phosphorylated residue
- NL scan of (+) 49.0 for 2+ charged phosphorylated Ser and Thr
- MRM scan for a specific modified peptide m/z value and a diagnostic fragment m/z value

In the automated PTM workflow, when an ion is detected in the high sensitivity precursor ion or neutral loss scan, the instrument performs an enhanced resolution scan to determine the accurate mass and charge state of the peptide. This is followed by an MS/MS scan using the high sensitivity afforded by the linear ion trap mode of the 4000 Q TRAP® system. An example of this is shown in Figures 2A and 2B for the selective identification of phosphorylation sites on Tyr using a true triple quadrupole precursor ion scan at m/z 216, the immonium ion for phosphorylated Tyr. As shown in Figure 2B, the precursor ion scan (signal shown in red) provides the selectivity to find even a very low level phosphopeptide in a complex sample.

Another example of a PTM-specific scan using the PTM discovery method uses a precursor ion scan for m/z 79 in the negative ion mode, followed by polarity switching to positive ion mode followed by enhanced resolution and enhanced product ion (MS/MS) scans. An example of this type of experiment is shown in Figure 3. This figure shows results from the analysis of approximately 100 fmol of a protein, that was isolated as an active kinase from an E. coli expression system. This sample was isolated by tryptic digestion from a 1D gel. LC/MS analysis was performed on the sample after digestion out of the gel. The most abundant protein identified in this sample was identified as human maternal embryonic leucine zipper kinase (MELK), a phosphoprotein involved in serine/threonine phosphorylation of other proteins. Figure 3 shows the detailed results from a singly phosphorylated peptide that was identified in this sample. In fact, 14 different previously unknown phosphopeptides, including singly and doubly phosphorylated peptides, were identified at high confidence for this protein using the PTM discovery method.
The resulting MS/MS data was searched with Pro ID software which identified the peptide sequences which belonged to MELK. In addition, the software automatically assigned the exact sites of phosphorylation(s) on each of the peptides. Figure 4 shows the detail of how the theoretical ions for the phosphopeptide sequence from the spectrum shown in Figure 3 (residues 492LMT(PO4)GVISPER501) match to the actual ions in the MS/MS spectrum (Figure 3) in Bio Analyst™ software, which is directly linked to the Pro ID software. The bold and yellow highlighted masses indicate the matching ions. The phosphorylation-specific ions, including theoretical b-H3PO4 and y-H3PO4 ions, are highlighted by the red boxes. The matching of only the higher mass y-H3PO4 ions combined with all the other b-ions and y-ions that match to the spectrum (within 0.1 Da) indicates that the phosphorylation site is at the threonine residue near the N-terminus of the peptide and eliminates the possibility of phosphorylation at the serine residue. The high quality of quadrupole-based MS/MS fragmentation consistently allows automatic identification of the phosphorylation sites.

**Targeted PTM Discovery for Highest Sensitivity**

Although precursor ion and neutral loss scans are extremely useful for identifying unknown PTMs from a complex mixture, they do not take advantage of any prior knowledge about the protein being investigated. Quite often a significant amount of information about a protein being characterized is available and can be leveraged to perform more targeted, hypothesis-driven experiments using the MS system. This additional information may include the primary amino acid sequence, type of phosphorylation (serine/threonine vs. tyrosine), or predicted phospho-acceptor sites (e.g. MAP kinase consensus sites). This information can be used to predict precursor and fragment ion m/z values for targeted MRM experiments. Using these fast (10-100 msec), highly sensitive MRM experiments to trigger dependent product ion (MS/MS) scans on the 4000 Q TRAP® system to analyze complex biological samples, often more, lower level peptides, phosphorylated peptides and other modified peptides can be identified than with precursor ion or neutral loss PTM discovery experiments.

When the sequence of a target protein is known, targeted PTM discovery can be performed by looking for all possible phosphopeptides using these extremely fast scans throughout the LC/MS/MS analysis.
For example, this targeted method was used to identify phosphopeptides in the muscle-specific transcription factor 2A (MEF2A). The activity of this protein is tightly controlled by phosphorylation at specific sites on this protein. Although NL and PI scans were successful in identifying several phosphopeptides, the targeted PTM discovery method was able to identify even more phosphopeptides for this protein. Table 1 shows the phosphopeptides for this protein that were identified using PTM discovery with a neutral loss of 49 as the survey scan vs. the targeted MRM-IDA method. Two additional phosphopeptides were identified with this targeted MRM approach.

**Conclusions**

The PTM discovery and targeted PTM discovery methods, using precursor ion (PI), neutral loss (NL), or multiple reaction monitoring (MRM) scans in combination with sensitive MS/MS data from the linear ion trap, are unique automated tools for identifying PTMs on proteins during single run LC/MS/MS with unmatched specificity and sensitivity on the 4000 Q TRAP® system. The specificity and sensitivity of these scans make them excellent tools for identification and sequencing of low level modified peptides in complex mixtures—where standard full scan automated LC/MS/MS often fails.

No other MS system on the market today has these combined features for the highest sensitivity identification and characterization of the sites of post-translational or chemical modifications on proteins.

**References**


We gratefully acknowledge Dr. Nick Morris and Dr. Brian Williamson for analysis of the MELK sample.

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**Table 1.** MEF2A phosphopeptides identified with a neutral loss of m/z 49 method vs. the targeted PTM discovery method. (red type indicates phosphorylated residues)

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>MH+</th>
<th>Neutral Loss 49</th>
<th>Targeted MRM</th>
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<tbody>
<tr>
<td>SEPlpSPPPR</td>
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**Identified Phosphopeptides**

<table>
<thead>
<tr>
<th></th>
<th>4</th>
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