Multiplexed Quantitative Peptide Assays for Protein Biomarkers of Cardiovascular Disease in Human Plasma

Overview

As the study of protein biomarkers increases in importance, extensive lists of candidate markers are being developed based on protein pathway information and discovery-based proteomics experiments. Even larger numbers of candidate genomic markers exist from DNA transcriptional profiling based on microarray-based gene expression studies or other genomic information.

While these genomic markers can be validated at the RNA level using real time PCR assays such as TaqMan® kits, and at the protein level using Western blots, validation at the protein level requires specific antibodies to each protein which is a very time consuming and expensive task if hundreds of markers are of interest. A much higher throughput and more universal strategy is needed to narrow the huge number of candidate biomarkers that are being generated by both genomic and proteomics today at the protein level. The MIDAS™ workflow combined with multiplexed high throughput quantitative MRM assays on a MIDAS™ TRAQ system addresses these challenges.

Many technical challenges exist in the preliminary validation of putative protein biomarkers, such as the detection of low abundance proteins in complex tissue or biological fluids,

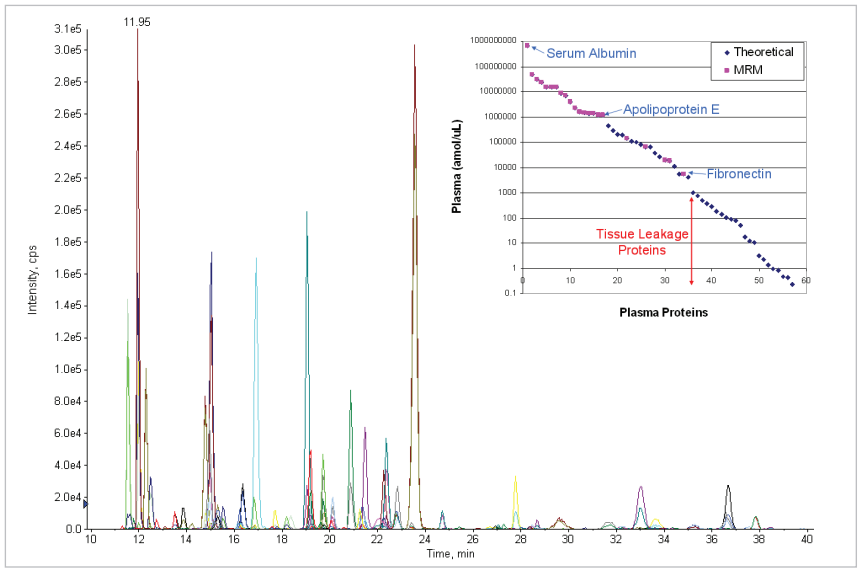

Figure 1. Dynamic range of protein abundance in plasma is extremely large[1] (inset, blue). This multiple reaction monitoring (MRM) multiplexed assay profiles 53 plasma proteins (137 MRM transitions) over the first 5 orders of abundance with minimal sample preparation.

as well as high-throughput, high precision quantitation. Validation of these markers for clinical use will require analysis of extensive candidate marker panels in thousands of clinical serum and plasma specimens. In this study, an assay was developed using multiple reaction monitoring (MRM) and MIDAS™ workflow on the MIDAS™ TRAQ system to detect and quantitate selected tryptic peptides, where each peptide represents a specific candidate protein biomarker identified for cardiovascular disease.

This application note demonstrates the specificity, sensitivity and reproducibility of this MRM based strategy in human plasma.

A number of strategies were employed to develop high quality MRM methods to obtain the best sensitivity and dynamic range for peptides from a panel of putative protein biomarkers for cardiovascular disease. For candidate proteins previously detected in proteomics experiments, MRMs were designed based on peptide MS/MS spectra. For the remaining candidates, MRMs were developed either by in *silico* design, based on gene or protein sequences, or by targeted, direct detection in plasma using the MIDAS™ workflow.

The MIDAS™ workflow provides a straight-forward link between the genomics/proteomics biomarker discovery experiments and high throughput, quantitative MS validation, because no previous experimental data on the individual proteins of interest is required to develop an assay.

This quantitative LC-MRM method profiles 53 high and medium abundant proteins in human plasma (Figure 1). Proteins down to ~1 µg/mL concentration in plasma can be reliably detected in both digested and depleted/digested human plasma, producing a dynamic range of ~5 orders of magnitude in this single method (Figure 1, inset). Peptide MRM measurements in plasma digests thus provide a robust platform for quantitative protein biomarker early stage validation.

MIDAS™ Workflow for Method Development:

During an MRM experiment, the triple quadrupole capability of the mass spectrometer transmits the parent ion and subsequent fragment ion with high sensitivity and selectivity. The first mass analyzer is set to transmit only the mass of the peptide parent ion (Q1 at unit resolution) into the collision cell. Only one of the sequence ions of the peptide, generated by fragmentation in the collision cell, is passed through the second mass analyzer (Q3 at unit resolution) to the detector (Figure 2).

This MRM experiment can be used as a survey scan in a MIDAS™ workflow to selectively detect low level peptides in complex mixtures. The detection of the peptide by MRM drives the acquisition of MS/MS to confirm the peptide sequence and thus definitively identity of the detected peptide

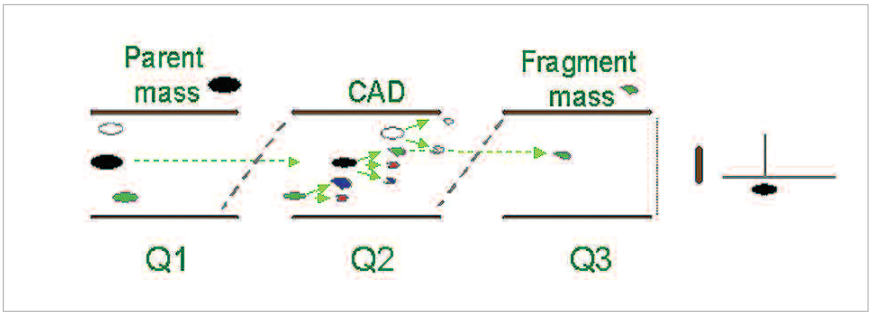

Figure 2. Multiple reaction monitoring (MRM) provides the highest sensitivity and specificity for quantitation in complex mixtures.

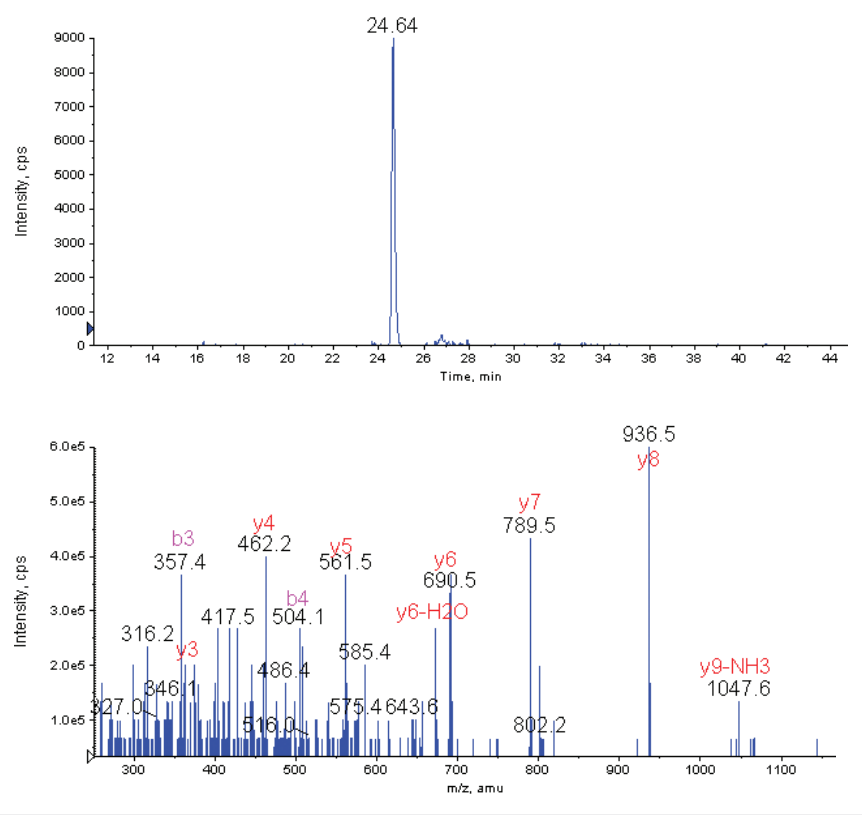

Figure 3. MIDAS™ Workflow using MRM as a survey scan to detect peptide DLQFVEVTDVK from fibronectin (~1 µg/mL) in depleted plasma.

(Figure 3). Because of the high specificity and sensitivity of MRM, these experiments possess better signal/noise than full scan MS signals and can be used to dig deeper into a sample. The hybrid triple quadrupole linear ion trap technology of the 4000 Q TRAP® system is the only platform that enables this combination of specific MRM detection with high sensitivity ion trap MS/MS.
The peptide MRM transitions can be determined either experimentally from previously obtained proteomics data or in *silico* by using the MIDAS™ workflow software designer. From the protein sequence, theoretical tryptic peptides are determined and the fragmentation pattern of the peptide by MS/MS is predicted (Figure 4). This strategy is key to developing MRMs with good S/N to low abundance proteins that are often not observed in regular LC/MS/MS methods. In

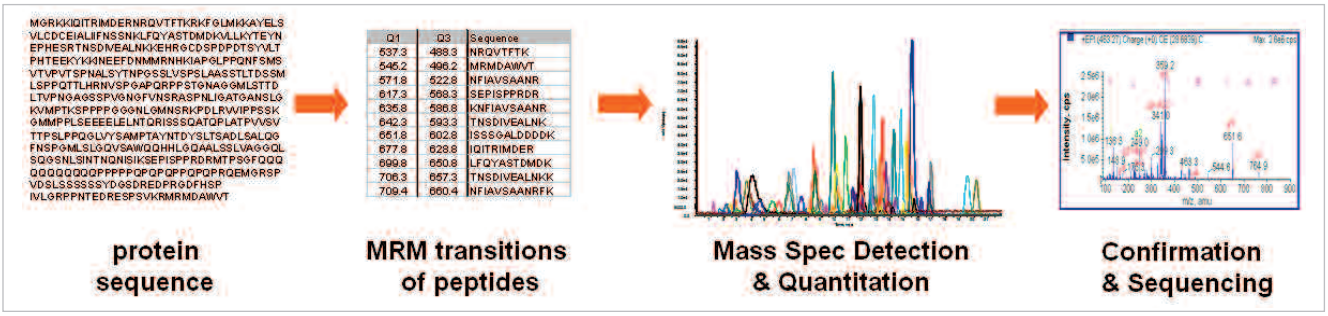

Figure 4. From the protein sequence, MRMs are designed in *silico* and used to detect and confirm peptides in complex mixtures using the MIDAS™ Workflow Designer.

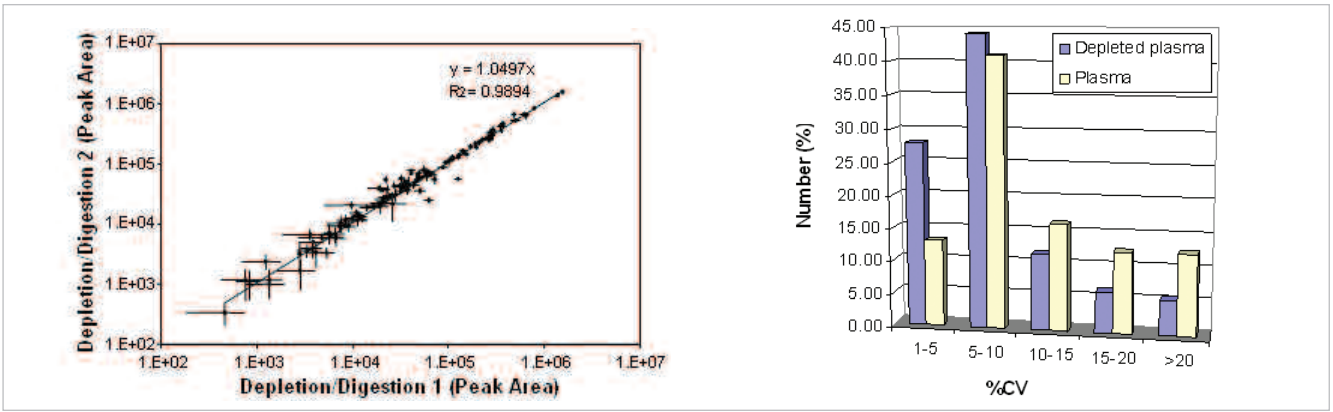

Figure 5. Depletion/digestion reproducibility (left) and replicate reproducibility (right) are very high across most peptides.

addition, putative biomarkers identified from a genomics based discovery platform can also be included in this validation strategy as all that is required is the sequence of the protein. No peptide or protein standards are required for method development.

High Quantitative Reproducibility:

A key component to the early and late stage validation of biomarkers in any body fluid is the ability to prepare and analyze many samples in parallel in a highly reproducible manner. The reproducibility of the LC-MRM method was assessed by measuring 10 LC-MRM replicates on the same sample. With no internal standard correction, the reproducibility of the peak areas was better than 10% for the majority of the MRM transitions (Figure 5, right). The average %CV for all MRM peaks was 8.6% for depleted plasma (loading equivalent of 10 nL of plasma) and 11.7% for undepleted plasma (loading 1 nL equivalent).

The reproducibility of both the depletion and digestion step of the plasma preparation was explored by taking one sample, splitting it in two and performing parallel depletions, followed by a further split of each to perform parallel digestions. All samples were then assayed and correlated (Figure 5, left). Initial results suggest that these types of sample preparation techniques can be performed in a highly reproducible manner.

MIDAS™ TRAQ System – optimized for the MIDAS™ Workflow

To enable the MIDAS workflow at optimum performance levels, Applied Biosystems/MDS SCIEX has introduced the MIDAS TRAQ LC/MS/MS system. The system combines the reproducibility and ease-of-use of the new Tempo™ nano MDLC system with the high sensitivity and dynamic range of the 4000 Q TRAP® hybrid triple quadrupole linear ion trap system. The system is also fully compatible with iTRAQ™ reagents for protein biomarker discovery.

Conclusions

- A single period multiplexed MRM assay consisting of 137 MRM experiments representing 53 plasma proteins (including 38 cardiovascular disease biomarkers) has been developed and tested in digested human plasma.[2] Very good reproducibility between replicates and across sample preparations was obtained, highlighting the potential of this method for higher throughput quantitative biomarker early stage validation.

Figure 6. The MIDAS™ TRAQ system has been designed with maximum performance to run the MIDAS™ workflow.

A key strategy to the design of these methods was the use of the MIDAS™ workflow for developing the MRM transitions to previously undetected proteins and improving MRM selection and sensitivity to others. With this straightforward strategy, proteins in the top 5 orders of the protein abundance curve in human plasma can be quantitatively profiled in a high throughput manner with minimal sample and minimal sample preparation.

This same strategy can be applied to proteins of lower abundance, using additional upfront sample preparation.

The MIDAS™ workflow provides a straight-forward link between the genomics/proteomics biomarker discovery experiments and high throughput, quantitative MS validation. Low cost, rapid method development allows for the MRM validation of many candidate biomarkers without the need for protein or peptide standards, relieving a key bottleneck. This strategy can be employed to narrow candidates to a smaller set to which labeled synthetic peptides are made as internal standards for further quantitative MS analysis. Results from this MS assay should be further validated using biological assays.

References

1. Anderson, N.L. and Anderson, N.G., Molecular & Cellular Proteomics, 2003, 2(1): 50.

2. Anderson, N.L. and Hunter C.L., Molecular and Cellular Proteomics, published December 6, 2005.

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