Improving Throughput for Targeted Quantification Methods by Intelligent Acquisition

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Overview

Automated remote multiplexed targeted protein quantification utilizing real-time qual/quan processing for increased quantitative accuracy over large dynamic ranges.

Introduction

Targeted quantification has become a very popular technique to verify putative biomarker candidates in large clinical cohorts of samples. These candidates are usually generated following a biomarker discovery experiment or derived from a biological hypothesis, for example, a pathway or biophysical interaction. These lists are usually large, containing upwards of 100–1000 proteins spanning several orders of magnitude dynamic concentration range. This presents analytical challenges for conventional SRM assays both in terms of method development and throughput. We propose using high-resolution, accurate-mass (HRAM) mass spectrometry (MS) and MS/MS schemes in conjunction with validated spectral libraries for automated method building, data acquisition, verification, and quantification in real-time using novel acquisition schemes.

Methods

K562 colon carcinoma cells were grown in heavy and light media, collected and mixed at different ratios to cover a 20-fold dynamic range. All samples were digested and analyzed on a Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap mass spectrometer equipped with a nanospray ion source. Data was acquired in two steps to simulate traditional workflows. Initial experiments employed unbiased data-dependent MS/MS acquisition resulting in peptide/protein identification as well as building of a spectral library. The spectral library contains relative retention time, precursor charge state distribution, and product ion distributions, creating a unique verification/quantification scheme. A highly multiplexed, targeted protein list was created from the spectral library and used for automated data acquisition and processing real time to facilitate changes to the acquisition scheme.

The scheme in Figure 1 describes the methodology in more detail. The first step is to characterize the LCMS parameters using the Thermo Scientific™ Pierce™ PRTC Mixture Kit. The next step is to build a list of proteins that we are interested in. This will typically come from a pathway study or a discovery experiment. The next is to build a spectral library for this list of proteins. This can be built via predictive algorithm or empirical observations. This turns into a spectral library lookup table. The look-up table includes the precursor *m/z* values for the defined charge state as well as the expected retention time window, which are used to initiate product ion spectral acquisition based on the presence of multiple precursor isotopes during the expected elution window. Once the signal for multiple precursor isotopes surpasses the user-defined intensity threshold, a higher-energy collision dissociation (HCD) spectrum is acquired and immediately compared against the spectral library generating a dot-product correlation coefficient to determine spectral overlap and to check if the targeted peptide has been detected previously. If the calculated correlation coefficient surpasses the user-defined acceptance value, HCD product ion spectra will continue to be acquired across the elution profile. This is shown in Figure 2.

FIGURE 1. Strategy for large-scale targeted quantification based on high IQ data acquisition scheme
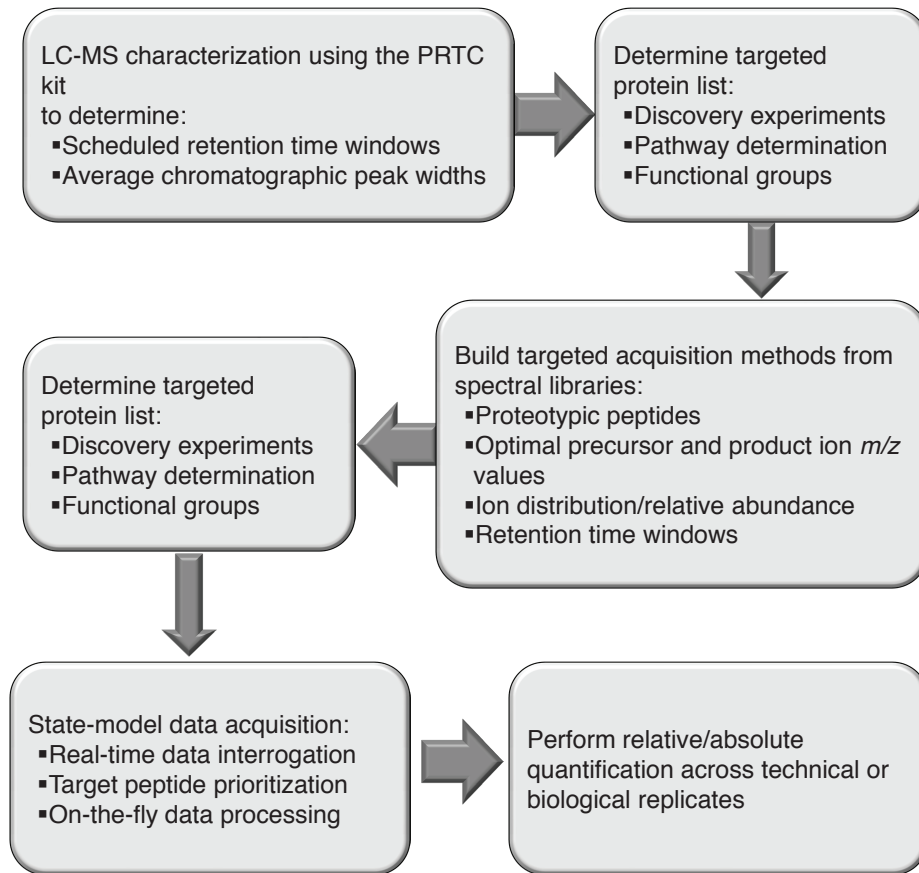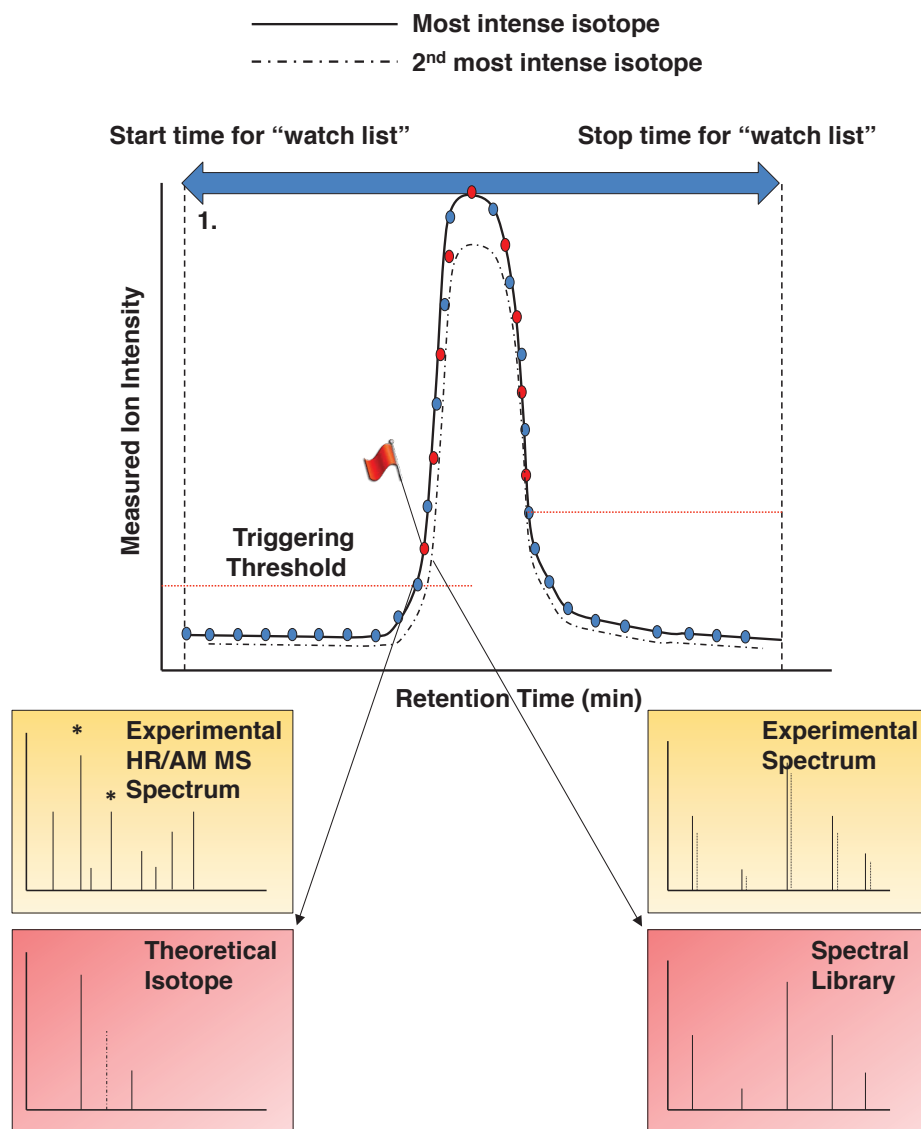
Scheme

FIGURE 2. Pictorial representation of high IQ data acquisition schemes for targeted peptide quantification using a targeted scanning window, target elution identification, and real-time product ion spectral acquisition. Both precursor and production spectral matching is performed to increase the analytical selectivity of data acquisition.

Results

Highly multiplexed targeted protein quantification requires significant steps of method refinement prior to implementation. While the determination of proteins is relatively straightforward based on biology, the selection of peptides as surrogate biomarkers and corresponding *m/z* values (precursor and product ions) used to uniquely identify and quantitate the peptide targets becomes challenging. Generally, retention times and acquisition windows must be determined to maximize instrument cycle time to achieve robust quantification. To expedite complex experimental method development, we have created a unique spectral library procedure based on an analytically rigorous discovery data acquisition scheme. The local spectral library contains both LC and MS information that can be readily enlisted to build robust methods requiring few refinement steps.

To first test our methods, a protein mix was spiked in equine plasma (containing PTRC kit). Spectral library was first built on the neat protein mixture. Experiments performed on the quadrupole Orbitrap mass spectrometer facilitate unique product ion collection and detection schemes to not only increase data acquisition, but perform state-model data acquisition, increasing the ability for quantification. Figure 3 shows the CV distribution for the peptides over four acquisitions (by summing the area of top eight product ions).

K562 Cell Line

2,100 proteins were selected from the K562 cell line and imported into the new algorithm. The algorithm utilizes the spectral library information to select unique peptides and create precursor and product ion information used to perform real-time qualitative and quantitative analysis. In total, 3,800 peptides were chosen and 20-fold range digest was created.

Figure 4 shows an example where the ratio of 1:10 could not be calculated using the full-scan MS1 (panel A), but could be calculated in tandem MS/MS scan (panel B, and zoom-in, panel C).

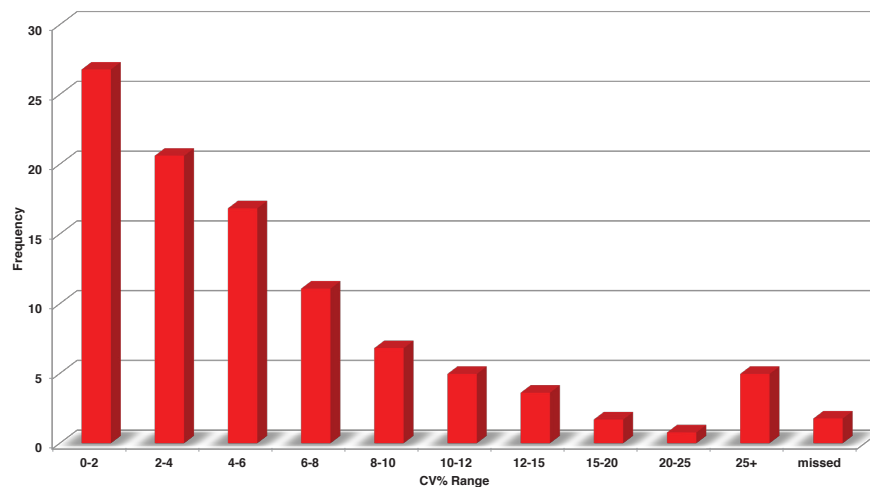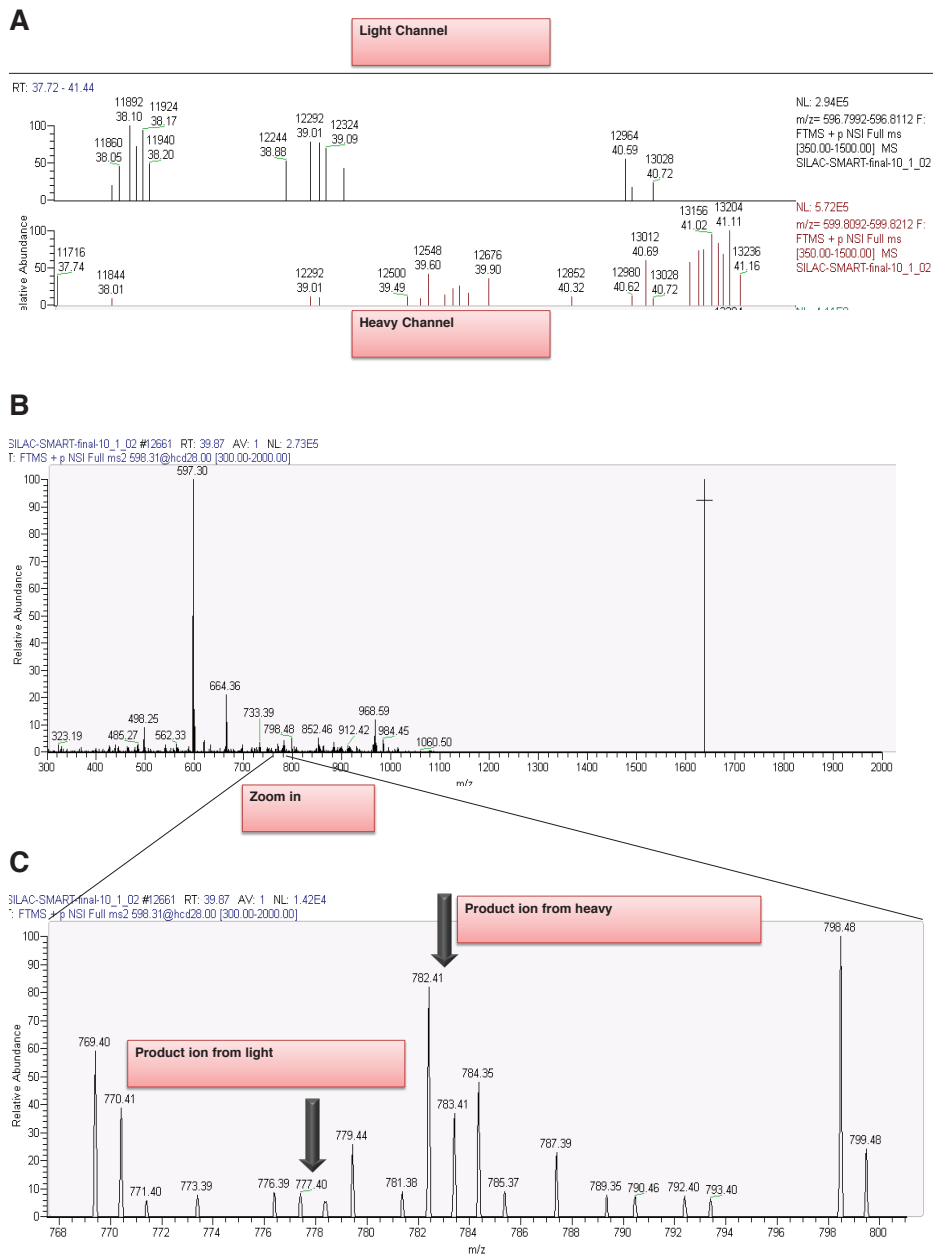**FIGURE 3. CV distribution for the initial peptide list.**

FIGURE 4. The benefit of MS/MS scan (with higher S/N) compared to full scan. Ratio of 1:10 could not be calculated in full scan (panel A), but it could be calculated in tandem MS/MS scan (panel C).

Conclusion

The developments here resulted in the successful qualitative/quantitative analysis for over 3,000 peptides representing over 2,000 proteins in this complex leukemia cell digest. Successful quantification was determined for proteins spiked at over a 20-fold range and the ability to change instrument acquisition parameters for increased analytical sensitivity.

References

1. Peptide retention standards and hydrophobicity indexes in reversed-phase high-performance liquid chromatography of peptides. Krokhin et al., Analytical Chemistry **2009**.

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