Automated Creation and Refinement of Complex Scheduled SRM Methods for Targeted Proteomics

Brendan MacLean[1], D. Tomazela[1], G. Finney[1], M. Chambers[2], N. Shulman[1], A. Prakash[3], S. Peterman[3], M. J. Maccoss[1]

[1]University of Washington, Department of Genome Sciences, [2]Vanderbilt University, [3]Thermo Fisher Scientific

Overview:

Selected Reaction Monitoring (SRM) is a technique widely used for the quantitative measurement of target compounds in complex mixtures. Increasingly it is being used for the hypothesis driven analysis of protein differences across large numbers of biological samples. One difficulty in making targeted proteomics routine is the complexity and labor involved in producing an optimized instrument method that measures many target peptides in a single analysis. Unfortunately the generation of these methods and their refinement is still largely a manual process. We have developed the software program Skyline that greatly shortens the path from hypothesis to a fully optimized instrument method.

Introduction:

Skyline presents an intuitive user interface for working with proteins, peptides and fragment ions, with rich support for in silico digestion and transition prediction.

Using ProteoWizard [1] to fully support instruments from:

- Agilent
- Applied Biosystems
- Thermo Fisher
- Waters

Figure 1: Peptide modifications and isotope labeling strategies

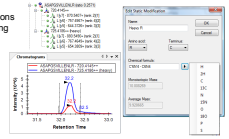
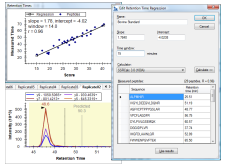


Figure 2: Retention time prediction [2]

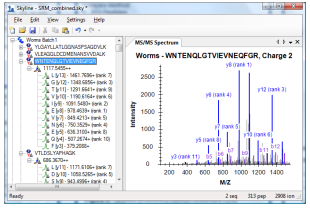


Methods:

Initial Complex Method:

- 313 Peptides with matching MS/MS library spectra
- 2908 Transitions ($y_3 - y_{N-1}$)
- 55 Sample injections

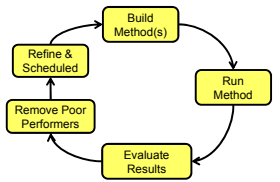
Figure 3: The unrefined method with transitions ranked by ion Intensity in the matching BiblioSpec [3] library spectra



Injections performed as follows on a TSQ Ultra (Thermo Fisher) :

- 2 µL (5ug) of the *C. elegans* digest was loaded onto an in-house packed capillary column with a ~5 µm pulled tip, 30 cm length, and 0.075 mm internal diameter using an Eksigent nanoLC-1D.
- The column was packed with Jupiter Proteo (C12, 4 µm particle size, 90 A pore size) using an in-house constructed pressure bomb.
- A binary solvent system used reverse-phase buffers consisting of an A buffer containing 95% water, 4.9% acetonitrile, and 0.1% formic acid and a B buffer composed of 20% water, 79.9% acetonitrile and 0.1% formic acid.
- The nanoLC was operated at a flow rate of 350 nL/min.

Figure 4: Targeted proteomics method refinement cycle

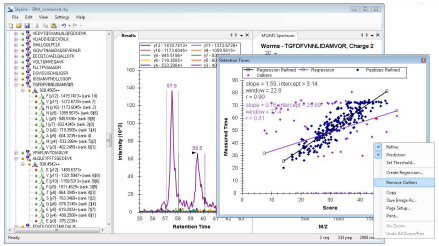


Results:

Monitoring 313 Peptides by SRM:

The resulting 55 Thermo RAW files were imported into the original Skyline document, beginning the process of refinement.

Figure 5: A single command to remove retention time outliers, and peptides without peaks, reduced our list to 243 peptides.



Refining for measurable peptides:

The Skyline document editor proved effective for further refining the list. Within ½ hour, the list had been reduced to the three best transitions for 135 peptides with clear signal.

Figure 6: The editable tree view, combined with the chromatogram and MS/MS library spectrum views proved a powerful combination for refining a method after collecting results.

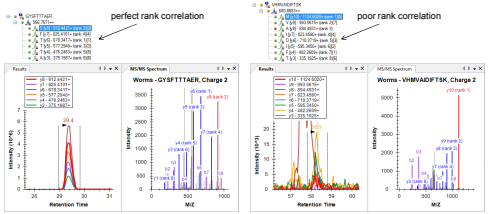
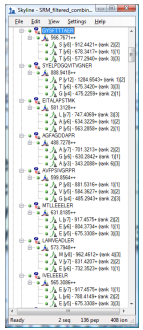


Figure 6: The first cycle of refinement complete, with the method reduced to 135 peptides and 405 transitions, ready for scheduled SRM.

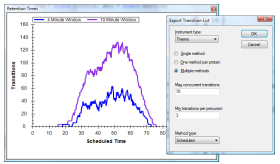


Scheduling:

For a final scheduled method we chose the constraints:

- 1.5 second cycle time
- 70 concurrent transitions maximum at any time
- >20ms dwell time

Figure 7: Skyline shows that the chosen constraints will require 2 sample injections with a 10 minute scheduling window, and only 1 sample injection with a 4 minute window, and presents the form for creating the lists.



Further Refinement:

After running 2 sample injections unscheduled on a new column, the list was reduced to 121 peptides, but had to be further reduced to 105 peptides due to an instrument limitation of the TSQ Ultra. The reduced list was run in single method with a 4 minute window, over 4 more replicates to test for stable chromatography.

http://proteome.gs.washington.edu/software/skyline

Preparing for a Quantitative Assay:

With a proven set of 105 peptides, and stable chromatography that will allow us to further reduce the scheduling window to 2 minutes, we are ready to order peptides for a high-repetition quantitative assay.

Figure 8: Skyline's flexible layout and retention time replicate view allowed detailed inspection of the scheduled single injection methods.

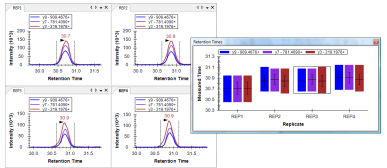
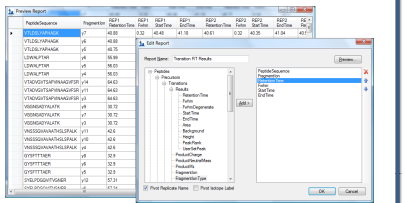


Figure 9: The report editor helped create a comma separated value (CSV) report for further statistical analysis before choosing the final set of peptides and schedule window



Conclusions:

- Skyline radically streamlines the process of refining complex SRM methods for targeted proteomics experiments.
- This streamlining allows optimized methods to be created by refining initial broad measurements in an actual biological matrix.

Future work includes:

- Open source in ProteoWizard [1] project (June, 2009)
- Final release of v0.5 (July, 2009)

References:

(1) Kessner D, Chambers C, et al. Bioinformatics. 2008/05;24(21):2534-6.
(2) Krokhin OV, Craig R, et al. Mol Cell Proteomics. 2004/09;3(9):908-19.
(3) Frewen B, MacCoss M. Curr. Protoc. Bioinform. 2007/12;20:13.7.1-13.7.12.

This work is funded by a subcontract from Vanderbilt University under NIH/NCI grant number U24-CA126479 for the CPTAC program.