# Signature Peptide MRM Optimization Made Easy for Therapeutic Protein and Peptide Quantification

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## Key Challenges in MRM Optimization for Therapeutic Protein and Peptide Quantification

1. **Choosing a unique peptide** – A peptide that is unique to the protein of interest within a given background and is also sufficiently sensitive and selective.
2. **Choosing most sensitive MRM for the peptide** – Multiple charge states are possible for a given peptide which in combination with the many product ion possibilities leaves many MRMs to be screened.
3. **Optimizing best MS parameters** – Manual tuning can be tedious, and optimizing via LC injections is time consuming, particularly when monitoring multiple MRMs per peptide and multiple peptides per protein.

## Key Benefits of Using DiscoveryQuant™ Software for Signature Peptide Optimization

1. **Increase productivity with reduced method development time** –infusion or flow-injection based tune with DiscoveryQuant™ software instead of LC with step parameters.
2. **Save time with automated workflow** –Predicted transitions from the Skyline software are validated on a real digest sample quickly. DiscoveryQuant™ software is used to automatically optimize DP, CE, CXP and EP.

## Unique Features of DiscoveryQuant™ Software for Signature Peptide Quantification

1. **Precise MRM optimization** – Optimize and fine tune MRM conditions using real time labeling of y and b ions for extra confirmation. Handle custom amino acid side chain modifications with new peptide editor.
2. **ChromaTune - On-column MRM optimization to eliminate false positives**-Validate MRM's from QuickTune and FineTune on column in matrix
3. **Automatic MRM method generation** – Using data from the database and DiscoveryQuant™ Analyze optimized MRM methods can be generated automatically.
4. **Global database sharing –** The data from multiple systems can be shared enterprise wide through the global DiscoveryQuant™ database connectivity.


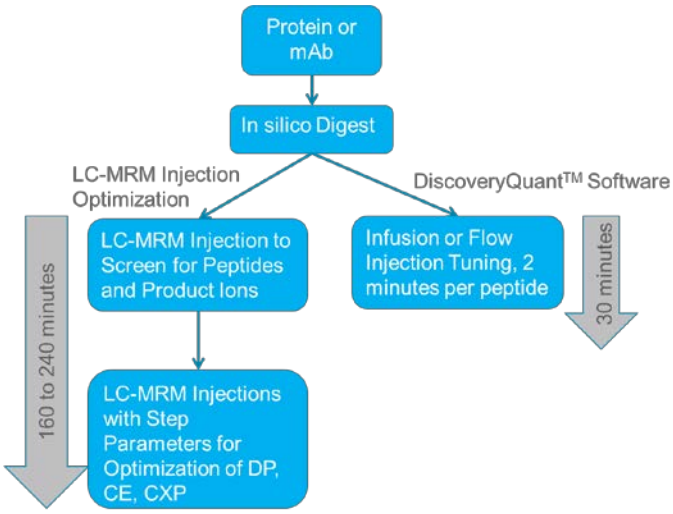


**Figure 1.** Time saving advantages of peptide optimization using DiscoveryQuant™ software.

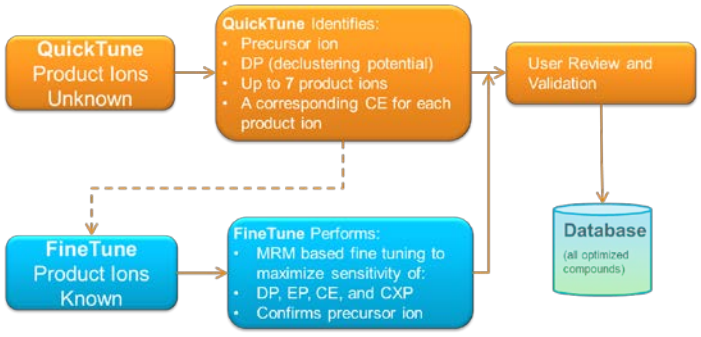


**Figure 2.** Schematic overview of the DiscoveryQuant™ software tuning and optimization workflow.

## Introduction

Protein based therapeutics are a rapidly expanding component of many pharmaceutical companies' drug portfolio. Monoclonal antibodies (mAb) used in the treatment of cancer are one class of protein therapeutics that has achieved success. In addition to proteins, smaller therapeutic peptides have achieved approval for a wide variety of indications in metabolic, cardiovascular and infectious diseases. In order to support this rapidly expanding new class of drug molecules, the rapid development of sensitive and selective bioanalytical methods are required.

Historically, protein and peptide quantification has been done using ligand binding assays (LBA) but LBAs 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 like trypsin followed by direct analysis of one or more of the proteolytic peptides. Unlike bioanalytical method development for small molecules the product ions of a peptide analyte can be predicted using known ion types (a, b, c, x, y, z). An excellent starting point for the development of an LC-MS/MS method for peptides is 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. The next step in the method development is to determine which proteolytic peptides are actually produced by the digestion reaction, and which product ions are actually formed in the collision cell and for a given peptide. Lastly, for the product ions formed the CE and CXP needs to be optimized to achieve maximum sensitivity. DiscoveryQuant™ software is the ideal tool to perform this optimization. DiscoveryQuant™ Optimize software allows for optimization of compound dependent parameters via flow injection or infusion and then populates a database with these parameters.

DiscoveryQuant™ Optimize software offers two options for tuning and optimization: QuickTune and FineTune. 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™ database. The FineTune experiment can then optimize DP, CE, CXP and EP (Figure 2) using MRM transitions loaded from the DiscoveryQuant™ database for a seamless and automated optimization that provides maximum quantitative sensitivity. The DiscoveryQuant™ database can also be manually populated with MRM information loaded from an external source like Skyline. In this way FineTune can be used to optimize DP, CE, CXP and EP without running a QuickTune experiment first.

This technical note describes the results of experiments where DiscoveryQuant™ software was used to optimize compound dependent parameters and improve upon the sensitivity of methods obtained from the output of Skyline software for the quantitation of peptides.

## Materials and Methods

### Sample Preparation

Trypsin digested *E. coli* BGAL from the AB SCIEX mass spectrometer standards kit, Part No. 4368624 was diluted to 0.5 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.

### Data Workflow

The BGAL peptide sequence (UniProt #P00722 ) was pasted into Skyline and in-silico digested with trypsin. Tryptic peptides between 9 and 25 amino acids were selected while excluding cysteine containing sequences. Skyline was setup to export up to six 'y' ions with masses above the doubly charged parent *m/z*. The molecular weight range of the peptides ranged from 1098.55 to 2445.97 Da. Only doubly charge peptides were selected and a list of doubly charged peptides and their 'y' ions was exported as an Analyst method in the .csv format. Using excel, this .csv file was formatted into a table that could be imported to the DQ database and saved as a .txt file.

### LC Conditions

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

Gradient conditions

| Time (min) | Mobile phase A% | Mobile phase B% |
|---|---|---|
| 1.0 | *97* | *3* |
| 10.0 | *50* | *50* |
| 11.0 | *5* | *95* |
| 13.0 | *5* | *95* |
| 14.0 | *97* | *3* |

### MS Conditions

| | |
|---|---|
| MS System | QTRAP® 4500 system with a Turbo V™ Ion Spray Source |
| Ionization Mode | ESI with Positive Mode |

### Software

| | |
|---|---|
| Data acquisition | DiscoveryQuant™ 2.1.2<br>Analyst® 1.6.1 Software |
| Quantitation | MultiQuant™ Software |

## Results and Discussion

*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.

| 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 |

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

Skyline software assigned DPs in the range of 71 to 120 V for the precursor ions of Table 1 and CEs in the range of 27 to 66V for the 6 product 'y' ions of each precursor. These values were used to construct an LC-MRM method that was used to analyze a sample of the BGAL tryptic digest (0.10 pmol/µL). The LC peak areas for each peptide MRM were calculated and used for comparison.

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. 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.

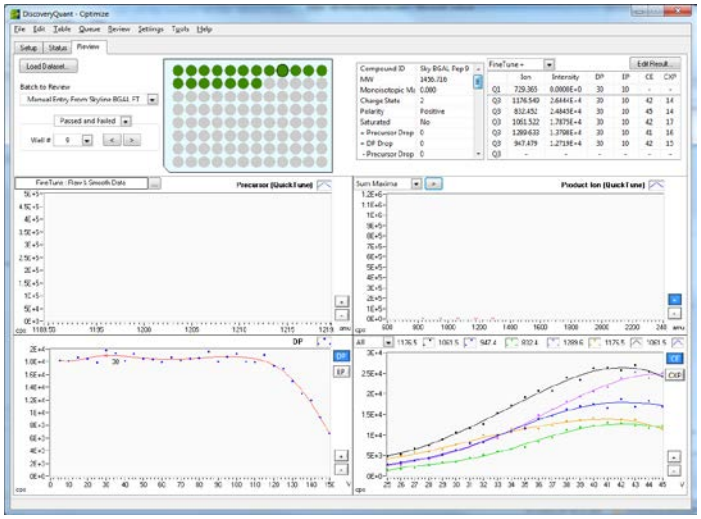

**Figure 3.** DP and CE optimization data from the peptide APLDNDIGVSEATR using DiscoveryQuant™ FineTune.

The CXP ramping data is shown below in Figure 4.

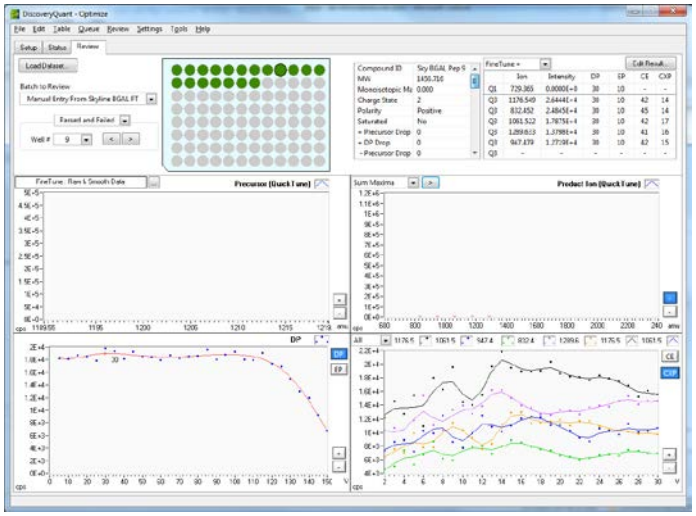

**Figure 4.** DP and CXP optimization data from the peptide APLDNDIGVSEATR using DiscoveryQuant™ FineTune.

DiscoveryQuant™ software ranks product ions in the database based on intensity of the CE ramping experiments. Therefore, the most intense product ion is known prior to starting

LC-MS/MS analysis. In the absence of library matching spectra this information is not known using Skyline alone. With the intensity of the product ions determined by the DiscoveryQuant™ software an LC-MS/MS method can be made for the digest sample including only the most intense MRMs for each peptide. This decreases the overall cycle time of the method and allows an increase in dwell time to improve S/N for each transition.

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.

| Peptide | Q1/Q3 | Retention Time (min) | 0.10 pmol/µL BGAL | |
|---|---|---|---|---|
| | | | Avg. Area Gain (N=3) | 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% |
| 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.

Of the 18 peptides tested 11 showed an increase in signal to noise ratio and peak area >10% and 7 were unchanged (±10%). The average gain for the 10 peptides showing improvement was 94%. Calibration standards of peptides IDPNAWVER and APLDNDIGVSEATR were prepared (figure 5) and a partial calibration curve constructed over 3 orders of magnitude. Both the signal to noise and peak area gains was consistent across all standards.

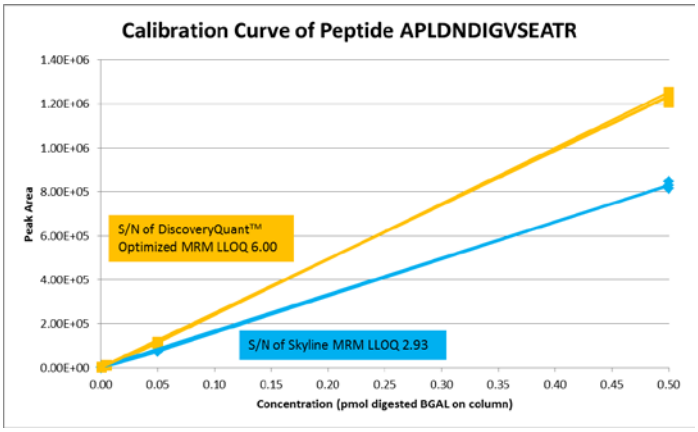


**Figure 5.** Partial calibration curves of peptide APLDNDIGVSEATR. Maroon squares represent data from the DiscoveryQuant™ software optimized LC-MRM method and blue diamonds represent data from the Skyline MRM method.

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 6.

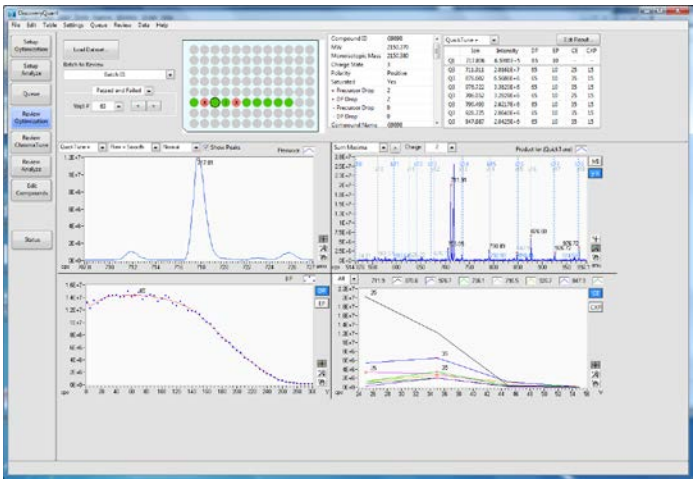

**Figure 6.** QuickTune results and product ion spectrum with b and y ion labeling for easy data review

The most intense product ion in the product ion mass window is 740.1 amu. The product ion spectrum was visualized with PeakView® 2.0 software using Biotools 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.

| Peptide | Q1/Q3 | Retention Time (min) | 0.10 pmol/µL BGAL | |
|---|---|---|---|---|
| | | | Avg. Peak Area (N=3) | Avg. S/N (N=3) |
| QSGFLSQMWIGDK | 748.9 / 964.5 | 7.37 | 4.30E+04 | 3.65E+02 |
| QSGFLSQMWIGDK | 748.9 / 740.1 | 7.37 | 8.59E+04 | 7.48E+02 |
| | | **Gain** | **2.00** | **2.05** |

**Table 3.** Gains in peak area and signal to noise ratio for peptide QSGFLSQMWIGDK based on a product ion pair identified using QuickTune.

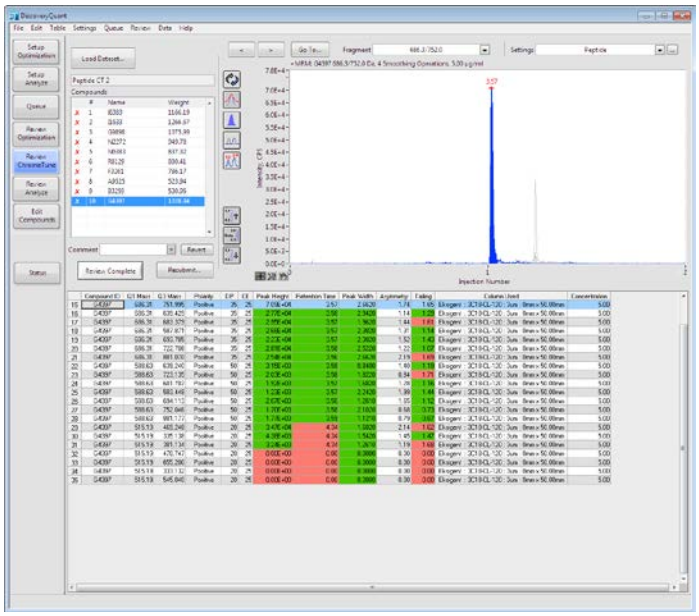

**Figure 7:** ChromaTune - On-column MRM optimization to eliminate false positives

New ChromaTune on column MRM optimization helps validate MRM's from QuickTune and FineTune on column. Screen compounds based on chromatographic properties such as sensitivity, retention time, peak width and peak tailing. Performs linear response experiments across multiple plates. Comprehensive compound summary panel for quick and easy review

## 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. Increase confidence by on column optimization using new ChromaTune feature



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