Designing Targeted Detection of Peptides in Complex Matrices - Combining the MIDAS™ Workflow with the Skyline Software

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INTRODUCTION

Developing a good targeted quantitative assay for MRM quantitation of proteins requires the selection of good tryptic peptides to monitor followed by the development of specific, sensitive MRMs for peptides in matrix. While some tools apply simple rules to help with the selection of peptides, testing each peptide experimentally in the matrix remains the best way to ensure a good assay. The MIDAS™ Workflow[1] leverages the unique hybrid nature of the QTRAP® System and enables the MRM triggered acquisition of full scan ion trap MS/MS. This allows the user to predict peptide MRMs, then detect and confirm these in matrix. This workflow has been implemented in Skyline Software to streamline the selection of peptides for targeted assays.

The implementation of this workflow is described in detail in this work. From a protein sequence, peptides and MRMs are generated *in silico* by Skyline and used to build a peptide detection method (MIDAS Workflow acquisition method, Figure 1) which is then run on the QTRAP® system on the sample matrix containing the endogenous peptides. Resulting data is imported back into Skyline which analyzes both the MRM data and the MS/MS data to determine which peptides are detected. The MS/MS spectra is then used to further refine MRM transitions for the detected peptides. Assay development then can proceed as normal through the other optimization steps such as collision energy optimization, MRM scheduling etc.

MATERIALS AND METHODS

Sample Preparation: Protein digests of Beta-Galactosidase (SCIEX) and yeast proteome were analyzed.

HPLC Conditions: Separation of a trypsin digest of yeast cell lysate was performed on a NanoLC™ 425 System (SCIEX) operating in trap elute mode at microflow rates. A 0.3 x 10 mm ChromXP™ C18CL trap column (5 µm, 120 Å, SCIEX) was used for loading the protein, then it was eluted onto a 0.3x150 cm ChromXP™ column (SCIEX) using a short gradient (4-32% solvent B in 30 min, B: 95% ACN , 0.1% formic acid in water) at 5 µL/min. Total protein injected on column ranged from 4 µg.

MS/MS Conditions: The MS analysis was performed on a QTRAP® 6500 system (SCIEX) using a IonDrive™ Turbo V source with a 25 µm I.D. hybrid electrodes (SCIEX). MIDAS™ Workflow methods, MRM methods and *Scheduled* MRM™ Algorithm methods were all used during data collection.

Data Processing: Skyline Software was used for building and processing the MRM and MIDAS data files.

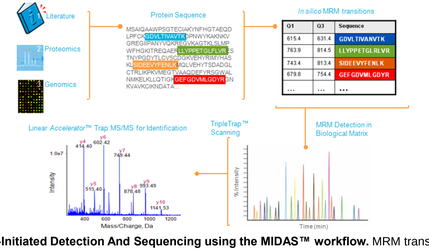

Figure 1. MRM-Initiated Detection And Sequencing using the MIDAS™ workflow. MRM transitions for peptides to desired proteins are predicted *in silico* then used as a survey scan for targeted detection of peptides. Once MRM signal is detected, high quality, high sensitivity MS/MS spectra are acquired for confirmation of sequence and peptide / protein identification. Once detected, optimized MRM assays for quantitation can be developed.

Typically in the Skyline workflow, a protein ID search is required to begin assay development, to aid in peptide and MRM selection. The workflow prototyped here allows a user to start assay development directly from a protein sequence, not requiring a previous in-depth analysis of the matrix. This provides an orthogonal path to choosing good peptides without having to synthesize peptides up front, saving time and cost. Previous work has demonstrated that MRM triggered MS/MS can detect lower level proteins and therefore will be used here as an assay development strategy.

The steps in the Skyline workflow were first prototyped and optimized using a simple protein digest Beta Galactosidase. The workflow was next applied to complex matrices (yeast, plasma, E. *coli*) to further optimize workflow efficiency. Five proteins C1TM, ADT2, SEC 31, SYTC and CPR5 were chosen from a tryptic digested yeast sample and Skyline workflow is described in detail here.

Figure 2. MRM Development Workflow Including the MIDAS™ Workflow. The protein sequence was entered into Skyline, followed by *in silico* digestion and MRM computation. Skyline then builds a set of MIDAS Workflow methods using a MIDAS starter method. These method can be viewed in the Analyst® software then used on the QTRAP system to analyze the sample matrix. Import and review of peptide detection results in Skyline allows the fragment selections to be optimized and the workflow to be streamlined. MS/MS spectra from the MIDAS file are used to build a standalone MIDAS spectral library file, which can then be used for further MRM assay fragment selection and MRM optimization similar to a library generated from a library search, but no additional upfront MS acquisition was required.

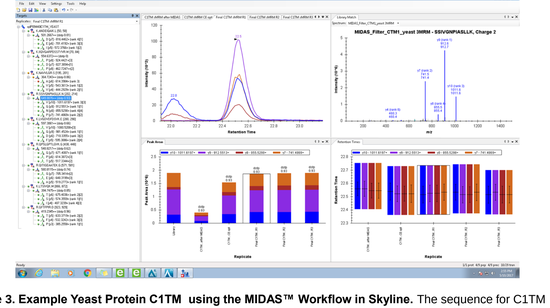

Figure 3. Example Yeast Protein C1TM using the MIDAS™ Workflow in Skyline. The sequence for C1TM was *in silico* digested and nine peptides were chosen (automatic and manual selection) with 3-4 MRM transitions to test the streamlined workflow. See peptide transitions on the left hand side. The upper two panels show the overlay of the MRM transitions next to the spectral library match, two levels of evidence for confirmation of correct peptide detection. The MIDAS Library is then built from this full scan MS/MS data. After review of MRM transition overlay and selection of more abundant fragment ions based on the MS/MS data, a *Scheduled* MRM Algorithm method including CE optimization was generated to further improve the MRM assay. The middle bottom pane highlights the improvement in MRM peak area across the workflow steps. Three replicates of the final MRM method were acquired and good peak area and retention time consistency (bottom right) was observed.

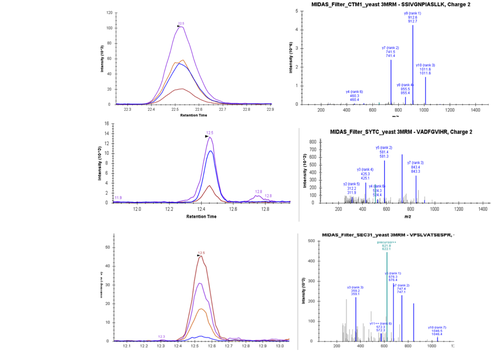

Figure 4: MIDAS Workflow for Peptide Detection Confirmation. Using the MIDAS workflow, two levels of confirmation information is obtained, the MRM data where peak shape and overlap can be reviewed, and the MS/MS data where matching to an expected fragmentation pattern can be assessed. These are example MRM transitions and MS/MS matches for yeast proteins CTM1, SYTC and SEC31 after the MIDAS workflow detection step. In each case, the MRM overlay and MS/MS fragment matching provides good confidence that the correct peptide has been detected, even for lower abundant peptides (middle pane).

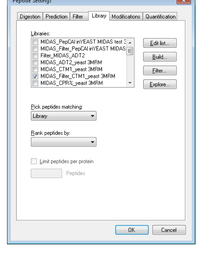

Figure 5. Generation and Use of the New MIDAS Generated Spectral Library.

The library generation in Skyline is different when using in the MIDAS workflow.
Typically, Skyline builds spectral libraries using a separate program called BiblioSpec, which has two main components. BlibBuild is called to build the redundant library, which is then filtered by a BlibFilter to create a non-redundant library. Skyline has had the ability to build BiblioSpec spectral libraries from experimental peptide search results. Now we can skip the search results step and start with an *in silico* digested protein sequence. Therefore the spectral library creation was changed and a "Filter" button appearing indicates that a MIDAS library is used, exporting a single canonical spectrum per peptide. In this MIDAS workflow, the user skipped the typical redundant library build when importing the MIDAS results.
The new "Filter" button appears under the "Build" button on the Peptide Settings - Library tab. This is only visible when a MIDAS library associated with MIDAS data was created. This ensures that the MIDAS workflow stays associated with the Library list on that tab.

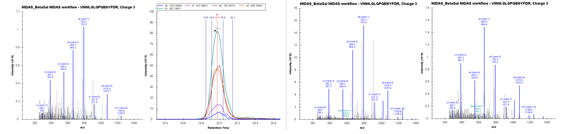

Figure 6. Skyline Automatically Chooses the MS/MS Spectrum Closest to the MRM Peak Apex. Times at which MS/MS spectra have been triggered from the MRM survey scan are shown as vertical lines on the MRM traces. Each line can be selected and that MS/MS is shown. Automatic selection is based on the spectrum closest to the MRM peak apex and matched ions are automatically indicated. This shows an example of an actual MIDAS data file. Shown are the MS/MS spectra of the vertical lines from front , center to end of MRM peak. The spectra and the spectra quality will vary across the peak. Fragment ion matches between the peptide sequence expected and the acquired MS/MS is shown, so user can easily determine if the peptide is correct.

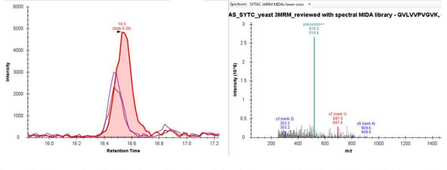

Figure 7. Peptide Detection Rejection. Review of the full scan MS/MS with the MRM transition overlays provide two levels of evidence. Example SYTC protein: This MRM overlay of a SYTC protein peptide is not aligned and the MS/MS evidence is weak, therefore this peptide detection is rejected.

CONCLUSIONS

- A workflow for performing assay development directly from protein sequence using the MIDAS workflow (Figure 1) on QTRAP® system has been implemented into the Skyline software. No previous MS/MS data is required.
- Having both MRM overlays and full scan MS/MS provides added confidence that the peptide detected at the selected retention time is the correct peptide (Figure 3)
- Workflow merges seamlessly into the full MRM assay development workflow (Figure 2)

REFERENCES

1. Unwin RD *et al.* (2005) A sensitive mass spectrometric method for hypothesis driven detection of peptide post-translational modifications: multiple reaction monitoring-initiated detection and sequencing (MIDAS). *Nature Protocols,* **4 (18),** 1134-1144.

TRADEMARKS/LICENSING

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