Peptide MRM Optimization Made Easy for Therapeutic Protein and Peptide Quantification

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ABSTRACT

Development of bioanalytical LC-MS methods for the detection of therapeutic peptides and the proteolytic fragments of larger proteins and antibodies is growing along with the increasing number of peptide and protein drugs entering clinical development. With multiple charge states possible for a given peptide and the abundance of product ion possibilities plus the large number of proteolytic fragment possible from a protein digest, development of these methods present challenges that are different to small molecule method development. In this poster we present a workflow using DiscoveryQuant™ software to overcome some of these challenges and make the optimization of peptide MRM methods easier and less time consuming.

INTRODUCTION

Protein based therapeutics including: monoclonal antibodies (mAb) and smaller therapeutic peptides are a rapidly expanding component of many pharmaceutical companies' drug portfolio. Ligand binding assays (LBA) used for protein and peptide quantification suffer from inherent variability, lack of specificity, narrow dynamic range, and time consuming method development. As an alternative to LBAs LC-MS/MS methods are both sensitive and selective, have a wide dynamic range, and have been a staple in the quantitation of small molecule drugs. Bioanalytical methods for proteins and mAbs generally require digestion of the sample with a proteolytic enzyme followed by direct analysis of one or more of the proteolytic peptides. The first challenge of this type of assay is choosing a peptide that is unique to the protein of interest yet is sufficiently selective and sensitive. The second challenge is that unlike bioanalytical method development for most small molecules, multiple charge states are possible for a given peptide which in combination with the many product ion possibilities leaves many MRMs to be screened. Manual tuning is tedious, and optimizing via LC injections is time consuming, particularly when monitoring multiple MRMs per peptide and multiple peptides per protein.

DiscoveryQuant™ software is an automated MRM tuning and optimization application and is the ideal tool to perform this optimization to achieve maximum sensitivity. DiscoveryQuant™ Optimize software allows for optimization of compound dependent mass spectrometer parameters (DP, CE, CXP and EP) via infusion or flow injection analysis. The MRM transitions along with optimized parameters are stored in the DiscoveryQuant™ software database and are made available for incorporation into LC-MRM methods using DiscoveryQuant™ Analyze software.

This poster describes the results of experiments where DiscoveryQuant™ software was used with infusion to tune and optimize MRM parameters of a protein digest and improve the sensitivity of MRM the methods generated.

MATERIALS AND METHODS

Mass Spectrometry Conditions:
An AB SCIEX QTRAP® 4500 system operating in ESI positive mode was used for tuning and LC-MS/MS analysis. Trypsin digested *E. coli* BGAL from the AB SCIEX mass spectrometer standards kit, Part No. 4368624 was diluted to 0.50 pmol/µL in 50% acetonitrile in water with 0.1%formic acid. Infusion was performed at 2 µL/min using an Eksigent microLC electrode (25 µm) in the Turbo V™ ion source. LC-MS/MS injections were performed on a 0.10 pmol/µL (5 µL injection) sample at 0.25 mL/min with the standard AB SCIEX Turbo V™ electrode.

HPLC Conditions:

LC System:	Shimadzu LC-30 Nexera System
Analytical column:	Phenomenex Aeris Peptide XB-C18, 3.6 µ, 2.1 mm x 150 mm, 0.25 mL/min
Mobile Phase A:	Water (0.1 % formic acid)
Mobile Phase B:	Acetonitrile (0.1 % formic acid)
Gradient:	

Time (min)	%B
1.0	3.0
10	50
11	95
13	95
14	3.0

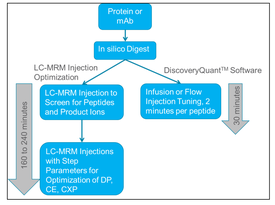

Figure 1. Workflow for peptide optimization by LC-MRM injection or using DiscoveryQuant™ software and infusion.

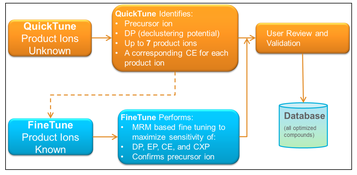

Figure 2. The DiscoveryQuant™ software tuning workflow that utilizes two experiments to achieve maximum sensitivity.

RESULTS

DiscoveryQuant™ Optimize software offers two options for tuning and optimization: QuickTune and FineTune (Figure 2). The QuickTune experiment is used to identify product ions and is comprised of a precursor ion scan, a DP optimization for the precursor and product ion scans at user defined CEs. The product ion masses and an associated CE are then stored in the DiscoveryQuant™ software database. The FineTune experiment can then optimize DP, CE, CXP and EP using MRM transitions loaded from the DiscoveryQuant™ software database for a seamless and automated optimization that provides maximum quantitative sensitivity. The DiscoveryQuant™ software database can also be manually populated with MRM information loaded from an external source like Skyline software (MacCoss Lab Software), which will provide a list of the possible product ions of a given peptide plus an estimate of the DP of the precursor ion and a CE for the product ions. In this way FineTune can be used to optimize DP, CE, CXP and EP without running a QuickTune experiment first.

Both the QuickTune plus FineTune workflow and the FineTune with external data workflow were used to optimize tryptic peptides between 9 and 25 amino acids (excluding cysteine containing sequences). The optimized parameters from both of these workflows were used to create LC-MS/MS methods and the sensitivity of the methods were compared to a LC-MS/MS method constructed using unoptimized parameters from an external data source (Skyline).

E. Coli BGAL (1024 amino acids) was digested *in silico* with trypsin using Skyline and the tryptic peptides selected for optimization are shown in Table 1.

Table 1. List of the *E. Coli* BGAL peptide sequences chosen for optimization.

Peptide	Monoisotopic Mass	Charge State	m/z
IDPNAWVER	1098.546	+2	550.3
TDRPSQQLR	1099.573	+2	550.8
HQQQFFQFR	1264.610	+2	626.8
ELNYGPHQWR	1298.616	+2	633.3
VDEDQPFPAVPK	1340.661	+2	650.3
LWSAEIPNLYR	1360.714	+2	671.3
LPSEFDLSAFLR	1393.724	+2	681.4
APLDNDIGVSEATR	1456.716	+2	697.9
QSGFLSQMWIGDK	1495.713	+2	729.4
YSQQQLMETSHR	1506.689	+2	748.9
LSGQTIEVTSEYLFR	1741.889	+2	754.4
VNWLGLGPQENYPDR	1756.853	+2	872.0
IENGLLLLNGKPLLIR	1775.103	+2	879.4
WSDGSYLEDQDMWR	1786.726	+2	888.6
LQGGFVWDWVDQSLIK	1889.968	+2	894.4
DVSLLHKPTTQISDFHVATR	2264.191	+2	946.0
YGLYVVDEANIETHGMVPMNR	2407.130	+2	1133.1
YDENGNPWSAYGGDFGDTPNDR	2445.973	+2	1204.6

The Skyline information (product ion masses, DP and CE) was imported into the DiscoveryQuant™ software database. A FineTune experiment was then used to optimize: the DP between 5 and 150 V, the CE between ±20V of the Skyline assigned CE and the CXP between 2 to 30V. EP was not optimized and was kept at 10 V. Total infusion time for each peptide was 1.0 minute. At a flow rate of 2.0 µL/min ~40 µL of sample was consumed or approximately 2.3 µg of protein. An example of the Optimize FineTune data (DP, CE) for peptide APLDNDIGVSEATR is shown in Figure 3 (left panel). The Skyline CE for the product ions of this peptide was 35 V and the actual optimized CE was between 41 and 45 V for the 5 product ions. The CXP ramping data is shown below in Figure 3 (right panel).

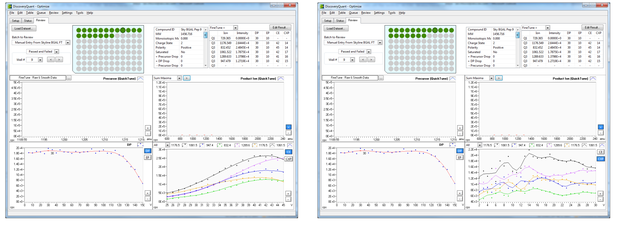

Figure 3. DP, CE and CXP optimization data from the peptide APLDNDIGVSEATR using DiscoveryQuant™ software FineTune.

The QuickTune feature in DiscoveryQuant™ software includes a product ion scan. Since not all peptide product ions can be described by a, b, c or x, y, z ion types the feature was used to analyze the BGAL digest for peptide product ions that are not supported in Skyline. In this workflow, only the peptide sequences need to be entered into the DiscoveryQuant™ software batch setup table. The QuickTune settings were set to scan for product ions from 700 amu up to the mass of the singly charged precursor ion using collision energies of 15, 25, 35, 45 and 55 V. In addition to the product ion scans a DP ramp and an enhanced resolution precursor ion scan were performed. The data from peptide QSGFLSQMWIGDK are displayed in figure 4.

Figure 4. QuickTune results and product ion spectrum for peptide QSGFLSQMWIGDK.

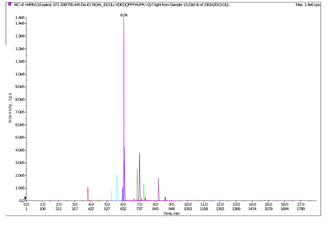

Figure 5. LC-MS/MS chromatogram of *E. coli* BGAL digest using an MRM method created from the DiscoveryQuant™ software database.

DiscoveryQuant™ Analyze software was used to build an LC-MRM method. Only the MRM of the most intense precursor/product ion pair was selected from the database. The 0.10 pmol/µL sample was analyzed with this method and peak areas were compared to peak areas generated from the Skyline MRM method. The data in Table 2 shows the changes in peak area and signal to noise from the LC-MRM method generated using DiscoveryQuant™ FineTune compared to the Skyline MRM method. A representative chromatogram of the protein digest is shown in Figure 5.

Peptide	Q1/Q3	Retention Time (min)	0.10 pmol/µL BGAL Avg. Area Gain (N=3)	0.10 pmol/µL BGAL Avg. S/N Gain (N=3)
DVSLLHKPTTQISDFHVATR	1133.1 / 1472.7	4.78	308%	296%
YGLYVVDEANIETHGMVPMNR	1204.6 / 1713.8	6.88	164%	157%
TDRPSQQLR	550.8 / 728.4	6.68	153%	147%
IENGLLLLNGKPLLIR	888.6 / 1023.7	6.89	91%	86%
APLDNDIGVSEATR	729.4 / 832.5	7.02	53%	55%
IDPNAWVER	550.3 / 660.3	6.04	49%	46%
YDENGNPWSAYGGDFGDTPNDR	1224.0 / 1754.7	3.81	48%	49%
YSQQQLMETSHR	754.4 / 760.3	7.37	31%	33%
LQGGFVWDWVDQSLIK	946.0 / 1289.7	7.15	31%	34%
ELNYGPHQWR	650.3 / 780.4	7.28	16%	13%
LPSEFDLSAFLR	697.9 / 1184.6	8.61	10%	11%
VDEDQPFPAVPK	671.3 / 755.4	8.20	9%	11%
WSDGSYLEDQDMWR	894.4 / 979.4	7.37	4%	4%
QSGFLSQMWIGDK	748.9 / 964.5	6.08	1%	0%
QSGFLSQMWIGDK	748.9 / 740.1	7.37	100%	105%
VNWLGLGPQENYPDR	879.4 / 1075.5	5.99	0%	2%
HQQQFFQFR	633.3 / 1000.5	5.30	-1%	1%
LWSAEIPNLYR	681.4 / 1062.6	5.98	-3%	-4%
LSGQTIEVTSEYLFR	872.0 / 1143.6	5.62	-10%	-10%

Table 2. Changes in peak area and signal to noise ratio of peptides that were optimized with DiscoveryQuant™ software compared to un-optimized mass dependent parameters from Skyline. The row highlighted grey contains data from an MRM pair selected from the product ion spectrum in figure 4.

The product ion spectrum of Figure 4 was visualized with PeakView® 2.0 software using Bio Tool Kit and ion 740.1 was not assigned to either a, b, c or x, y, z ion types. The product ion was included in an LC-MRM method and used to analyze the same 0.10 pmol/µL BGAL digest. The peak area and signal to noise ratio of the 748.9/740.1 pair was 2.02 fold greater that the 748.9/964.5 pair. In addition to maximizing the sensitivity of product ion types supported by Skyline, DiscoveryQuant™ software can be used to increase the sensitivity of target peptides by identifying product ions not of the a, b, c or x, y, z type.

CONCLUSIONS

1. Optimizing the mass dependent parameters with DiscoveryQuant™ software for transitions generated by Skyline increases the peak area and signal to noise ratio of the majority of peptides from a protein digest.
2. Optimizing peptides with DiscoveryQuant™ software using infusion is fast, at 1 min per peptide, while requiring little sample, ~2 µL per peptide.
3. The QuickTune feature of DiscoveryQuant™ allows for the identification of product ions not of the traditional a, b, c or x, y, z ion types which can boost sensitivity for certain peptides.

TRADEMARKS/LICENSING