Targeted, Hypothesis-Driven Mass Spectrometry:
MRM Initiated Detection and Sequencing using the MIDAS™ Workflow for Faster, More Intelligent and Sensitive Protein Discovery and Characterization

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In the field of proteomics, most of the information needed on biological samples of interest extends beyond just the identity of the protein. Once the protein identification is made from a variety of MS and orthogonal experiments, a more in-depth study is necessary to characterize isoforms, sites of post-translational modification, or even sites of cleavage after activation or secretion. Additionally, constructing methods to determine the presence of a specific protein or peptide in a complex mixture is of greater importance as the field of biomarker discovery and validation grows. Robust and sensitive techniques for this targeted discovery and characterization of peptides and proteins are necessary.

The information known about the sample, such as the protein sequence or a hypothesized post-translational modification, allows more specific questions to be addressed. Normal information dependent acquisition techniques will not always detect the components of interest if they are of low abundance, or are poorly amenable to MS analysis. A more hypothesis-driven acquisition approach is often more effective such as the MIDAS™ workflow (Figure 1 and 2)∗. The utility and power of this approach is explored here.

Figure 1. Schematic of the Multiple Reaction Monitoring (MRM) scan for high selectivity and sensitivity. Q1 is set to transmit only the parent m/z of the peptide, the collision energy is optimized to produce a diagnostic charged fragment of this peptide in Q2, and Q3 is set to transmit this diagnostic fragment only. Because of the short dwell times required (10-50 ms) and the ability to change rapidly between MRM transitions, many components (transitions) in a mixture can be monitored simultaneously in a single LC/MS/MS run.

Key Features of the MIDAS™ Workflow on the 4000 Q TRAP® System

- Extremely high selectivity and sensitivity for detecting peptides in complex mixtures.
- High sensitivity and high quality MS/MS for confirmation of peptide sequence.
- Multiple Reaction Monitoring (MRM) can provide quantitative information between samples.

Figure 2. Using the MIDAS Workflow for Discovery by prediction of MRM transitions from a known protein sequence. The sequence of the protein of interest is digested into theoretical peptides. The precursor ion m/z (Q1) is determined by the size of the peptide and the prediction of several charge states (typically 2+ and 3+). The fragment ion m/z (Q3) is determined from the peptide sequence and simple MS/MS fragmentation rules.

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Predicted MRM ‘Scans’ Provide Lower Levels of Detection than Generic Full Scan MS Scans

Traditionally, full scan MS data is utilized to detect peptide peaks and generate precursor peak lists for MS/MS. This approach is limited in its ability to detect peaks that possess high signal relative to other co-eluting species and more commonly to the chemical background noise. The MRM scan has extremely low noise because the mass spectrometer only transmits the parent ion of interest for fragmentation and then only detects a single daughter ion (very few co-eluting species meet this criteria). As a result of this, peptides can be detected at significantly lower concentrations, and maintain excellent S/N ratios as Figure 3 indicates. In this experiment, a sample consisting of 1 fmol of a bovine serum albumin (BSA) tryptic digest and 20 fmol Cytochrome c tryptic digest was run using MRM and EMS to detect the BSA peptides. The S/N in MRM is always much better than that of the same ion in full scan MS mode. This allows for a lower level of detection of specific components in a complex mixture, allowing one to ‘dig deeper’ into a biological sample.

The MIDAS™ Workflow

Typically in an information dependent acquisition (IDA), peaks are selected from full scan MS data, and are then used for dependent MS/MS experiments. The MRM scan can also be used as a survey scan which enables the user to influence the choice of precursor ion selection for MS/MS. In combination with ab initio prediction of theoretical fragments and modifications of interest, MRM driven IDA provides all of the advantages of MRM sensitivity, S/N gains, and high selectivity, with full scan MS/MS data for confirmation, and even database searching. Figure 4 highlights the general schema utilized in these experiments. Since each MRM transition is rapid, many of these can be combined in a single survey scan spanning hundreds of potential peptides and modifications. After a peak is detected by MRM, an Enhanced Resolution (ER) scan can be performed to obtain accurate charge and m/z information, and then MS/MS is performed. In many cases the detection of an MRM transition can be sufficient for confirmation, but the MS/MS data can provide additional validation of identity.
High Sensitivity MS/MS Confirms Peptide Identification

A series of samples were prepared in which increasing concentrations of BSA (0.025 – 20 fmol/µL) was mixed with 25 fmol/µL each of cytochrome c (CytC), carbonic anhydrase (CA) and lactate dehydrogenase (LD). A volume of 1µL of the mixture was loaded onto a 75 µm column. MRM scans were created for 15 BSA peptides and used as the MRM survey scan in the IDA method. MS/MS spectra were obtained down to 25 amol of BSA on-column, with 3 peptides confirmed by database searching (Figure 5). As the top panel shows, S/N for predicted peptides is high, and even at low concentrations, the quality of the MS/MS data in the linear ion trap is excellent (Figure 5, lower panel).

MRM Builder Software Tool for Identification of Predicted Proteins

Although focused MS collection efforts are common, such as on a particular mass range, or for certain ions on inclusion lists, translating predicted fragments into a predefined LC/MS/MS method is often tedious. In order to make targeted, hypothesis-driven LC/MS/MS methods easy to generate and enhance the quality and throughput of data obtained using MRM-driven MS/MS experiments, the MRM builder tool for biomolecules was developed. This tool takes amino acid sequences of the proteins of interest, and performs a user-defined digestion in silico. In the example highlighted from the software in Figure 6, a method was created to include just the peptides present in a protein without modification. Many post-translational modifications can also be specified. The user simply has to select which modifications and residues meet their criteria and the predicted Q1 and Q3 MRM transitions are built into a method with appropriate collision energies and masses.

Figure 5. BSA digest (25 amol) in 25 fmol of CytC, CA, and LD on-column. 15 MRM transitions predicted from BSA peptides were used as a survey scan for triggering MS/MS. Shown is the MRM transition for one BSA peptide and the MS/MS obtained at 25 amol on column.

Figure 6. Build MIDAS™ Workflow methods automatically with the MRM Builder Tool. This software automatically populates acquisition methods for detecting predicted peptides. Using the sequence of the protein and some basic rules for selecting Q1 and Q3 masses for peptides, the tool determines a list of MRM transitions, calculates the proper collision energy and builds an acquisition method.
Detecting Predicted Proteins in Plasma

Because of the extreme complexity of plasma and the large dynamic range in concentration of proteins within plasma, it is often difficult to find the proteins of interest by normal MS acquisition strategies. In this example, specific protein biomarkers in human plasma were of interest and needed to be detected. Using the known protein sequence of each protein, the MRM builder script was used to automatically design MIDAS™ workflow experiments for each protein of interest. These acquisition methods were then used to specifically detect the peptides for the targeted protein fibronectin in human plasma (Figure 7). Human plasma which had been depleted of the six most abundant proteins (Multiple Affinity Removal System, Agilent Technologies) was run using a 2 hour LC gradient. The specificity and sensitivity of the MRM scan allowed the peptides from the protein of interest to be detected in the complex mixture. In addition, the sensitivity in MS/MS mode of the 4000 Q TRAP system generated high quality fragmentation spectra to easily confirm the peptide predictions from the script.

Conclusions

The unique hybrid nature of the triple quadrupole linear ion trap 4000 Q TRAP® system allows for targeted powerful workflows. Combining the specificity and sensitivity of Multiple Reaction Monitoring (MRM) with the high quality MS/MS allows for the targeted discovery of peptides and post-translational modifications. Good MS/MS can be obtained on peptides at extremely low amounts on column (25 amol), easily tracking the very high sensitivity of the MRM scan.

Applications of the MIDAS™ Workflow

- Targeting low level phosphorylation sites on a protein of known sequence to identify or confirm modification location
- Validating weak protein identifications by specifically obtaining more MS/MS on additional peptides for that protein
- Detection of low level proteins in complex mixtures
- Quantitation of proteins/peptides with accompanying MS/MS for identity confirmation

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