Application Note: 554

# HR/AM Targeted Peptide Quantitation on a Q Exactive MS: A Unique Combination of High Selectivity, Sensitivity and Throughput

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**Key Words**

- Q Exactive
- Targeted peptide quantitation
- msx tSIM
- High resolution, accurate mass

## Q Exactive HR/AM targeted peptide quantitation workflow at a glance:

- A two-in-one qual-quan instrument
- Four orders of quantitative dynamic range
- Low-attomole detection limits
- Ultra-high selectivity with 140K mass resolution and sub-ppm mass accuracy
- Increased throughput and quantitative precision offered by unique multiplexed SIM scan function

## Introduction

Mass spectrometry has become an essential tool for understanding aspects of biological systems such as proteome dynamics and signaling regulation.[1] When coupled with multi-dimensional liquid chromatography, modern mass spectrometers, such as the Thermo Scientific LTQ Orbitrap Velos hybrid ion trap-Orbitrap MS, are capable of identifying thousands of peptides and post-translation modifications per hour on a routine basis. Relative quantitation, through a label-free approach or via incorporation of heavy isotopes such as SILAC[2] or isobaric tagging (TMT)[3], enables the identification of candidates displaying biologically interesting dynamics on a global scale in the early discovery phase. It is now common to find several hundred biomarker candidates, including many novel ones, in a complex biological system. Putative biomarkers can also be generated through hypothesis or via literature knowledge.

Putative biomarkers need to be quantitatively analyzed across large numbers of samples from multiple biological sources and conditions, either for understanding of signaling regulation or for verification and selection of final biomarkers. A fast, robust and cost-effective platform is required at this verification stage. The presence of large numbers of diverse candidates makes it impractical to use traditional antibody-based immunoassays. A targeted MS approach, in particular selected reaction monitoring (SRM), has become the preferred platform for quantitative analysis of tens to hundreds of peptide candidates.[4,5] Detection of target proteins at low micrograms per liter has been reported from depleted human plasma using SRM.[6,7]

SRM approaches on triple-quadrupole mass spectrometers can be very cost-effective, have higher selectivity, and be easier to develop than traditional ELISAs, yet several major challenges remain:

- The balance between duty cycle and sensitivity limits the number of peptide targets that can be analyzed in a single experiment. This problem can be partially solved by incorporating retention time windows.
- The low resolution of quadrupoles limits their ability to distinguish targets from complex backgrounds. As a result, the detection limit is often in the low milligrams per liter in untreated plasma samples.
- The development of an SRM-based assay for a given protein is time consuming. It has been estimated that only ~100 such assays could be developed over a year even in an expert laboratory.[8]
- Some peptide targets, especially those with higher charge states, higher mass, or modifications, do not fragment well and are therefore difficult to analyze by SRM. This poses a major problem for many functional-oriented analyses because there are no alternative peptide targets to select.

High-resolution, accurate-mass (HR/AM) MS methods provide an attractive alternative for targeted peptide analyses.[8] High resolution on an Orbitrap™-based mass spectrometer can resolve peptide species differing in mass by as little as 5 ppm, providing high selectivity. The combination of accurate mass, isotope pattern recognition and elution time provides confident confirmation of targets in complex mixtures. The application of an HR/AM approach has been explored recently as an efficient way to reduce initial candidates to smaller lists for further quantitative assay development.[9] Two recent studies using Thermo Scientific LTQ Orbitrap Velos and Orbitrap Elite hybrid mass spectrometers presented a targeted workflow of simultaneous quantitation and identity verification of candidate peptides with reported LODs at the low amol level.[10, 11]

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In this study, the performance of a newly developed Thermo Scientific Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Figure 1) was evaluated for targeted protein quantitation. In addition to common attributes of an Orbitrap mass analyzer such as high mass accuracy and high intrascan dynamic range, several major improvements have been made on the Q Exactive™ MS when compared to an Exactive MS:

- Incorporation of the S-lens greatly increases ion flux, boosting sensitivity
- Deployment of a quadrupole mass filter between the source and the C-trap enables precursor selection for SIM or MS/MS. The exclusion of ions outside of the targeted mass range leads to an enrichment of the ions within the targeted range, which directly leads to lower LOQs in both the full and SIM scans.
- A new advanced signal-processing algorithm processes Orbitrap data on the fly resulting in effective resolving power of up to 140,000 FWHM at *m/z* 200.
- Faster scan speed, up to 12 MS/MS events per second, allows in-depth analysis of complex peptide mixtures.
- Precursor isolation and accumulation is concurrent with Orbitrap scanning, which greatly improves duty cycle.
- A newly developed multiplexing scan function allows isolation of multiple precursors prior to Orbitrap detection for either MS or MS/MS spectra, which greatly improves throughput and LOD/LOQ of complex biological samples.

Here we describe a targeted HR/AM peptide quantitation workflow on a Q Exactive mass spectrometer. Peptide targets present in complex mixtures were quantified using multiplexed selected ion monitoring (msx-SIM). A detection limit of 10 amol was achieved. Dynamic range was observed to be linear across 4 orders of magnitude.

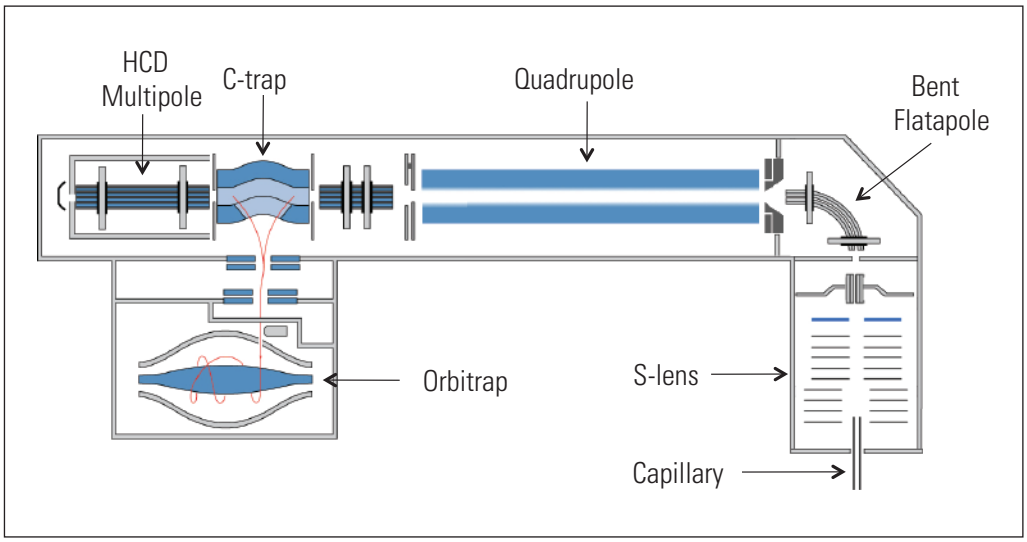


Figure 1. Q Exactive instrument configuration

## Experimental

### Sample Preparation

Thermo Scientific Pierce Peptide Retention Time Calibration Mixture (heavy isotope labeled peptide retention standards) was spiked into either 5 ng/µL or 500 ng/µL yeast whole-cell tryptic digest (kindly provided by Dr. Steven Gygi at Harvard Medical School) to reach final concentrations of 0, 5 amol/µL, 25 amol/µL, 50 amol/µL, 500 amol/µL, 5 fmol/µL, and 50 fmol/µL.

### Liquid Chromatography

One hundred fmol of heavy retention peptide standards in 10 ng or 1 µg of yeast tryptic digest were loaded onto a Thermo Scientific Acclaim PepMap C18 column (75-µm ID x 15 cm, 5-µm particle size). The peptides were separated with a 1-hr gradient (Table 1) at a flow rate of 300 nL/min. The retention times of the heavy peptide standards were extracted and used to set target monitoring windows. The column was then thoroughly cleaned with 10 blank runs using a 2-µL injection of 30% MeCN, 30% isopropanol, 0.1% formic acid.

Table 1. Gradient table

| Time (min) | Duration (min) | %B* |
|---|---|---|
| 0 | 0 | 5 |
| 2 | 2 | 5 |
| 42 | 40 | 30 |
| 44 | 2 | 95 |
| 46 | 2 | 95 |
| 47 | 1 | 5 |
| 53 | 6 | 95 |
| 54 | 1 | 95 |
| 55 | 1 | 5 |
| 65 | 10 | 5 |

*B: acetonitrile with 0.1% formic acid

### Mass Spectrometry

A method that consisted of full scans and targeted multiplexed SIM scans was generated. Full scans were acquired with AGC target value of 1E6, resolution of 70,000 FWHM at 200 *m/z*, and maximum ion injection time (IT) of 100 ms. Each target was monitored with a 4-minute window (retention time ± 2 min), 4-amu isolation window (target *m/z* ± 2 amu), AGC target value of 2E5, 140k resolution and maximum ion injection time of 500 ms.

When retention times of multiple targets overlapped, up to four targets were isolated and accumulated in the C-trap first, then all accumulated ions were transferred to the Orbitrap mass analyzer for HR/AM detection. Increasing amounts of heavy peptide standards (0 amol, 10 amol, 50 amol, 100 amol, 1 fmol, 10 fmol, 100 fmol) within a constant amount of yeast tryptic digest (10 ng or 1000 ng) were analyzed in triplicate.

## Results and Discussions

### High resolution and high mass accuracy lead to better selectivity and more accurate quantitation

Sufficient separation of target peptides from other species is the most important requirement for accurate identification and quantitation of a peptide target. When the peptide target is present in a complex background, LC separation is routinely incorporated to reduce the number of species that are co-eluting with the target. Even with chromatographic separation hundreds, even thousands, of peptides are still present at any given time when the target is detected by the mass spectrometer. A mass spectrometer with higher resolving power is able to provide better selectivity and, as a result, more accurate quantitation.

Figure 2 shows the detection of two species with a mass difference as small as 0.015 amu at different resolution settings on the Q Exactive instrument. The two species are not resolved at 35,000 FWHM, which results in a mass shift and an inaccurate detection and quantitation for both species. The two species are separated to 50% valley at 70,000 resolution, and baseline-separated at 140,000 resolution. Therefore, higher resolution (in this case 140,000 FWHM) allowed for more reliable identification and accurate quantitation. **At such high resolution, more than two hundred species can be separated in a unit-mass window.** This translates into resolving, in theory, >100,000 species in the mass range of 300-1000 amu at any one point in time. This greatly reduces background interference and enables confident identification and quantitation of the target peptides in the presence of very complex backgrounds.

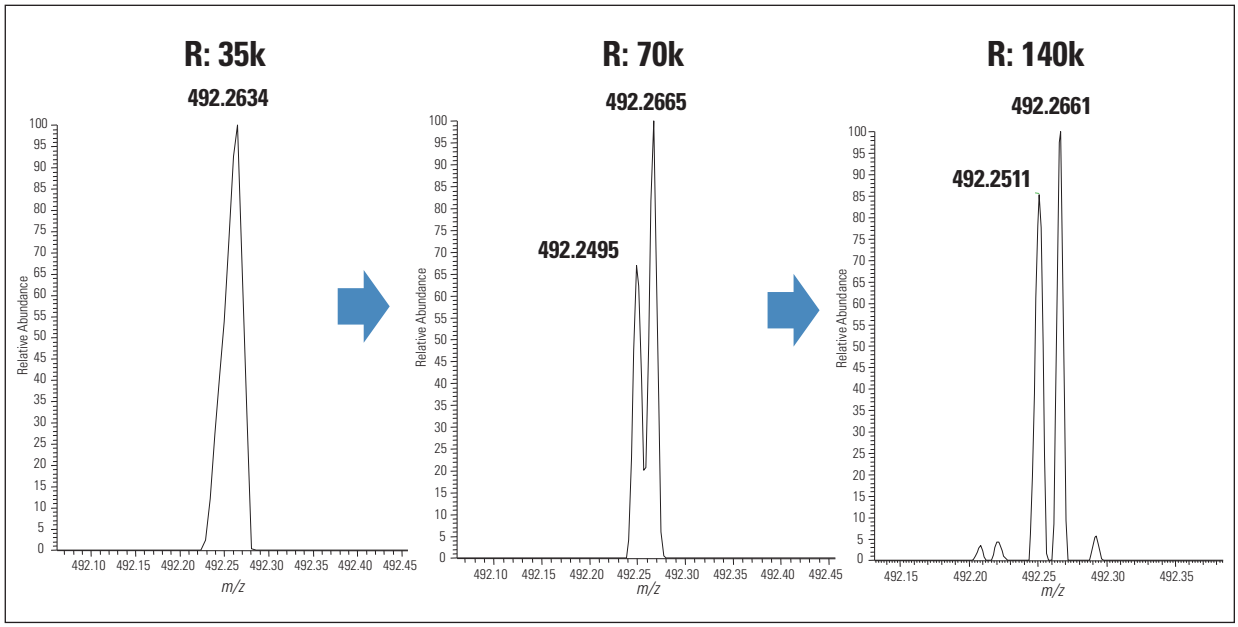


Figure 2. Two closely spaced isobaric species were measured at 35k, 70k, and 140k resolution. Higher resolution provided better separation and selectivity.

### Lower detection limit with tSIM scan

The ability to detect low-abundance targets in the presence of high background signal is another crucial requirement for any targeted quantitation approach. To obtain accurate quantitation, sufficient numbers of target ions need to be detected to ensure precise measurement.

On a triple-quadrupole mass spectrometer, this may be achieved simply by allowing longer transition or "dwell" times. However, this can reduce the duty cycle to impractical levels when a large number of targets are monitored, particularly those with three or more transitions per target.

In the Q Exactive MS and other mass spectrometers that employ mass analyzers that temporarily "trap" ions, the total detectable ion population is defined by automatic gain control (AGC). Depending on the intensity of the incoming ion beam, the ion injection time is varied to allow the accumulation of a pre-defined number of ions (AGC target) in the Orbitrap mass analyzer. Two approaches can be used to improve detectability of the ion of interest in the complex mixture. The first approach is to increase the AGC target value so that more ions are accumulated in the trap. This approach results in better detection of all species in the mass range. The second approach is to *selectively accumulate* ions in a narrow mass range centered on the target, which is referred to as a targeted SIM (tSIM) scan. The target ion in the latter case will account for larger and larger percentages of total ion population as the mass range narrows. This leads to an improved signal-to-noise ratio and to lower detection limits.

The incorporation of a quadrupole mass filter on the Q Exactive MS allows efficient selective isolation of ions with an isolation window as narrow as 0.7 amu. This has a significant positive impact on the detection limit of the target in complex backgrounds. Figure 3 shows the detection of a heavy peptide standard (GLILVGGYGTR*, *m/z* = 558.326) at 100 amol load in the presence of a 1-µg yeast tryptic digest with both full scan (Figure 3A, 300-100 amu, AGC target: 1E6) and tSIM scan (Figure 3B, *m/z* 571.3-575.3, AGC target: 2E5) that were acquired sequentially. Although the AGC target for full scan was 5 times larger than SIM scan, barely any signal was detected in the mass range of *m/z* 558.2–559.2 (insert in Figure 3A). However, close to 50 peaks were detected in the same mass window in 4-amu-wide SIM scan. Both the mono-isotopic peak and the C13 peak were easily detected with S/N of 48 and 22, respectively (insert in Figure 3B). This was due to the selective isolation of a narrow mass range (4 amu) by the quadrupole, which resulted in a greater proportion of target ions being accumulated before reaching the AGC target of 2E5.

A total of thirteen measurements of the mono-isotopic peak were achieved across the LC peak which ensures accurate quantitation of the target at 100 amol (total load amount). A detection limit of 10 amol was achieved using SIM for this peptide target which compares favorably to triple quadrupole based SRM approaches. A detection limit for the same peptide was ~1 fmol, which is 100 times higher, using full scan. This clearly demonstrated the advantage of the quadrupole-based high-resolution SIM scan; a unique feature of the Q Exactive instrument. In addition, the high resolution of 140,000 resulted in full separation of the target from other species and ensures accurate identification and quantitation.

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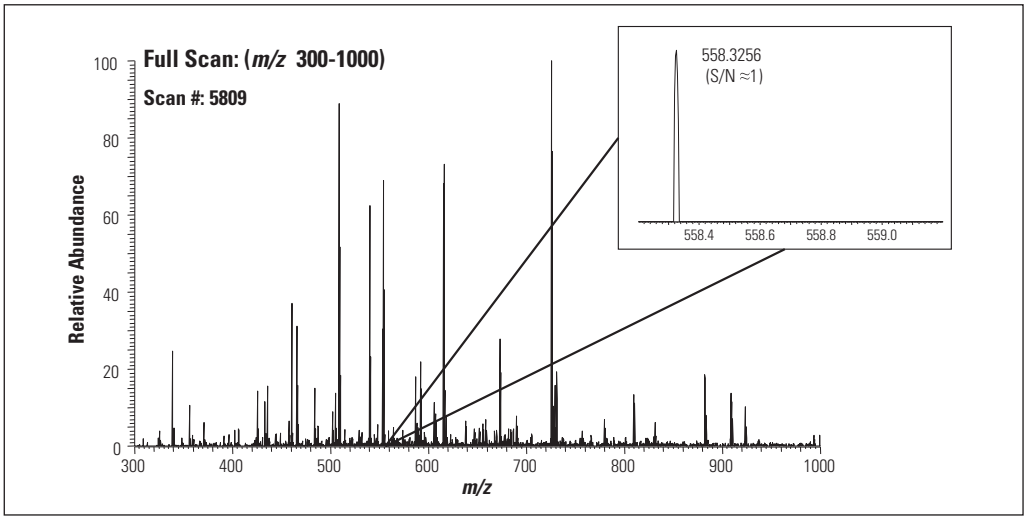


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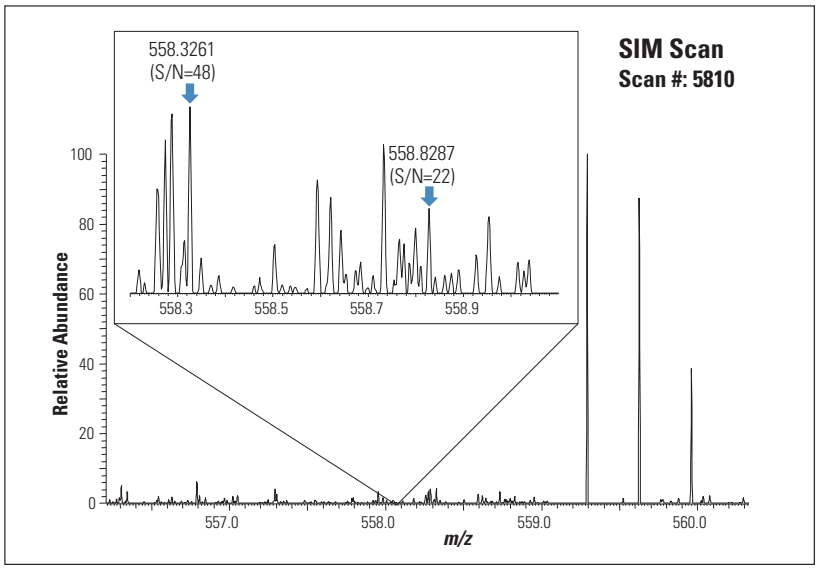


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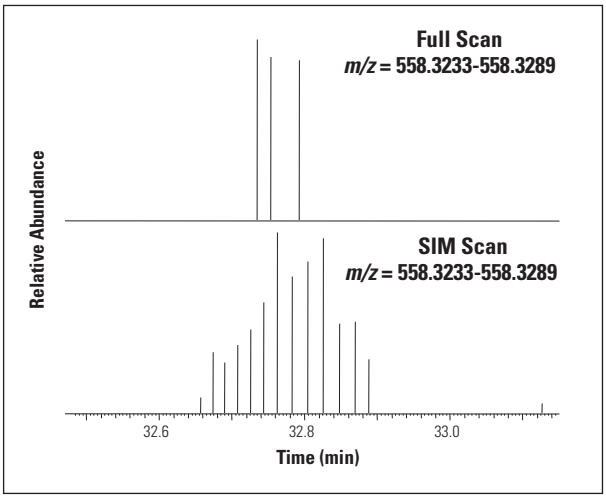



Figure 3. Detection of 100 amol GLILVGGYGTR* (*m/z* = 558.326) in full scan and SIM scan. A) Full-scan (#5809) spectrum with AGC target of 1E6, 70K resolution. The mass range [*m/z* 558.2-559.2] is shown in the insert. B) SIM scan (#5810) spectrum with AGC target of 2E5, 140,000 resolution. The mass range [*m/z* 558.2-559.2] is shown in the insert. The monoisotopic and $C_{13}$ peaks of the target were highlighted and labeled with measured *m/z*, S/N. C) Extracted ion chromatogram of the target with 5-ppm tolerance is shown for both full scan and SIM scan.

### Detection limit and linear dynamic range

The detection limit and linear dynamic range for analysis of the heavy peptide standards were evaluated in the presence of either a 10-ng or 1-µg yeast tryptic digest background. The detection limits and S/N values in both SIM and full scans are listed in Table 2. The samples with a 10-ng background resemble situations where the targets have been efficiently isolated from the original complex background. The absence of intense background species makes it easy to identify and quantify the target at much lower amounts.

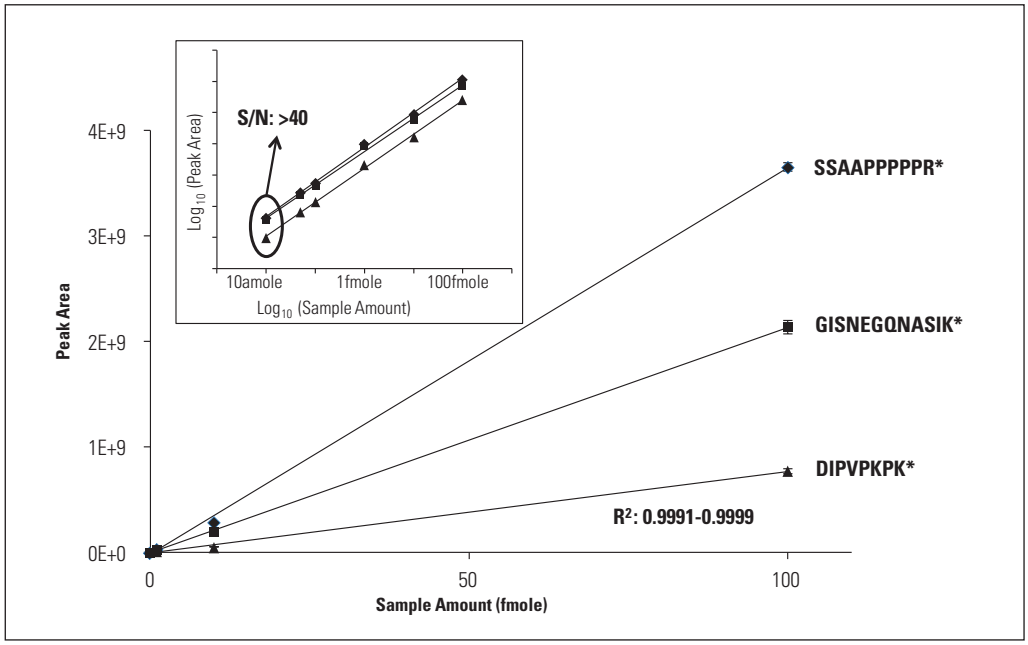
With tSIM, we easily detected all ten heavy peptide targets at 10 amol (Table 2). The high S/N values (10-60) for a few targets suggest that detection at limits lower than 10 amol would have been possible. In full scan, we detected six peptide targets at 10 amol, three at 50 amol and one at 1 fmol. For those targets that were detected at 10 amol in both scan types, the S/N value was generally 5-10 times higher in the SIM scan. Linear regression analysis, both in regular scale and in $\log_{10}$ scale, showed that four orders linear dynamic range, spanning 10 amol to 100 fmol, was achieved for at least six targets with SIM scan and three targets with full scan. Plots for three peptide standards are shown in Figure 4A. High quality MS/MS spectra unambiguously confirmed the presence of the target peptides. The HCD spectrum for GISNEGQNASIK* (*m/z* = 631.3130, 2+) at the 10 amol loading level is shown in Figure 4B. More than seven y ions were identified with <10 ppm mass deviation.

Table 2. Detection limits and S/N of heavy peptide standards in 10 ng and 1000 ng yeast digest background

| | 10-ng Yeast Digest | | | | 1000-ng Yeast Digest | | | |
|---|---|---|---|---|---|---|---|---|
| | SIM | | Full | | SIM | | Full | |
| | LOD amole | S/N | LOD amole | S/N | LOD amole | S/N | LOD amole | S/N |
| SSAAPPPPPR* | 10 | 40 | 10 | N/A | 1000 | 200 | 1000 | N/A |
| GISNEGQNASIK* | 10 | N/A | 10 | N/A | 50 | 2 | 1000 | 10 |
| DIPVPKPK* | 10 | 60 | 50 | N/A | 1000 | 2 | 1000 | 2 |
| IGDYAGIK* | 10 | 9 | 10 | N/A | 1000 | 80 | 1000 | 2 |
| TASEFDSAIAQDK* | 10 | 12 | 10 | 9 | 1000 | 7 | 10000 | 15 |
| SAAGAFGPELSR* | 10 | N/A | 10 | 30 | 100 | 2 | 10000 | 60 |
| ELGQSGVDTYLQTK* | 10 | 18 | 10 | 2 | 1000 | 6 | 1000 | 6 |
| GLILVGGYGTR* | 10 | 3 | 50 | 15 | 10 | 6 | 1000 | 80 |
| SFANQPLEVVYSK* | 10 | 17 | 1000 | N/A | 50 | 30 | 1000 | 3 |
| LTILEELR* | 10 | 8 | 50 | N/A | 1000 | 15 | 1000 | 40 |

N/A: S/N value is not reported if no peaks are detected in blank runs.

A

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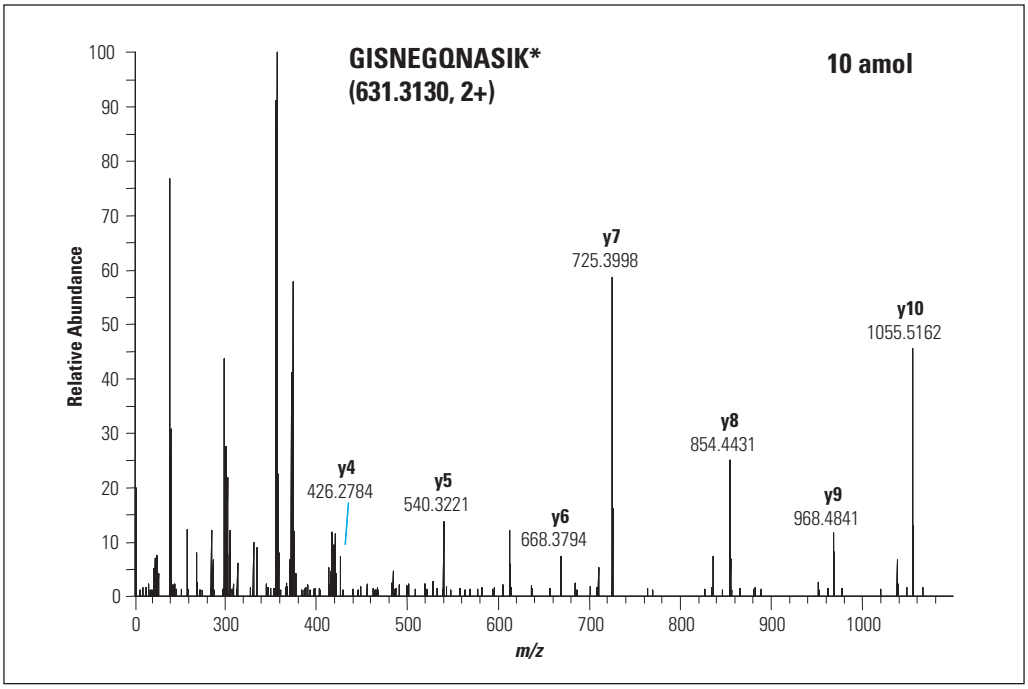


Figure 4. Amounts of heavy peptide standards and measured peak area in SIM scan were plotted and analyzed by linear regression fitting with 10-ng yeast tryptic digest as background. A) Linear regression fittings and standard deviations for three heavy peptide standards in 10-ng yeast tryptic digest. The $R^2$ of linear fitting is above 0.9991. The insert shows linear fitting of the same data in $\log_{10}$ space. B) HCD spectrum for GISNEGQNASIK (m/z 631.3130, 2+) is shown with major y ions labeled.

The samples with 1-µg background resembled more challenging situations where the targets are poorly isolated from the complex background. Under these conditions, the targets overlap with a large number of intense isobaric species. With tSIM, we detected and quantified two out of eleven heavy peptide targets at 10 amol. The linear regression fitting plots, both in regular scale and in $\log_{10}$ scale, are shown in Figure 5A. The remaining targets were detected at 50 amol – 1 fmol, well within the concentration range of most biomarkers. In full scan, the lowest detection level was 1 fmol or higher for all ten peptide targets, which was 50–100 times higher than that obtained via the tSIM. Similarly, high-quality MS/MS spectra unambiguously confirmed the presence of target peptides. The HCD spectrum for NGFILDGFPR* (*m/z* = 573.3025, 2+) at a 50-amol loading level is shown in Figure 5B. More than seven b and y ions were identified with <5 ppm mass deviation.

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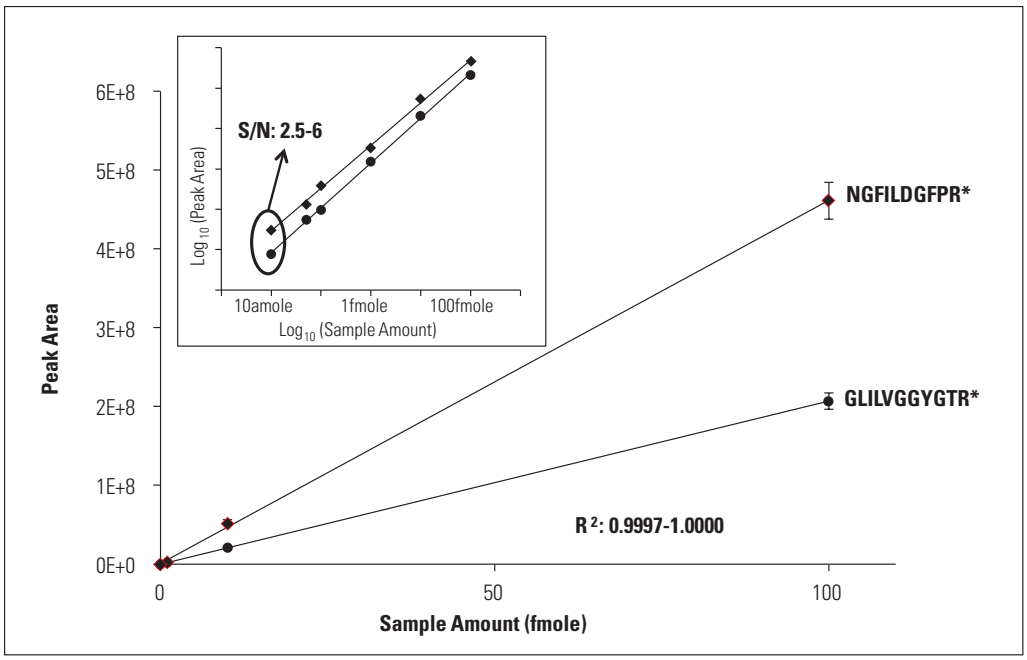


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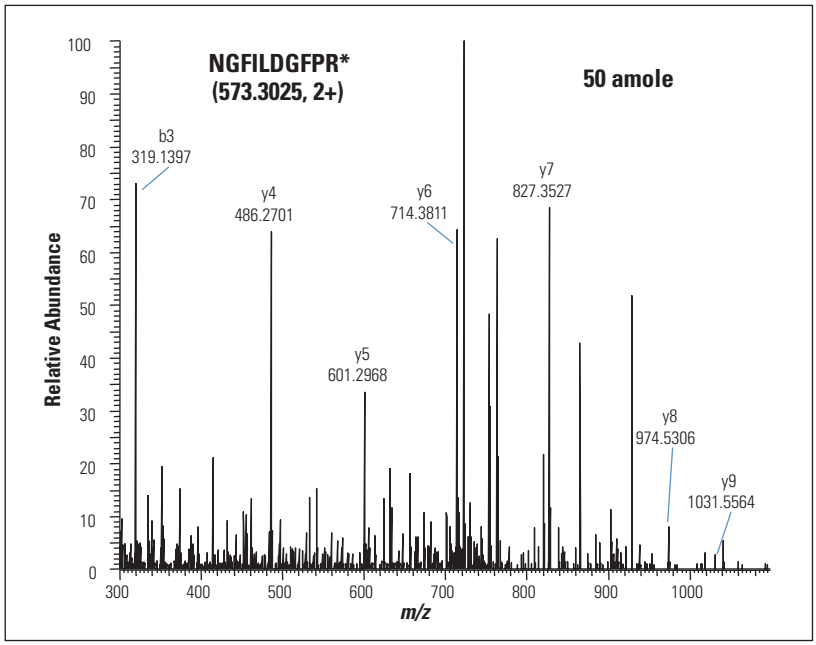


Figure 5. Amounts of heavy peptide standards and measured peak area in SIM scan were plotted and analyzed by linear regression fitting with 1 µg of yeast tryptic digest as background. A) Linear regression fittings and standard deviations for two heavy peptide standards. The $R^2$ of linear fitting is shown to be above 0.9991. The insert shows linear fitting of the same data in $\log_{10}$ space. B) HCD spectrum for NGFILDGFPR* (*m/z* 573.3025, 2+) with major y ions labeled.

Our quantitation results are comparable with previous observations on the best SRM-based assays where target enrichment, combined with HR/AM, is the key for obtaining sensitive and accurate quantitative analysis.[12, 13]

### Higher throughput with msx tSIM

It is generally accepted that at least seven measurements of the target across the LC elution profile are necessary in order to obtain accurate quantitation with acceptable RSDs. This means that the duty cycle of the MS measurement has to keep up with the LC peak width as more and more targets are monitored.

The multiplex tSIM scan function, available only on the Q Exactive instrument, greatly improves overall duty cycle while maintaining high mass accuracy and high resolution. In an msx tSIM experiment, up to ten targets can be isolated sequentially, accumulated in the C-trap, and then transferred to the Orbitrap mass analyzer for detection in a single spectrum. At 140,000 resolution, this leads to a ~6 second shorter duty cycle when compared to performing 10 sequential SIM experiments at 140,000 resolution. The Orbitrap detection is also concurrent with the isolation and accumulation of the next group of targets, which further reduces the overall scan time and thus improves the duty cycle.[14] These unique features enable the usage of high target values (2E5) and long injection times (500 ms) for detection of very-low-abundance targets while maintaining sufficient duty cycle for quantitation. For example, three targets were monitored with msx tSIM in the time window shown in Figure 3C. Thirteen SIM measurements at resolution of 140,000 with S/N > 5 were obtained for the target at *m/z* 558.3234 in a time window of 14 seconds, which also included 13 full scans acquired at 70,000 resolution. If those targets were monitored separately without the parallel C-trap fill feature, it would have taken over 3 seconds to finish one cycle if the average fill time was 250 ms. Without the parallel C-trap fill feature, only 5 measurements could have been achieved and this would be insufficient for accurate quantitation. With the parallel C-trap fill feature, it is now possible to monitor 10 targets and still obtain > 7 measurements over a 20-second LC peak, (provided the maximum fill time is below 250 ms for each target). Clearly, this unique msx tSIM feature greatly expands the number of targets that can be monitored on a Q Exactive instrument, without sacrificing resolution or mass accuracy.

### A full-SIM quantitation strategy for high dynamic range, high throughput analysis

The ability to perform HR/AM scans and data-dependent MS/MS makes the Q Exactive mass spectrometer a unique platform to accomplish both discovery and target verification. A complete qual-quan strategy is illustrated in Figure 6.

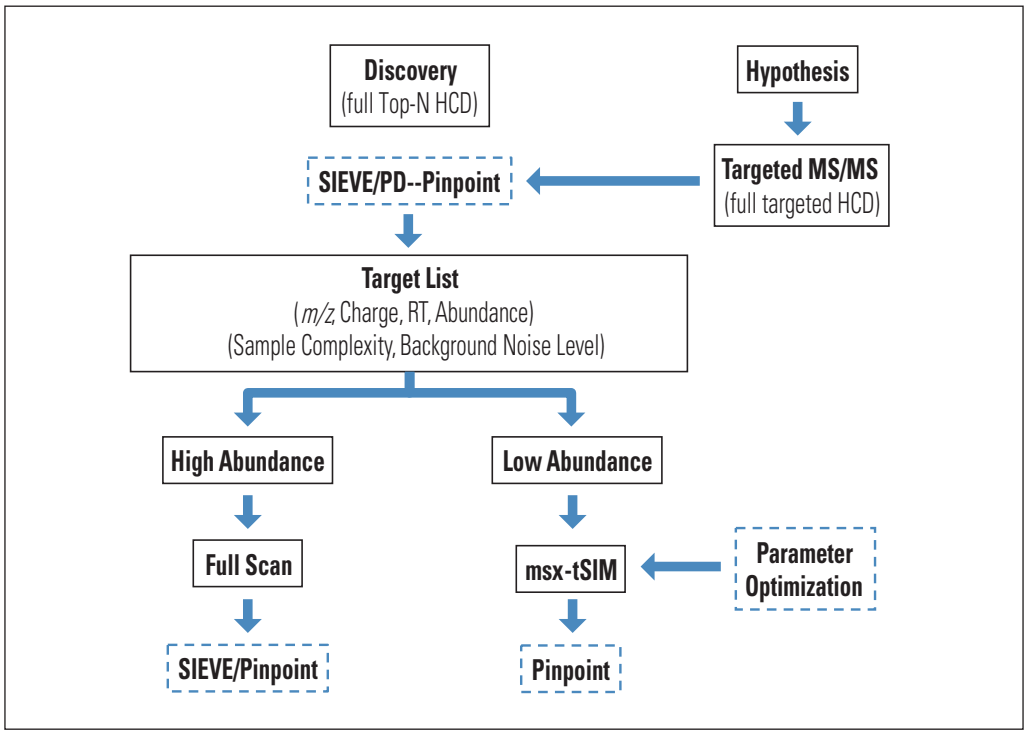


Figure 6. A discovery-to-quantitation flowchart on Q Exactive mass spectrometer

High scan speed enables the identification of thousands peptides and proteins with a traditional data-dependent acquisition method, as described in previous application notes. The discovery data are processed to identify peptide sequences and obtain quantitative information using Thermo Scientific Proteome Discoverer software and/or Thermo Scientific SIEVE software. Putative peptides targets are then identified either through the incorporation of heavy isotope labels, such as SILAC or TMT, or through other non-label approaches, such as spectrum counting or integrated peak area. The targets can also be generated through hypothesis, such as signaling pathway components, and validated through targeted MS/MS. The information on the targets can then be collected, including accurate *m/z*, retention time, signal intensity, charge state and MS/MS spectrum patterns.

If one were to use the traditional triple-stage quadrupole-based SRM workflow, those targets would have to be further validated, sometimes with synthetic peptides. Some targets might fragment poorly making them impossible to quantify. All of these problems can be avoided when an HR/AM targeted quantitation workflow is applied on the Q Exactive mass spectrometer. Furthermore, hundreds, even thousand of targets can be quantified using the full msx tSIM strategy on the Q Exactive MS. Low-level targets are grouped and monitored using msx tSIM. All of the other targets that are easily detected will be quantified using full scan along with all of the other species. By separating the targets into two groups, parameters for msx tSIM can be optimized for a small number of targets and still maintain a reasonable duty cycle to ensure accurate quantitation. The combination of high mass accuracy, high resolution, retention time, isotope pattern validation and HR/AM MS/MS allows confident identification of the targets. Furthermore, the co-elution pattern can also be used to confirm the identity of the targets. Thermo Scientific Pinpoint software can automatically quantify all the targets in both full and msx tSIM and allow user validation of the quantitative result.

## Conclusions

In this study, we described HR/AM targeted quantitation on a Q Exactive MS; a hybrid quadrupole-Orbitrap mass spectrometer. Using targeted SIM, detection limits of low attomole (~10 amol) or high attomole (~100 amol) were achieved in low- or high-level complex backgrounds, respectively. The high resolution of 140,000 and high mass accuracy afforded high selectivity in target detection. The unique feature of multiplexed tSIM scan allowed sequential isolation of up to 10 targets within one scan cycle, greatly increasing throughput.

The HR/AM full scan and MS/MS capability also make the Q Exactive MS well suited for qual/quan workflows. Extensive lists of target candidates can be generated through traditional data-dependent acquisition or hypothesis-driven targeted MS/MS in the early discovery phase. Hundreds, even thousands of target candidates can be immediately verified on the same instrument with a msx tSIM strategy over large number of samples with virtually no additional method development. This allows the efficient identification of most-likely candidates, on which effort can be focused in developing more-sensitive and higher-throughput quantitation methods.

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