SYSTEMATIC METHOD DEVELOPMENT FOR QUANTIFICATION OF PROTEINS VIA THE SURROGATE PEPTIDE APPROACH USING LC-MS/MS, MASSLYNX™ AND SKYLINE

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INTRODUCTION

Biotherapeutics are increasingly becoming a significant part of the pharmaceutical arsenal as more and more companies work towards using them individually or in combination with other large or small molecules as drugs of choice. This trend manifests itself in the growing number of bioanalytical laboratories across the globe incorporating technological and scientific expertise required to deal with the complexities such analyses bring. The diversity within biotherapeutics ranges from small linear or cyclic peptides, all the way to complex monoclonal antibodies and antibody drug conjugates. The instruments of choice for performing these types of analysis are largely tandem quadrupole mass spectrometers, like the Waters Xevo TQ-S.

Bioanalysis of large proteins brings unique challenges for scientists. Sample preparation for large molecules often involves denaturation, reduction, alkylation followed by digestion. Depending upon the analyte of interest and the matrix in which the analyte is being quantified, the need for reduction and alkylation also needs to be evaluated. Each one of these steps needs optimization. Determining the best MRM transitions for peptides generated after digestion is also complicated, as each peptide can have multiply charged precursors and products. MRM methods are optimized through LC injections, creating multiple acquisition methods, modifying one parameter at a time, effectively generating upwards to 15-20 acquisition methods. Once the data is acquired, manually mining through the data is very time consuming, and could take hours, or even days to distill down to the best acquisition method to be used.

Skyline is a freely-available, open-source Windows client application for building Selected Reaction Monitoring (SRM) / Multiple Reaction Monitoring (MRM) quantitative methods and analyzing the resulting mass spectrometer data. It aims to employ cutting-edge technologies for creating and iteratively refining targeted methods for large-scale quantitative mass spectrometry studies in life sciences. This software has been developed and is being constantly maintained and improved by the MacCoss Lab at University of Washington.

In this poster, we present a detailed workflow covering sample preparation, generating in-silico peptides, determining precursor and fragment ions, generating multiple acquisition methods optimizing MRM parameters, and eventually analyzing the data generated in an easy to interpret visual format, using Skyline and Waters MassLynx™ software. Interferon gamma will be used as a candidate molecule for this poster. However, a similar approach can be applied for the quantification of most proteins via the surrogate peptide approach.

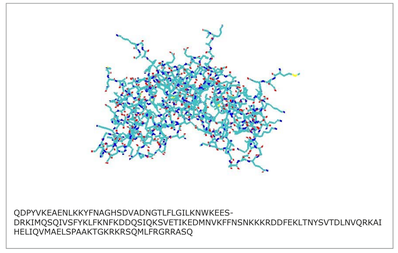


Figure 1. Interferon γ crystal structure[1] and amino acid sequence[2]

METHODS

The protein sequence for Interferon γ was inserted in Skyline. In the settings menu, peptide settings were chosen to allow for digestion using trypsin, allowing 1 miscleavage. No background proteome was chosen. The minimum and maximum length of the peptides was chosen as 5 and 25 amino acids long respectively, and no modifications were excluded. Skyline instantly performed an in-silico digest of the protein sequence based on the parameters selected in the peptide settings.

In the transition settings, Waters Xevo instrument was selected for the collision energy calculation. Precursor and product ions(b and y only) with 1, 2 and 3 charges were selected. The minimum m/z was set at 300 and the maximum was set at 2048.

MS acquisition method were then created for MassLynx™ using Skyline. In the File option on the menu bar, export method was selected. Waters Xevo was chosen as the instrument type. Minimum transitions per sample was 150. Multiple methods optimizing collision energy was selected. Skyline generated 9 acquisition methods varying the CE for each transition of each peptide, in a MassLynx™ compatible format.

Sample Preparation:
Quantification of proteins via the bottom up approach usually involves multiple steps of denaturation, reduction, alkylation and digestion. At each one of these steps, multiple variables, like grade/vendor of reagent, time, temperature have to be evaluated and optimized. A standardized, kit based approach to sample preparation in the form of ProteinWorks™ kits was evaluated for this assay.

Different digestion protocols described below were evaluated during method development.

ProteinWorks eXpress Digest

Step 1	• Denaturation
Step 2	• Digestion
Step 3	• Stop Reaction

Total time < 3 hours

ProteinWorks Direct Digest

Step 1	• Denaturation
Step 2	• Reduction
Step 3	• Alkylation
Step 4	• Digestion
Step 5	• Stop Reaction

Total time < 5 hours

Chromatographic conditions:
Mobile Phase A: 0.1% Formic Acid in Water
Mobile Phase B: 0.1% Formic Acid in Acetonitrile
Column: ACQUITY UPLC HSS T3 1.8 µm, 2.1 x 150 mm
Column Temperature: 60 °C
Injection Volume: 10 µL

Xevo TQ-S source conditions:
Cone Voltage (kV): 2.50
Capillary Voltage (V): 40
Desolvation Temperature (°C): 500
Desolvation Gas (L/Hr) 1000
Cone Gas (L/Hr): 150

Time (mins)	Flow (mL/min)	%A	%B	Curve
Initial	0.400	95	5	Initial
2.00	0.400	95	5	6
2.10	0.400	95	5	6
6.00	0.400	50	50	6
6.10	0.400	5	95	6
8.00	0.400	5	95	6
8.10	0.400	95	5	6
10.00	0.400	95	5	6

RESULTS

A high concentration sample of INF γ spiked in buffer was digested using the 5 step ProteinWorks™ protocol. A total of 9 injections were made, 1 using each of the 9 methods created by Skyline. The results were imported back into Skyline through the File -> Import -> Results -> Add New replicate -> Optimizing Collision Energy. Once the import is complete, Skyline detects the best peak for each transition of each peptide and plots the corresponding peak area for each of the collision energy tested in a bar graph format as shown below (Figure 2). This easy to interpret visual format allows the scientist to quickly review the data and pick the optimal peptide transitions for a given peptide. The transitions which do not yield a good signal can simply be deleted using the delete/backspace key. Once the initial review is complete and the weak transitions are deleted, the scientist can either choose to make a final MRM method directly, or further fine-tune the method. To further fine-tune, one can undergo another round of method generation using the export function, changing the collision energy step size and step count. This can be done in the transition settings section, in the Collision Energy drop-down by choosing to edit the current instrument settings. If the scientist is content with the results form the first set of data, they can choose to create a final MRM method by simply exporting a single method in the File -> Export -> Method dialogue box. On doing so, Skyline will automatically pick the optimal collision energy for all the transitions left in the transition list.

INF γ spiked in buffer was digested using the 5 step protocol, and used to generate the final acquisition method as listed below (Figure 3). Blank human plasma and human plasma spiked with different concentrations of INF γ were then digested using the 5 step digestion protocol. Results showed background interfering peaks from the matrix present at the retention time of the peptide of interest, having very close MRM transitions (Figure 4). To resolve the matrix issue, INF γ spiked in buffer was digested using the 3 step protocol. Data showed that a subset of INF γ peptides were generated without the need for reduction and alkylation (Figure 3). A calibration curve of INF γ spiked in buffer and digested using the 3 step protocol showed good linearity and precision (figure 5).

Thus, for this molecule, the reduction and alkylation steps can be skipped, simplifying the sample preparation protocol. This also reduces the total number of peptides being generated from the sample matrix, therefore reducing the possibility of ion suppression due to fewer ionisable species. This may result in higher sensitivity from more area counts for analyte of interest and better signal to noise due to lower matrix background.

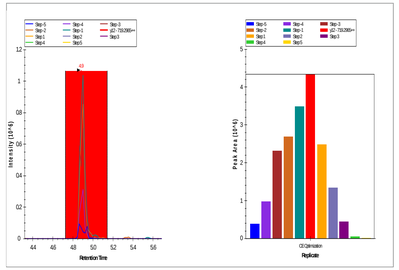


Figure 2. Data review pane for Collision Energy optimization within Skyline. The different bars indicate the different CE's for a single transition.

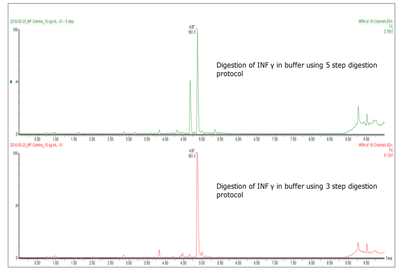


Figure 3. TIC of INF γ spiked in buffer and digested using the 5 step and 3 step ProteinWorks digestion protocol

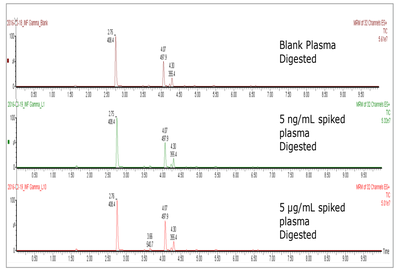


Figure 4. Blank human plasma digested using the 5 step protocol. Interference observed at the retention time of interest

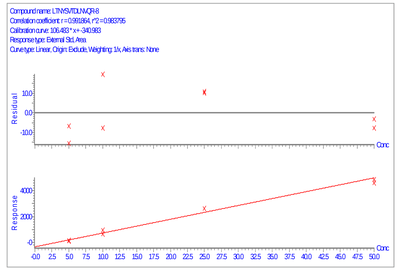
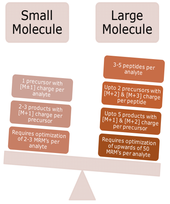


Figure 5. Calibration curve for INF γ spiked in buffer and digested using the 3 step protocol.

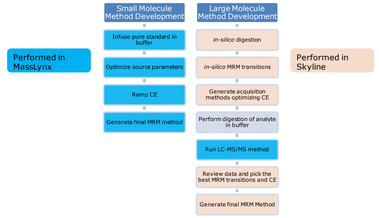
DISCUSSION

Quantitation of proteins using the surrogate peptide approach is rapidly becoming a key part of most bioanalytical laboratories have traditionally focused on performing small molecule quantification. Protein/peptide quantification brings with it unique challenges which are different from those typically experienced with a small molecule assay. Scientists are having to learn to navigate these challenges quickly. It has been shown multiple times that simple sample preparation techniques like protein precipitation, or liquid-liquid extraction rarely work for large molecules. The limited mass range of tandem quadrupole instruments, which are the instrument of choice for bioanalytical quantification, pose an additional challenge by requiring the proteins to be digested, to form small peptides which can then be detected within the mass range of these instruments.



Building an MRM method for a protein/peptide molecule is also very different compared to a small molecule. With small molecule method development, an infusion of the standard can be used to optimize all of the MS parameters and the spectrum is fairly clean. With peptides however, the spectrum gets extremely complicated. An infusion of even a pure synthetic peptide can generate a complex spectrum, making it impossible to fine-tune any parameters. The preferred option is to optimize MS parameters based on LC injections, varying one parameter at a time. For a large protein like a monoclonal antibody, there are anywhere between 3-5 possible peptides. Each peptide can have 8-10 MRM pairs which show some amount of signal. Optimizing the collision energy for each one of those MRM pairs would mean generating multiple acquisition methods, one by one. This process can get confusing and tiresome pretty quickly.

Using an open source software like Skyline removes many barriers associated with MRM method development. Its ability to not only generate in-silico peptides, but also to generate in-silico MRM transitions, and then generate acquisition methods optimizing collision energies for each one of those MRM transitions in minutes saves a lot of time and energy for the scientist. The ability to import the data into Skyline, review it in minutes, and then choose to either make a final MRM method with a few clicks can be extremely powerful. The software also affords ample flexibility to choose the type of in-silico digestion and MRM transitions based upon information about the specific analyte of interest.



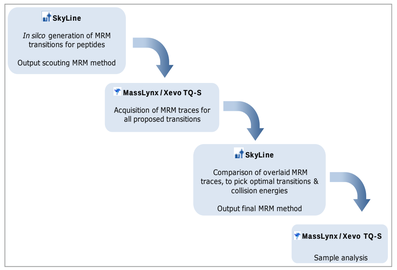


Figure 6. Workflow for MRM method generation using Skyline and MassLynx™ softwares

CONCLUSIONS

- **Skyline works seamlessly with MassLynx™ software to make method development for the analytical scientist simple, easy and quick**
- **ProteinWorks™ kits provide a simple, standardized sample preparation kit for large molecule bioanalysis reducing the time required for method development and ensuring reproducibility across multiple laboratories**
- **Reduction and alkylation may not always be needed prior to digestion. Eliminating these steps may lead to increased area counts for analytes and/or reduced matrix background resulting in better sensitivity**
- **Elimination of these steps also result in a simpler sample preparation protocol, thus reducing the total error within an analysis, reagent cost and total assay time**

References

1. Thiel DJ, le Du MH, Walter RL, D'Arcy A, Chène C, Fountoulakis M, Garotta G, Winkler FK, Ealick SE (September 2000). "Observation of an unexpected third receptor molecule in the crystal structure of human interferon-gamma receptor complex". Structure8 (9): 927–36. doi:10.1016/S0969-2126(00)00184-2.PMID 10986460
2. CAS Registry Number: 82115-62-6: CAS, A Division of the American Chemical Society

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