Development of Quality Assurance/Quality Control Specifications for Robust SRM-Based Assays: Increasing Reproducibility from Laboratory to Laboratory

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Overview

Purpose: To establish a protocol to relate experimental data across instrument and laboratories for targeted protein quantitation.

Methods: Incorporation of a well-characterized peptide set that is used to train method set up for SRM acquisition by predicting retention times as a function of hydrophobicity factors.

Results: Successful detection, verification, and quantitation of 220 peptides in one experiment was accomplished using the predicted retention times based on the peptide trainer set. The assay was built from data acquired using a 90-minute gradient on an LTQ Orbitrap hybrid mass spectrometer while only a 25-minute gradient was used on a TSQ Quantum Ultra triple quadrupole mass spectrometer.

Introduction

The use of selected reaction monitoring (SRM) assays on a triple quadrupole mass spectrometer to perform targeted peptide quantitation has become the standard method in biomarker verification and validation. The benefits of using SRM assays on a triple quadrupole include increased sensitivity and selectivity in the presence of the biological matrix and an increased capability of multiplexing protein quantitation through the use of large SRM tables. To increase the effectiveness of setting up targeted SRM assays for multiplexing protein quantitation and producing reproducible data across laboratories, optimization of the mass spectrometer and HPLC system is mandatory. We propose the incorporation of a set of synthetic peptides into the method development pipeline. The peptide trainer set can be used to establish optimization protocols and standardize both the mass spectrometer via direct infusion as well as the HPLC for system performance. In addition, the selection of the proper trainer set can be used to predict retention times for targeted peptides cutting down the verification steps required for method refinement.[1-4]

Methods

Samples:

A set of 9 Thermo Scientific HeavyPeptide labeled peptides were pooled together and used as a peptide trainer set.[3] The peptides were selected based on *m/z* values, most abundant precursor charge state and hydrophobicity factors. A yeast cell lysate was reduced, alkylated, and digested resulting in a stock solution of 2 µg/µL. The sample was made by diluting yeast cell lysate digest into the heavy labeled peptides for a final concentration of 200 ng/µL yeast lysate and 80 fmol/µL of the heavy labeled peptide solution. A total of 5 µL were injected per analysis.

Data Acquisition:

Discovery: Initial experiments were performed on a Thermo Scientific LTQ Orbitrap XL hybrid mass spectrometer in a standard data dependent/dynamic exclusion analysis. The one sample was acquired in 4 technical replicates for statistical analysis to generate the list of proteins and peptides, product ion information and retention time information. A binary mobile phase of A) 0.1% formic acid B) MeCN (0.1% formic acid) on a packed capillary column 150 x 0.075 mm was used for the discovery phase.

Verification/Quantitation: Verification/quantitation experiments were performed on Thermo Scientific TSQ Quantum Ultra triple quadrupole that had been upgraded to facilitate iSRM applications and over 1000 SRM transitions in one experiment. Initial experiments used to characterize the LC-MS/MS via the trainer set peptide kit was done using a 20 msec dwell time and 4 SRM transitions per trainer peptide to determine the relationship between hydrophobicity factors and retention time. A plot of the results are show in Figure 1. All experiments on the TSQ used the same mobile phase as that described above and separations were done using a Thermo Scientific Hypersil Gold 250 x 1 mm column (5 µm particle size) at a flow rate of 200 µL/min and a 1.5% per minute gradient (8 → 45% B).

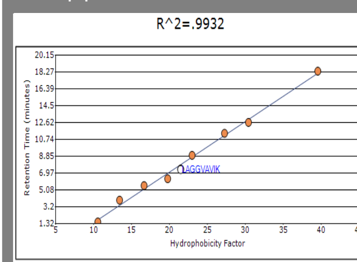
The hydrophobicity factors for all peptides (targeted and trainer set) were determined using the SSRCalc algorithm (http://hs2.proteome.ca/SSRCalc/SSRCalc.html)[1] to help establish expected RT as a function of hydrophobicity factor. The trainer set was used to establish the linear equation used to predict the RT for all yeast digest peptides and set the detection window for timed SRM (T-SRM) experiments to help establish expected RT as a function of hydrophobicity factor. The trainer set was used to establish the linear equation used to predict the RT for all yeast digest peptides and set the detection window for timed SRM (T-SRM) experiments

Data Processing:

LTQ Orbitrap XL™ discovery data was processed using Thermo Scientific SIEVE 1.2 software that produced a list of confidently identified proteins, peptides, and transition information that was used for the SRM assays loaded into the TSQ Quantum Ultra™.

Thermo Scientific Pinpoint software was used to read in the SIEVE™ experimental results that were used to build the resulting SRM assays to target all possible peptides from 20 yeast proteins. The TSQ Quantum Ultra data was also brought back into Pinpoint™ for processing.

FIGURE 1: Establishing the relationship between calculated hydrophobicity factors and experimentally determined retention time for the set of heavy labeled peptides used as a trainer set.



Results

The workflow adopted by most laboratories performing targeted protein quantitation is to utilize the abundance of shotgun proteomics data to help determine the peptides and SRM transition information used to build SRM assays. The primary limit is transferring the method from one lab to another or across instruments. To help address this, we have used a unique set of heavy labeled peptides that contain desired physiochemical properties to help create a test mixture to compare triple quadrupole and HPLC performance as well as predicting elution times for T-SRM method development and verifying targeted peptide detection by bracketing the measured elution time with the trainer set landmarks. The first step is to ensure the trainer set selected satisfies the necessary criteria of ionization and chromatographic behavior. The peptides were selected based on the calculated hydrophobicity factors that range between 10 and 40 which covers most digested peptides. Figure 1 shows the experimental data obtained for the trainer set spiked into yeast cell lysate digest. Of primary interest is the trainer peptide with the fastest elution time under the experimental set up used for the study. The second aspect is chromatographic reproducibility for the trainer set peptides. Figure 2 shows the observed retention times for each of the 9 peptides for 12 technical replicates. Each of the trainer set peptides have a standard deviation less than 3 seconds giving confidence to the experimental set up. In addition, the ion ratio calculations are readily available in Pinpoint for additional verification. Figure 3 shows an exported figure from Pinpoint showing the ion ratio calculations and automatic peak integration performed for the trainer peptide DGYLFQLLR* for the technical replicates.

Using the trainer set, the equation to relate retention time and hydrophobicity factor can be determined by 1 experiment. The results from the first experiment can also be used to evaluate triple quadrupole performance based on peak shapes, integrated area, RT, and ion ratios prior to performing a targeted peptide quantitation assay. Following the characterization of a yeast cell lysate digest. Twenty proteins identified from a shotgun proteomics study on the LTQ Orbitrap XL were imported into Pinpoint and a method was created to identify the best peptides and transitions. The resulting SRM list contained 301 peptides and 2107 SRM transitions with seven SRM transitions per peptide for quantitation and verification. The predicted retention time was calculated based on the trainer set response and programmed for a timed detection window of ±3 minutes.. Pinpoint software broke the list down into 4 different experiments containing ca., 75 peptides and 526 transitions.

FIGURE 2: Evaluation of the retention time stability for the 9 heavy labeled trainer peptides spiked into the yeast cell lysate digest.

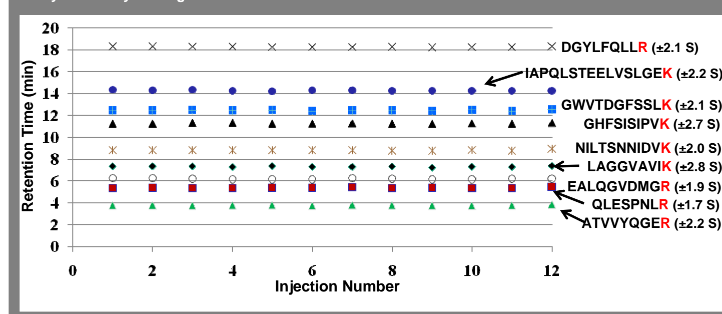


FIGURE 3: Evaluation of intensity measurements, peak shapes, and ion ratio calculations for the targeted peptide DGYLFQLLR as a function of technical replicates. The data was processed using Pinpoint software.

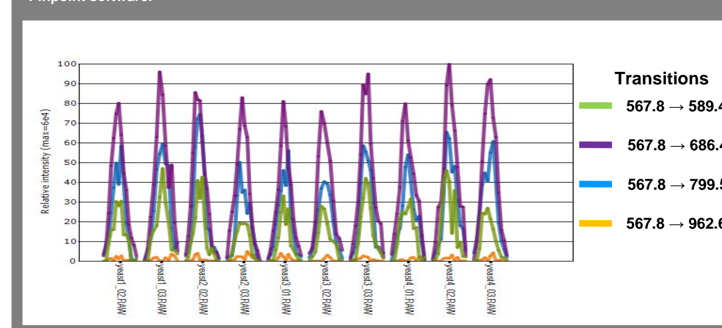


Figure 4 shows the results from the 4 experiments used to probe the 20 selected proteins from the yeast cell lysate. Four trainer peptides covering retention time windows were used to align and combine the data in Pinpoint. The reproducibility enabled the trainer set to be used as a retention time bracket used to help verify the targeted peptide detection.

FIGURE 4: Use of four trainer peptides to compare the instrument performance across the four experimental methods used to probe the yeast cell lysate digest. The symbols are taken from Figure 2 to represent the specified trainer set peptide sequence.

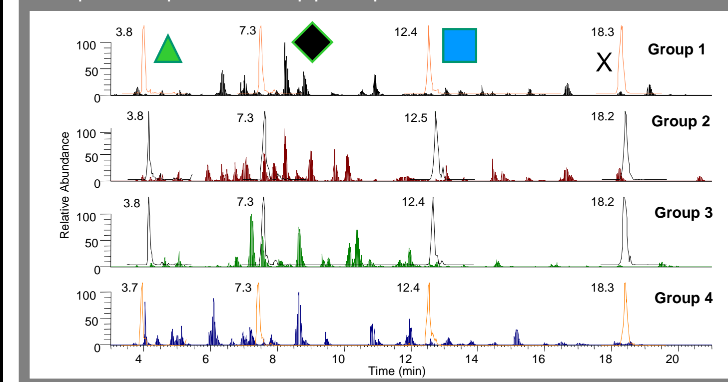
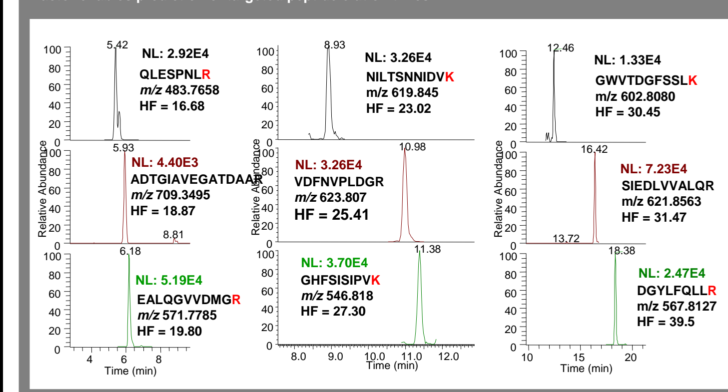


Figure 5 shows three different examples of confirming targeted peptide detection using two trainer set landmarks to bracket the retention time. Since the initial experiment incorporated wide detection windows (±3 minutes for a 25 minute gradient) retention time confirmation was needed to verify targeted peptide detection. The results from the initial 4 experiments were processed using Pinpoint and the best peptides and SRM transitions for each protein were moved into a refined instrument method for a collective assay containing 209 peptides and the 5 best SRM transitions for a total of 1207 transition to be acquired in a 25 minute gradient. The acquisition of the 5 SRM transitions per peptide enabled spectral matching and evaluation of the probability scoring. The new method was created automatically in Pinpoint and reduced the acquisition time windows down from ±3 minutes to ±1.0 minutes to accommodate the increased number of peptides and transitions as compared to the first round of experiments. The detection time windows were centered around the experimentally determined RT values.

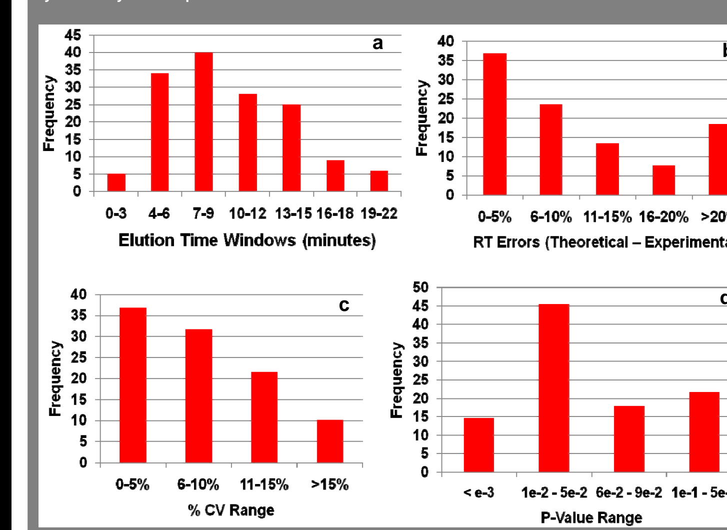
Figure 6 contains the processed results from Pinpoint for the 209 targeted yeast peptides. Despite a short chromatographic gradient, all 209 peptides were detected and verified by bracketing by landmark peptides, CV analysis, and probability scoring against the spectral library entry.. About 75% of the targeted peptides had retention times within 15% of the

FIGURE 5: Using the trainer set peptides as retention time landmarks to bracket targeted peptides for detection verification. The use of the linear response of retention time as a function of hydrophobicity factor enables prediction of targeted peptide elution times.



predicted times indicating tighter windows could be used (between 0.9 and 1.5 minutes) for the discovery experiment. The confidence in using the trainer set to predict targeted peptide retention times results in a greater number of candidates to be screened effectively per experiment. Figures 6c and 6d show the confidence in peptide measurements with greater than 75% of the peptides having a %CV less than 15% while maintaining verification through spectral matching of 5 SRM transitions.

FIGURE 6: Summary of experimental parameters determined for the 201 targeted peptides from the yeast cell lysate sample.



Conclusions

The use of a well-characterize peptide trainer set is essential to transferring SRM assays across instrument platforms as well as laboratories. Synthesizing peptide sequences enables control of the ionization, dissociation, and elution properties that provide a means to assess the performance and normalize biological samples. The use of trainer set peptides enables a dramatic reduction in method development time for large targeted protein quantitation assays. Some of the key benefits of using peptide trainer sets include:

1. Determination of the linear relationship between experimental retention time and calculated hydrophobicity factors.
2. Evaluation of the chromatographic reproducibility and peak shapes across the LC gradient used for the experiment.
3. Spiking the peptide trainer set in the sample for the LTQ Orbitrap XL discovery experiments to establish the elution relationships that can be carried over to SRM assays.
4. Evaluating collisional activation performance via ion ratio analysis of the targeted peptide trainer kit.
5. Confirming targeted peptide identity by bracketing experimentally determined retention times between neighboring trainer set peptides.

References

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2. A. A. Klammer et al. Modeling peptide fragmentation with dynamic Bayseian networks for peptide identification: *Bioinformatics*, 2008, *24(13)*, i348-56
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4. A. Prakash et al. Expediting the development of targeted SRM assays: using data from shotgun proteomics to automate method development, *J. Proteome Research*, on-line publication March 27, 2009, 10.1021/pr801028b

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