Introduction
Over the last several years proteomics labs have begun asking, “Is protein X measurable in these samples?” as opposed to “What proteins can be detected in this sample?” In our own lab, the progression began with collaborators asking to measure a specific target protein of interest when they could not obtain a suitable antibody. In essence they were looking for the mass spectrometry equivalent to a Western blot.

Because there was a specific target analyte of interest, the use of selected reaction monitoring (SRM, also referred to as multiple reaction monitoring or MRM) on a triple quadrupole mass spectrometer was an obvious solution. Less obvious were 1) what peptides to measure as a proxy of the target protein; 2) what transitions to measure from the target peptide; and 3) what specific optimizations (e.g. collision energy) to perform for each peptide. Addressing these issues with the tools available at the time proved extremely cumbersome, even for relatively small sets of peptides. To enable more complex experiments in this area we needed a software tool that made use of existing data to establish an initial set of peptides and transitions, but also allowed us to analyze data from “pilot” runs to further refine and optimize these initial instrument methods. Skyline was developed to achieve these goals.

Make use of available shotgun data
Many thousands of shotgun proteomics experiments have been performed, and many of the resulting data sets are publicly available. Shotgun proteomics data collected within the lab and by the broader community provide useful information for targeted proteomics experiments. Two separate papers have indicated high correlation between shotgun MS/MS y-ion peak intensities measured on ion trap instruments and peak areas from chromatograms on triple quadrupole mass spectrometers. These papers compared library spectra from Thermo LTQ data with peak areas from a Thermo TSQ and a Waters Quattro Premier, respectively.

Figure 1: The Skyline Spectral Library Explorer showing a MS/MS spectrum from a phosphorylation experiment. Several of the most intense peaks show loss of H3PO4 as -98.

While the results on the Quattro Premier suggest stability of relative fragment ion intensities across triple quadrupole instruments from different manufacturers, some skepticism of how widely this result may be applied is probably healthy. From our own experience with the NCI-CPTAC program, it is not necessarily easy to come up with a single set of product ion transitions to measure the same peptides on the instruments of four different triple quadrupole vendors. Skyline provides powerful tools for testing such assumptions, and investigators are encouraged to use them.

Use any public spectral libraries
Even if a tandem mass spectrum for a target peptide has not been collected in a prior shotgun experiment, a spectrum matched to the peptide may well exist in any of a number of public spectral libraries. Skyline supports all major publicly available spectral libraries from the Global Proteome Machine (GPM), National Institute of Standards (NIST), the Institute for Systems Biology (ISB), and the MacCoss Lab. Links to these repositories are available on the Skyline web site. Once down-loaded, a library file from one of these sources can be inspected with the Skyline Spectral Library Explorer, as shown in Figure 1.
The library can be used to help choose peptide precursors and product ions to monitor specific proteins of interest. After acquired SRM data is imported, Skyline provides dot-product scores between the library spectra and the transition chromatograms which can be used for identity confirmation.

**Build new spectral libraries**

Skyline supports building BiblioSpec spectral libraries from a growing number of peptide search output formats, currently including Mascot, Myrimatch & ID Picker, Protein Pilot, Scaffold, Spectrum Mill, Trans Proteomic Pipeline, and X! Tandem. Even if data made available publically has not been incorporated into spectral library projects, the search results can be incorporated into custom built libraries using tools within Skyline. A single laboratory can also build libraries from its own shotgun, discovery experiments to begin creating targeted methods based on those findings. Experiment specific libraries might include post-translational modifications (PTMs) unavailable in public libraries and might contain MS/MS spectra from instrumentation more similar to what will be used for the targeted investigation. If peptide abundance information, such as spectral counts, will be used to pick the peptides to monitor for the proteins of interest, then certainly experimental context is important.

Starting with a spectral library built from a shotgun proteomics experiment, the Skyline Spectral Library Explorer can add all of its peptides to a method as a starting point for method editing. Even with libraries containing hundreds of thousands of peptide spectrum matches, the Library Explorer allows quick peptide searching and adding individual peptides to a method. By providing Skyline with protein sequence information for the Background Proteome of an experiment, added peptides can be correctly associated with their proteins of origin.

**Start with broad coverage**

Whether or not library spectra are available for peptides originating from the proteins of interest, targeted experiments usually perform best if started by measuring more information than desired for a final quantitative assay. The combination of peptide precursors, product ions, instrumentation, and sample matrix introduce a great deal of variability. In creating a new targeted assay from scratch, direct empirical knowledge of the experiment is extremely valuable.

**Choose peptides carefully**

Predicting which peptides for a given protein will produce the best signal in a targeted experiment is not well understood. Predictive tools exist\(^4\), \(^5\), but only claim enough accuracy to limit initial measurements in search of the highest responding peptides for a protein. Abundance indicators in a public spectral library may not reflect the experiment sample matrix. In an experiment-specific spectral library, a targeted method may produce clean signal for peptides masked during shotgun acquisition by more abundant peptides that co-elute, even ones with similar precursor m/z values. If a spectral library contains several abundant peptides for a protein of interest, however, it is probably safe to start your measurements targeting that protein by measuring some of these. Skyline makes it easy to do this. If you are less sure, Skyline also makes it easy to measure every tryptic peptide in a protein, and later refine back to the ones that produced the best signal in the targeted experimental context.

**Start with more transitions than required**

Spectral libraries are most useful in predicting the product m/z values that will register the greatest y-ion signal. It is still a good idea to start a targeted method measuring more than just the desired final number of transitions. The noted high correlation between shotgun MS/MS spectra and targeted chromatogram peak areas means that the 3

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**Figure 2:** Skyline showing a refinement experiment including 225 peptides measured in 1347 transitions. Refinement started with the 6 most intense peaks matching y3 through y(n-1) in the MS/MS library spectra. The selected peptide VTLDSDLYAPHAGK has a 0.99 dot-product score between the peak areas of the 6 measured transitions and the matching peaks in the library spectrum.
to 4 most intense chromatogram peaks tend to be among the 6 most intense MS/MS peaks. Starting with smaller numbers of transitions will also reduce confidence in confirming the peptide identity of a chromatogram peak group. As shown in Figure 2, Skyline allows an investigator targeting peptides with existing library spectra to start out measuring the 6 most intense library y-ions for hundreds of peptides.

If no library spectrum can be found for a peptide of interest, a good place to start is simply measuring all y-ions from y3 to y(n-1); where n is the number of amino acids in the peptide. This method does not provide absolute confidence in the identity of the chromatographic peak, but can be used to exclude peptides that cannot be measured from further consideration.

**Prefer singly charged y-ions**

Singly charged y-ions are the ion of choice for targeted applications. In triple quadrupole instruments, b-ions tend to undergo more secondary fragmentation than they do in ion trap instruments using resonance excitation collision induced dissociation. This can result in strong signal at the m/z values for small b-ions, particularly b2, but these ions lack the specificity of larger y-ions. A b-ion with strong signal in a library spectrum measured on an ion trap may produce disappointing results when measured on a triple quadrupole instrument. This will be instrument specific. While doubly charged fragment ions can be used, we and others have found that the collision energy prediction algorithms optimized on singly charged y-ions may not perform as well for these ions\(^6,7\). Additionally, because doubly charged fragments are typically at lower m/z, there can be greater amounts of interference in complicated mixtures.

**Avoid transitions where b- and y- ions overlap**

Because of the mechanism of b-ion fragmentation, product ions where y- and b-ions overlap at the same m/z should be avoided. While these fragment ions may show strong signal in ion trap MS/MS spectra from shotgun experiments, they may be significantly underrepresented in a triple quadrupole mass spectrometer. Skyline can explicitly highlight these peaks in its spectral library graphs.

**Prefer longer product ions**

When choosing product ions to measure, fragment ions of higher m/z generally provide greater specificity and cleaner signal than ions of lower m/z. In a case where a y3 ion produces only slightly higher intensity than a larger ion, the larger ion should be chosen. The y1 and y2 ions should generally be avoided entirely, since y1 for tryptic peptides will always be either R (m/z 175) or K (m/z 147). It has been suggested that using product ions where product m/z is greater than the precursor m/z is preferable, since these transitions will be absent of interference from singly charged precursors\(^8\). Initial measurements might include smaller ions, but during refinement favor larger ions and approach peptides where y3 is the dominant peak with some skepticism.

**Refine the method using empirical results**

When starting with a broad set of candidate proteins, the optimal instrument settings for measuring them quantitatively and repeatedly are often unknown. Achieving a highly effective instrument method requires empirical measurements in the experimental context and subsequent method refinement based on these results. Whether the initial protein candidates come from a literature search or a previous shotgun, discovery experiment, Skyline was designed to facilitate this refinement process. Zeroing in on a limited set of higher confidence candidates for repeated measurement in high value samples will invariably take multiple phases of data collection, or iterations of what we call the Targeted Method Refinement Cycle, as depicted in Figure 3.

**Exclude peptides which cannot be measured**

Using Skyline in this workflow, we recently started with over 100 proteins from the literature, lacking sufficient library spectra. An initial exhaustive targeted method measured over 12,000 transitions in 136 sample injections, yielding
138 peptides with reliably measureable chromatogram peak groups. Other labs have covered even broader ranges of initial proteins, using library spectra, as described above, to narrow the initial set of transitions. If the target assay will be used to differentiate between diseased and healthy subjects, consider performing this phase on pooled sample from both groups, since valuable candidates may eventually turn out to be barely measurable in one group or the other.

**Build familiarity, run multiple replicates**

After an initial screen to exclude peptides with no discernable response in the sample matrix for the experiment, it is a good idea to become more familiar with the remaining peptides and transitions before using them to investigate high value samples. In the experiment mentioned above, the remaining 138 peptides were scheduled for acquisition in a single sample injection and acquired repeatedly. Skyline makes this scheduling easy. It also provides rich graphical support for investigating multi-replicate data and the issues that impact repetitive acquisition and quantification.

**Chromatography issues**

Precise quantitative measurement of peptides in scheduled SRM requires highly reproducible chromatography. Implementing system suitability standards as part of the acquisition process can help, but different peptides exhibit different chromatography. One peptide might elute within seconds of a single retention time over dozens of replicates while another in the same sample might drift by minutes or vary widely. Failing to capture the entire peak for a peptide during a comparative experiment can negatively impact the resulting data, especially if the peptide is not paired with a stable isotope labeled standard. The Skyline retention time replicate comparison graph can expose chromatography issues at a glance, and the peptide comparison view can show the retention time coefficient of variation for every peptide measured.

**Peak area issues**

Understanding the peak area precision characteristics of a targeted peptide is critical to knowing whether it can be measured accurately enough for the intended experiment. Many peptides with relatively stable peak area to sample abundance correlation may be quantified accurately enough using a shared set of internal standards to adjust for global effects during acquisition. A peptide that exhibits lower precision alone or even degradation over time in the auto-sampler may require a paired isotope labeled internal standard to measure, or it might be discarded in favor of a less intense but more stable peptide. Finally, injecting varying quantities of a synthesized peptide of interest can help determine whether any of the potential transitions for measuring a peptide are impacted by interference from other molecules. Only through multi-replicate analysis can these issues be detected. The Skyline peak area view can make such issues obvious at a glance.

**More Information**

Skyline is an open source software project, and may be freely installed from:

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

This page currently provides nearly 150 pages of tutorials and over an hour of instructional video on targeted proteomics experimentation using Skyline. The tutorials most directly related to the material in this article are:

- Targeted Method Editing
- Targeted Method Refinement
- Spectral Library Explorer

Available at:

http://proteome.gs.washington.edu/software/Skyline/tutorials.html

**Join the Discussion — US HUPO WIKI**

Share your ideas, questions, and experiences with Skyline and suggest future TechTalk topics. We hope to enliven and to create a resource for the Proteomics community with this feature. Go to US HUPO WIKI.

**Reference List**