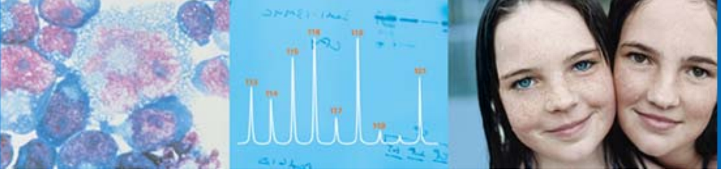


MRMPilot™ Software: Developing MRM Assays for Peptide Quantitation

Overview

Targeted peptide quantitation is a rapidly growing application within proteomics mass spectrometry due to its widespread utility in biomarker verification, protein/peptide confirmation and characterization, and pathway mapping (e.g., tracking changes in phosphorylation signaling networks during various cellular perturbations). A key requirement of this application is the existence of a robust, quantitative peptide MRM assay, that can stand-up to the rigorous demands for sensitivity, specificity, accuracy, and reproducibility in complex biological samples. The efficient development of these assays requires careful design of experiments to scout for and then optimize MRM transitions for the peptides or proteins of interest. Without dedicated software, this experimental design and subsequent data tracking is prohibitively tedious and time consuming.

MRMPilot™ Software is a program that streamlines the building and optimization of peptide MRM quantitative assays on QTRAP® systems (Figure 1). Because the third quadrupole of a QTRAP system can function as a high-sensitivity linear ion trap (LIT) or a standard quadrupole, the unique and powerful MIDAS™ Workflow is possible[1]. The MIDAS Workflow incorporates a full scan LIT MS/MS scan for the peptide after MRM detection. The MS/MS data both confirms the detection of the peptide in the sample and enables selection of abundant fragments when designing further MRM transitions. This allows efficient development of assays for even very low level peptides from complex real-world biological matrices. MRMPilot™ Software goes beyond the functionality of the MIDAS™ Workflow Designer software[2], leveraging protein discovery data to build MRM and MIDAS Workflow assays in addition to enabling iterative optimization of assays and storing and reporting qualitative and quantitative results. The end result is an optimized MRM based method.

Key Features of MRMPilot™ Software

- Supports all biomarker verification and peptide quantitation applications
- Creates robust peptide MRM assays using previously acquired discovery or identification data or protein sequence information
- Builds Analyst® Software MRM, Scheduled MRM™ Algorithm and MIDAS™ Workflow methods
- Allows iterative optimization of MRM assays
- Provides tools for reviewing and reporting the quality and reporting of large numbers of quantitative MRM and MS/MS ID results with links back to discovery and optimization data
- Supports use of stable isotope labeled peptides including the mTRAQ™ reagents
- Works directly with MultiQuant™ Software[3]

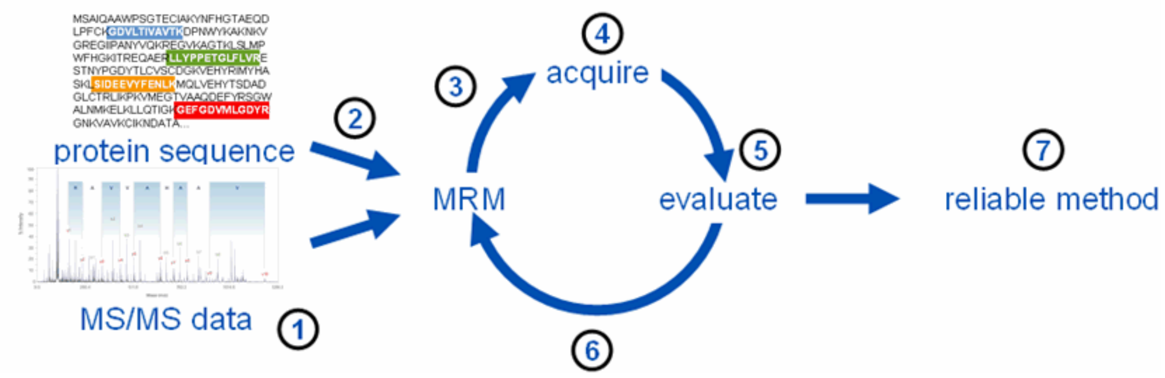
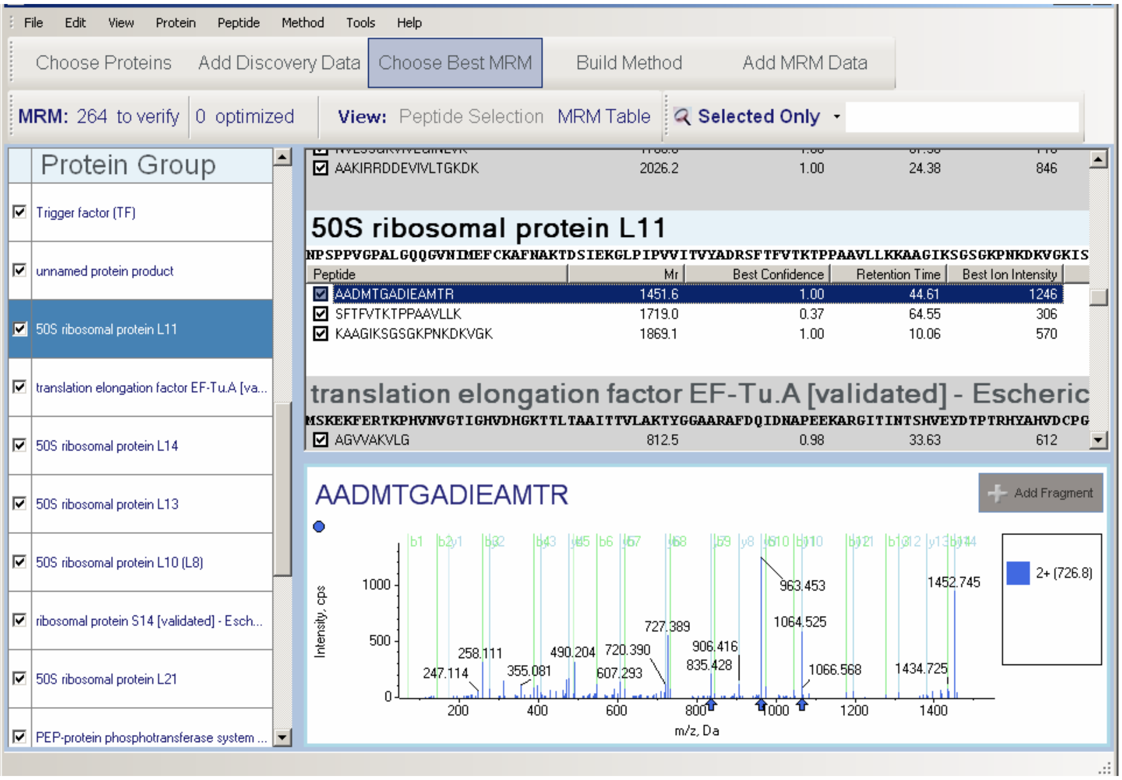


Figure 1: Creating a reliable MRM-based method for peptide quantitation is an iterative procedure that takes input from various sources. Choosing which peptides, and then which fragment ions, can be based on either ① previously acquired MS/MS data or from ② non-MS based techniques and peptide fragmentation prediction. The next step is to ③ create an instrument specific method (MRM-triggered MS/MS method otherwise known as a MIDAS Workflow method) and then use this method to ④ acquire data. The selected MRM transitions are then ⑤ evaluated for their suitability for quantitative measurements (i.e. signal/noise, intensity, etc.) and the MS/MS is evaluated for how well it confirms the peptide ID. This process is ⑥ repeated until ⑦ a set of MRM transitions are generated that reliably identify the peptides and can be used for quantitation.



Protein Group

- ☑ Trigger factor (TF)
- ☑ unnamed protein product
- ☑ 50S ribosomal protein L11
- ☑ translation elongation factor EF-Tu.A [va...
- ☑ 50S ribosomal protein L14
- ☑ 50S ribosomal protein L13
- ☑ 50S ribosomal protein L10 (L8)
- ☑ ribosomal protein S14 [validated] - Esch...
- ☑ 50S ribosomal protein L21
- ☑ PEP-protein phosphotransferase system ...

☑ AAKIRRDDEVIVLTGKDK	2026.2	1.00	24.38	846

50S ribosomal protein L11

NPSPPVGPALGQQGVNIMEFCKAFNAKTDSIEKGLPIPVVITVYADRSFTFVTKTPPAAVLLKKAAGIKSGSGKPNKDKVGKIS

Peptide	Mr	Best Confidence	Retention Time	Best Ion Intensity
☑ AADMTGADIEAMTR	1451.6	1.00	44.61	1246
☑ SFTFVTKTPPAAVLLK	1719.0	0.37	64.55	306
☑ KAAGIKSGSGKPNKDKVGK	1869.1	1.00	10.06	570

translation elongation factor EF-Tu.A [validated] - Escheric

MSKEKFERTKPHVNVGTIGHVDHGKTTLTAAITTVLAKTYGGAARAFDQIDNAPEEKARGITINTSHVEYDTPTRHYAHVDCPG

☑ AGVVAKVLG	812.5	0.98	33.63	612

AADMTGADIEAMTR

Figure 2: Once proteins are imported into MRMPilot Software, MRM transitions are created. In the example above, the best 3 transitions are chosen for the best 3 peptides for each protein by selecting Choose Best MRM. Users may configure the number of transitions and peptides within the Options pane under the Tools menu.

Build MRM Methods

The basic steps for creating a robust high quality MRM peptide assay are outlined in Figure 1. The first step is to create a starter assay containing MRM transitions for the peptides and proteins of interest. Protein ID results from either ProteinPilot™ Software, Mascot, or Sequest searches may be imported, or alternatively, protein sequences can be entered directly. MRMPilot™ Software is used to intelligently create a set of initial transitions based on real or predicted fragment data. Figure 2 shows an example of a set of MRM transitions prepared from a ProteinPilot Software group file of imported proteins. In this example, only the best three MRM transitions for the best three peptides for each protein are chosen for the initial assay. The numbers of MRM transitions and peptides chosen per protein are user configurable. The peptide fragment data is shown in the bottom pane for the selected peptide from 50S ribosomal protein L11 and the three product ions for the MRM transitions are highlighted with arrows.

The user has the option to add new proteins, select additional or different peptides, or edit transitions. Once the transitions are created, MRMPilot Software then builds the Analyst acquisition method. The data is then collected using this new method and processed with MultiQuant™ Software[3].

Track the Quality of MRM Transitions

MRM data that has been integrated by MultiQuant™ software can be imported into MRMPilot Software, for the purpose of determining whether further optimization is required. Figure 3 shows an example of MRM data integrated with MultiQuant Software imported into an MRMPilot Software MRM Table view. The peptide MRM transitions are listed in the top pane and the MRM data for the selected peptide transition is shown in the bottom pane along with the full scan MS/MS data acquired using the MIDAS™ Workflow for confirmatory purposes. Overlaid with the MIDAS Workflow MS/MS data is the MS/MS data from the discovery phase for comparison.

In order to assess the quality of the MRM transitions, a mean height and %CV are calculated. In addition, a Summary Graph is displayed (Figure 3, top right). A Summary Graph is a graph of mean intensity vs. %CV for the best MRM transition from each peptide in the MRM table. This graph allows one to easily visualize which peptides have the highest intensity and reproducibility for quantitative purposes.

Iteratively Optimize MRM Transitions

MRMPilot™ Software allows iterative optimization of MRM transitions and assays. Based on the qualitative and quantitative information for a particular transition, it can be further optimized by finding the best collision energy, or it can be completely discarded and new MRM transitions acquired and evaluated.

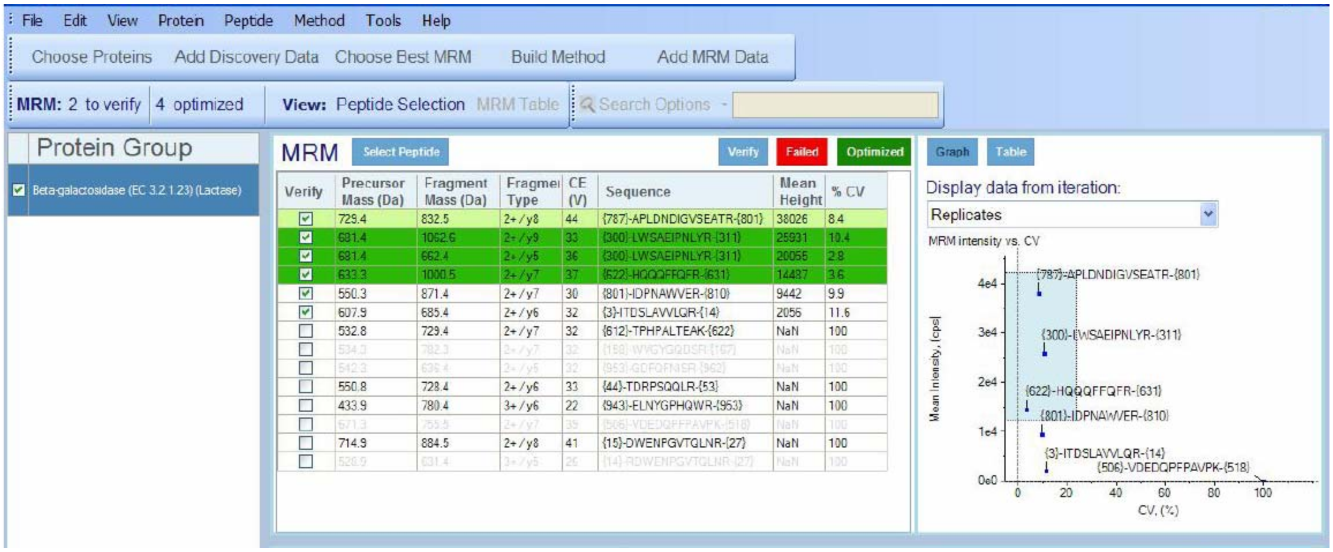
The final step in producing a reliable MRM based method is to acquire replicate samples and evaluate which MRM transitions are the most sensitive and reproducible. Here the Summary Graph comes in quite handy. By drawing a box around the peptides in the top left corner of the graph, only those peptides with the highest intensity and lowest %CV are selected (Figure 4). These transitions can then be set as “Optimized” indicating that no further iterative optimization is required and they are ready to be used in a real biological assay.

Once the retention time of the peptides is known, the multiplexing capabilities of the Scheduled MRM™ Algorithm^5 can be leveraged during the optimization or reproducibility testing, further improving the efficiency of assay development. With Scheduled MRM Algorithm, an MRM transition is only monitored across its expected retention time, reducing MRM concurrency and allowing for many more MRMs to be analyzed in a single run.

After all optimization is completed, a final MRM assay is built. This method can be an MRM only acquisition method, a MIDAS™ Workflow acquisition method or a Scheduled™ MRM Acquisition method for any AB SCIEX QTRAP® or Triple Quad™ based system.

Verify	Precursor Mass (Da)	Fragment Mass (Da)	Fragment Type	CE (V)	Sequence	Mean Height	% CV
☑	681.4	1062.6	2+ / y9	39	{300}-LWSAEIPNLYR-{311}	36581	50.1
☑	681.4	662.4	2+ / y5	39	{300}-LWSAEIPNLYR-{311}	27424	54.5
☑	714.9	884.5	2+ / y8	41	{15}-DWENPGVTGLNR-{27}	6291	31.6
☑	550.3	871.4	2+ / y7	33	{801}-IDPNAWVER-{810}	6234	32.8
☑	729.4	832.5	2+ / y8	41	{787}-APLDNDIGVSEATR-{801}	5135	43.7
☑	532.8	729.4	2+ / y7	32	{612}-TPHPALTEAK-{622}	2503	42.2
☑	433.9	780.4	3+ / y6	22	{943}-ELNYGPHQWR-{953}	2223	43.9
☑	633.3	1000.5	2+ / y7	37	{622}-HGQQFFQFR-{631}	1901	33.2
☑	550.8	728.4	2+ / y6	33	{44}-TDRPSQQLR-{53}	722	35.4
☑	607.9	685.4	2+ / y6	35	{3}-ITDSLAVVLQR-{14}	552	58.6
☑	534.3	782.3	2+ / y7	32	{158}-WVGYGQDSR-{167}	NaN	100
☑	542.3	636.4	2+ / y5	32	{953}-GDFQFNISR-{962}	NaN	100
☑	671.3	755.5	2+ / y7	39	{506}-VDEDQPFPAVPK-{518}	NaN	100
☑	528.9	631.4	3+ / y5	26	{14}-RDWENPGVTGLNR-{27}	NaN	100

Figure 3: After acquiring MRM data using methods created previously, the data is integrated by MultiQuant™ Software and imported back into MRMPilot™ Software for evaluation. The MRM Table view allows qualitative and quantitative visualization of all of the MRM transitions and MIDAS™ Workflow data. The MRM transitions are assessed for quantitative quality and the MS/MS data is assessed for identification quality with comparison back to original discovery ID data.



Display data from iteration: Replicates

Verify	Precursor Mass (Da)	Fragment Mass (Da)	Fragment Type	CE (V)	Sequence	Mean Height	% CV
☑	729.4	832.5	2+/y8	44	{787}-APLDNDIGVSEATR-{801}	38026	8.4
☑	681.4	1062.6	2+/y9	33	{300}-LWSAEIPNLYR-{311}	25931	10.4
☑	681.4	662.4	2+/y5	36	{300}-LWSAEIPNLYR-{311}	20055	2.8
☑	633.3	1000.5	2+/y7	37	{622}-HQQQFFQFR-{631}	14487	3.6
☑	550.3	871.4	2+/y7	30	{801}-IDPNAWVER-{810}	9442	9.9
☑	607.9	685.4	2+/y6	32	{3}-ITDSLAVVLQR-{14}	2056	11.6
☐	532.8	729.4	2+/y7	32	{612}-TPHPALTEAK-{622}	NaN	100
☐	534.3	782.3	2+/y7	32	{158}-WVGYGQDSR-{167}	NaN	100
☐	542.3	636.4	2+/y5	32	{953}-GDFQFNISR-{962}	NaN	100
☐	550.8	728.4	2+/y6	33	{44}-TDRPSQQLR-{53}	NaN	100
☐	433.9	780.4	3+/y6	22	{943}-ELNYGPHQWR-{953}	NaN	100
☐	671.3	755.5	2+/y7	35	{506}-VDEDQPFPAVPK-{518}	NaN	100
☐	714.9	884.5	2+/y8	41	{15}-DWENPGVTQLNR-{27}	NaN	100
☐	520.9	631.4	3+/y5	26	{14}-RDWENPGVTQLNR-{27}	NaN	100

Figure 4: A final step in creation of an MRM assay is to assess the reproducibility of an MRM transition. The Summary Graph shown in the top right of the MRM Table view is very helpful for this purpose. By selecting on a particular protein, only the best peptide MRM transitions are displayed for each peptide. This graph allows the user to quickly assess which MRM transitions are best since the highest intensity MRM transitions with the lowest %CV will appear closest to the top left corner. Selecting those peptides allows them to be set as "Optimized" so that no further optimization is required.

Conclusions

MRMPilot™ Software allows a user to create MRM based experiments to identify or quantitate peptides. The selection of MRM is based on either previously acquired MS/MS identifications, or predicted based on peptide sequence. MRMPilot Software then helps to iteratively optimize MRM transitions by allowing the evaluation of quantitative and qualitative results by calculated metrics as well as intelligent and user friendly graphs and tables. The final result is a set of high quality MRM transitions that can be used in a true biological assay.

References

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