Application Note: 532

High-Throughput Screening Using Multiplexed High-Resolution, Accurate Mass LC/MS

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Key Words

- Exactive MS
- Transcend TLX-4 System
- Pinpoint Software
- QuickCalc Software
- Phosphorylated Peptides

Introduction

High throughput screening (HTS) is a valuable part of identifying new leads and directing drug design in pharmaceutical research. The ability to quickly perform large numbers of analyses is critical to the success of any HTS assay. Traditionally, the quantitative components of these analyses are performed using fluorescence or radiolabeled techniques. While these techniques provide for rapid sample analysis, they also contain inherent limitations including the occurrence of false positives, increased time for method development, and costly and complex sample preparation. High-resolution, accurate mass (HRAM) liquid chromatography-mass spectrometry (LC/MS) coupled with chromatographic multiplexing technology provides a selective and sensitive alternative to traditional methods with comparable analytical speed and without the need for labeled substrates. Chromatographic multiplexing coupled with HRAM analysis was used to collect full scan MS data providing valuable spectral information in a high throughput format that is not possible when using traditional targeted MRM experiments.

Goal

To develop a high throughput screening method utilizing high-resolution, accurate mass LC/MS and chromatographic multiplexing with a Thermo Scientific Transcend TLX-4 multichannel U-HPLC system and a Thermo Scientific Exactive high performance benchtop mass spectrometer.

Experimental

Phosphorylated peptides were analyzed at various concentration levels in both neat and buffer solutions to determine signal response, linearity, reproducibility, and data quality, as represented by Z′ value.

Sample Preparation

Two powdered phosphorylated peptides, CREBtide 29865 and CREBtide 27018 (AnaSpec™, San Jose, CA), were each reconstituted in a 50:50 acetonitrile (ACN):water solution at 1 mg/mL. Two serial dilutions of CREBtide 29865 were prepared at concentrations of 10 nM, 25 nM, 50 nM, 100 nM, 150 nM, and 250 nM in an ACN:water solution and in a 50 mM HEPES buffer containing 50 mM $MgCl_2$. CREBtide 27018 was then added to each dilution level as an internal standard at a concentration of 150 nM.

Liquid Chromatography and Multiplexing

Samples were injected onto a C_{18} (4.6 x 20 mm, 3.5 µm) HPLC column. A step gradient elution was accomplished using water + 0.05% trifluoroacetic acid (TFA) (v/v) and acetonitrile + 0.05% TFA (v/v) with a one-minute step gradient at a flow rate of 1.5 mL/min. All methods were completed using a Transcend™ TLX-4 system with a dual injector arm and DLW (Dynamic Load and Wash, CTC Analytics, Switzerland).

Mass Spectrometry

MS analysis was carried out on an Exactive™ high performance benchtop mass spectrometer powered by Thermo Scientific Orbitrap technology.

The MS conditions were as follows:

Data acquisition mode:	Full scan
Scan range (*m/z*):	475-690
Vaporizer temp (°C):	350
Capillary temp (°C):	230
Sheath gas (AU):	60
Auxillary gas (AU):	30
Max injection time (ms):	250
Resolution:	25,000
Spectral speed (Hz):	4

The instrument was calibrated in positive ion mode before sample acquisition using ProteoMass™ LTQ/FT-Hybrid ESI Pos. Mode Cal Mix (Sigma-Aldrich, St. Louis, MO). The resolution procedure determined the optimal MS resolution settings by direct infusion of 50:50 mobile phase at 1500 µL/min and 1 µg/mL solution of compound at 50 µL/min. In all runs 384 sample injections were collected into one data file using Thermo Scientific Xcalibur and Thermo Scientific Aria software to control data acquisition start and stop times for each 384-sample set.

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Data Analysis Software

Thermo Scientific Pinpoint software was used for exact mass calculations of all peptides at each of the observable charge states (Figure 1). Exact mass information was saved in Microsoft® Excel® for later use. Thermo Scientific QuickCalc software, powered by Gubbs™ Inc. GMSU (Gubbs Mass Spec Utilities) was used for all chromatographic data review and report generation. Compound information was imported into QuickCalc™ software using an Excel import template containing the required *m/z* information and stored for later use. The compound information was then grouped into a "compound set" and saved allowing the exact *m/z* information for multiple compounds to be recalled easily and applied to an entire data set for simple chromatographic review. QuickCalc software was also used to automatically extract and sort each of the 384 injections contained in a data file. The individual chromatographic peaks were then displayed in a table view correlated with the sample information related to each individual injection.

Results and Discussion

Mass Spectrometer and LC Method Optimization

The optimal scan range for the MS experiment was selected based upon the most intense charge states observed for each phosphorylated peptide. Both CREBtide 27018 and CREBtide 29865 displayed the maximum signal intensity in the 3+ charge state under the previously specified experimental LC conditions. Additionally, the 4+ charge state was present at higher concentrations, although it was not quantifiable at lower concentrations. The scan range of *m/z* 475-690 was selected to provide for data collection of both the 3+ and 4+ charge states (Figure 2).

Using the MS full scan mode across a wide *m/z* range enabled data collection of all isotopic peaks in the charge envelope for each peptide and provided additional confidence for the reported data. Spectral information for each charge envelope was collected and analyzed with no additional MS scan time or overall duty cycle.

The LC method optimization determined that a 35% ACN + 0.05% TFA step for 25 seconds provided optimal separation of the phosphorylated peptide from other components in the sample matrix. A subsequent 95% ACN + 0.05% TFA step for 10 seconds flushed the remaining components from the column and resulted in robust and reproducible chromatographic separation and peak shape (Figure 3).

Protein/Peptide/Precursor/Product	
CREBtide 27018	
KRREILSRRPSY[Phosphoryl]RK	
642.3616->642.6959	
642.3616->642.3617	
642.3616->643.0302	
642.3616->643.3643	
482.0230->482.2737	
482.0230->482.0231	
482.0230->482.5244	
482.0230->482.7750	

Protein/Peptide/Precursor/Product	
CREBtide 29865	
KRREILSRRPS[Phosphoryl]YRKC	
676.6980->677.0323	
676.6980->676.6981	
676.6980->677.3663	
676.6980->677.7001	
507.7753->508.0260	
507.7753->507.7754	
507.7753->508.2765	
507.7753->508.5269	

Figure 1. Exact mass calculations for CREBtide 27018 and CREBtide 29865 using Pinpoint™ software. The 3+ and 4+ charge states are displayed in order of decreasing intensity.

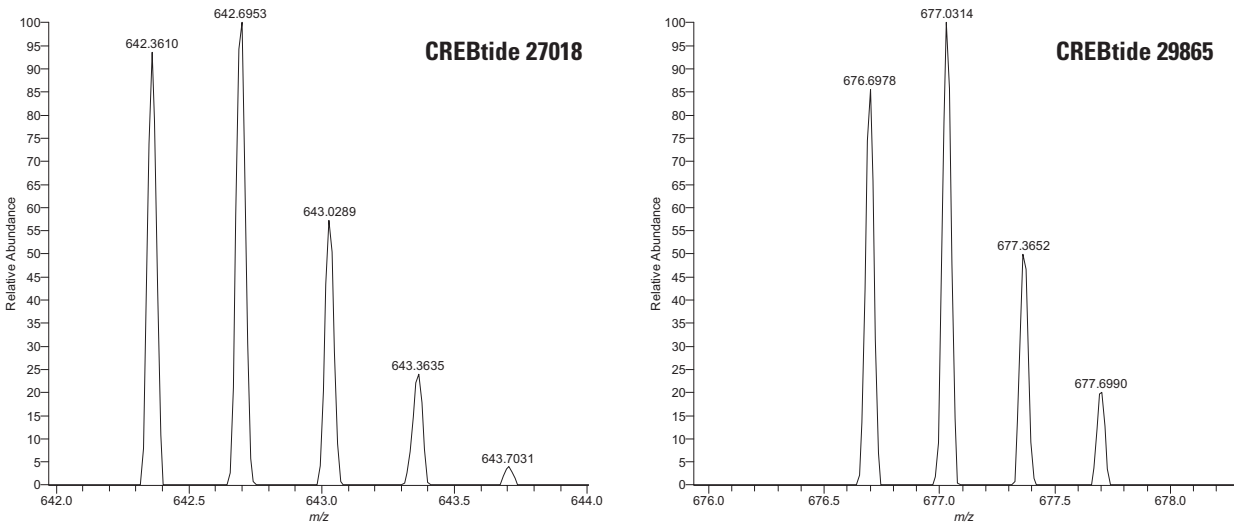


Figure 2. Isotopic charge envelopes for CREBtide 27018 (left) and CREBtide 29865 (right) in the 3+ charge state

High Throughput Data Collection and Review

Rapid sample analysis was achieved through a combination of chromatographic multiplexing and fast chromatography on a Transcend TLX-4 system. All 384 sample injections were written to a single data file, eliminating the need for MS or LC recovery time between sample injection.

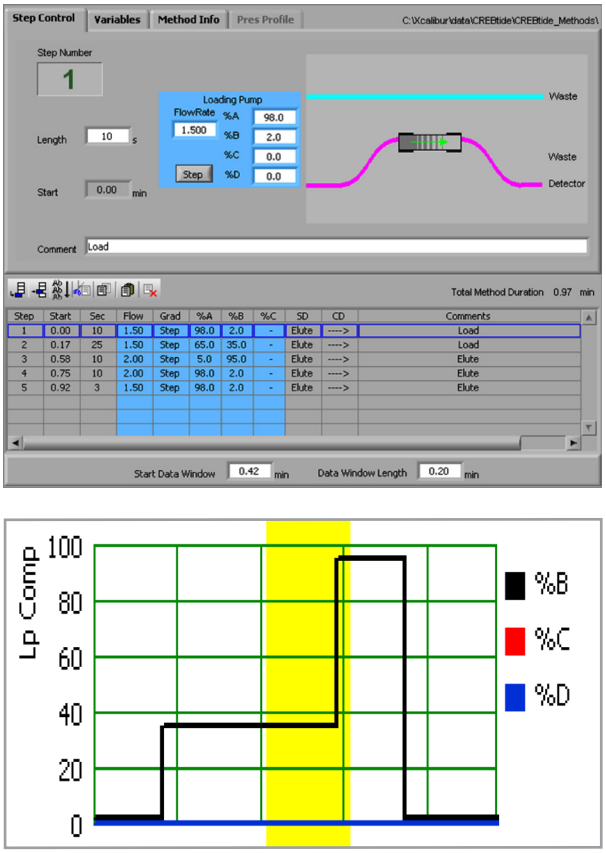


Figure 3. LC step gradient method (top) and Aria™ software multiplexing data window (bottom) allow for quick method development.

Sample collection speeds averaged 17 seconds per injection cycle. Each 384-injection sample set was collected in under 2 hours. An average of 8 to 12 scans were collected across each peak allowing for reproducible peak shape and area counts at the limit of quantification (Figure 4).

Data processing using QuickCalc software provided specific sample information correlated with each injection displayed in a standard single injection format. QuickCalc software was also used for data review based on user defined system suitability criteria, calibration curve generation and data report generation (Figure 5).

Data Analysis

All data collection was performed at resolution 25,000 resolution with an external calibration for mass accuracy and analyzed using a 3-ppm filter window. After the initial MS mass calibration, 10,000+ sample injections were completed over a period of 24 hours. All sample data remained with the initial 3-ppm filter window without requiring additional mass calibration.

The two sample sets analyzed were in either ACN and water solution or in HEPES buffer solution. The samples analyzed in the ACN/water solution demonstrated a linear response across the entire concentration range from 10 nM to 250 nM. All points in the calibration curve demonstrated good reproducibility with %CV values below 20% (Figure 6).

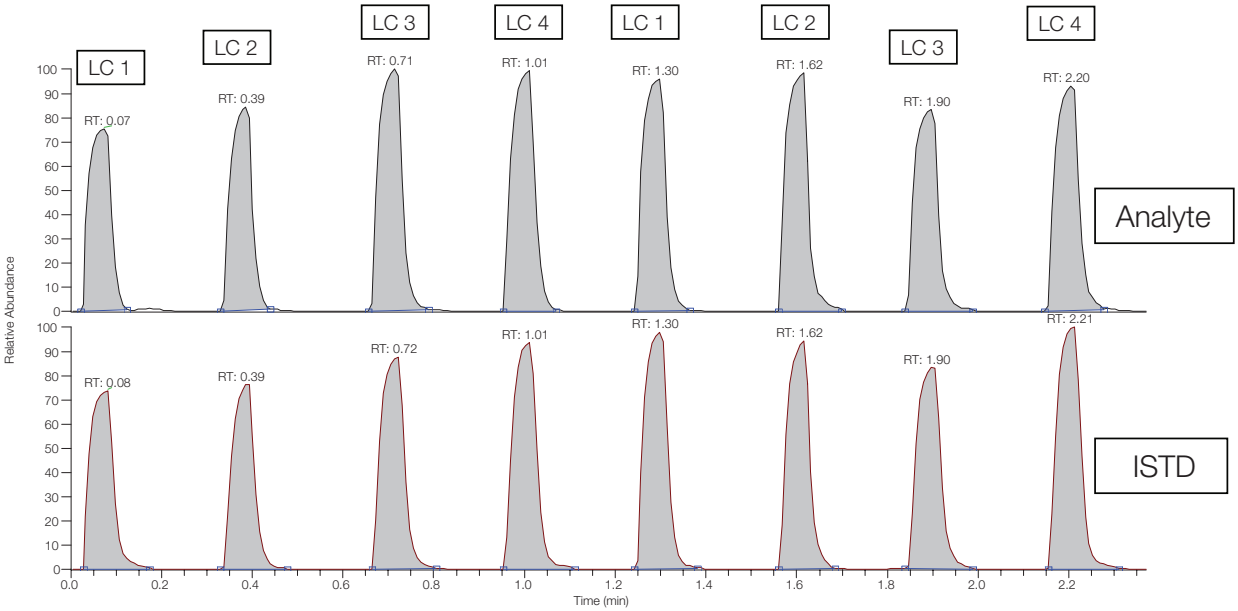


Figure 4. Zoomed-in extracted ion chromatograms for CREBtide 27018 and CREBtide 29865 in a 384-injection set. Injections were made sequentially across all available LC channels to provide maximum throughput.

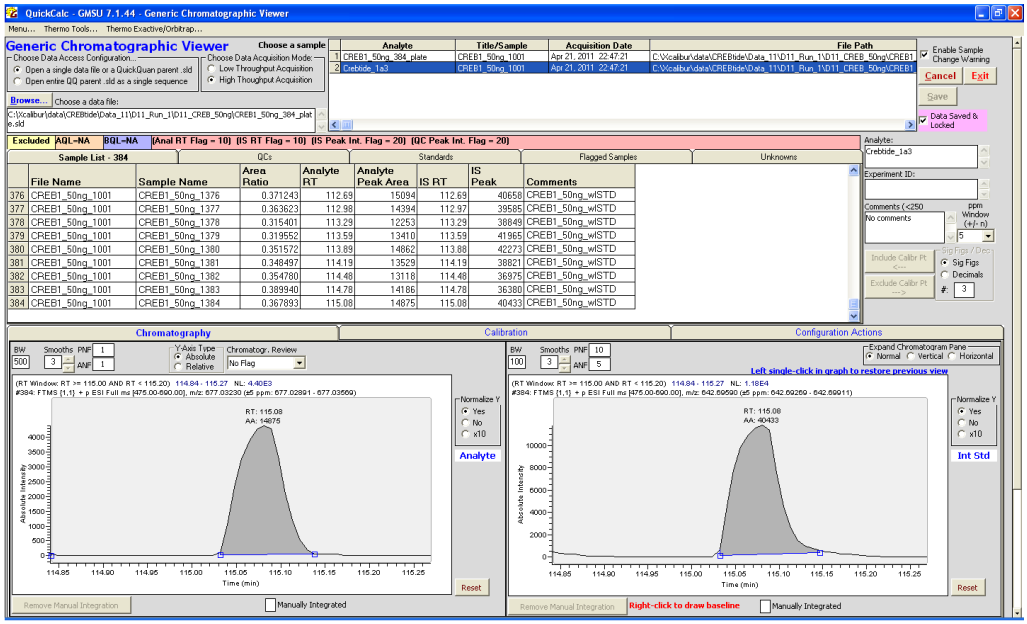
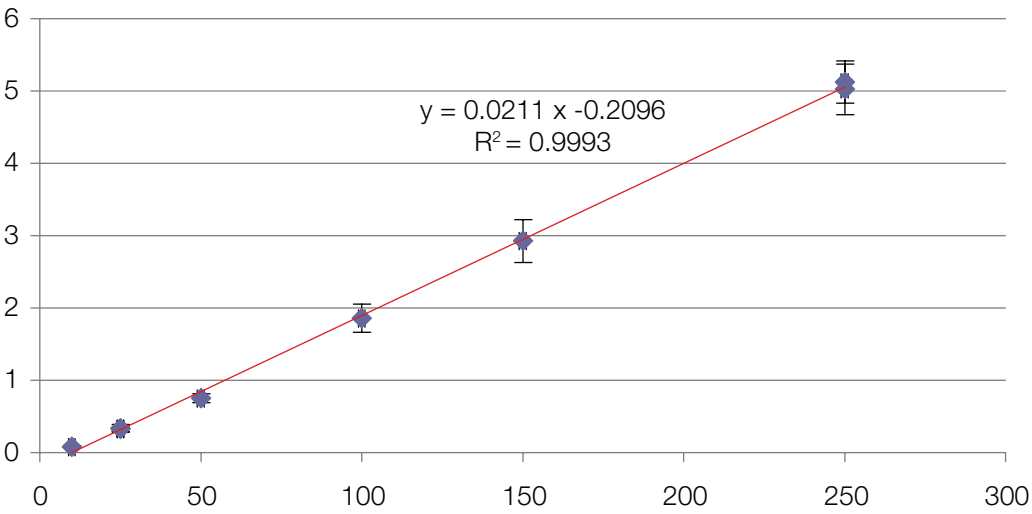


Figure 5. Files containing up to 384 individual injections were sorted and displayed in a standard review format. Extracted ion chromatogram for CREBtide 29865 (left) and CREBtide 27018 (right) using QuickCalc software.



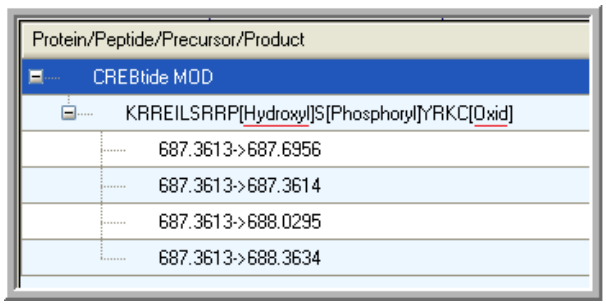
	10 nM	25 nM	50 nM	100 nM	150 nM	250 nM
# of Injections	384	768	384	384	384	768
%CV	18.37	12.13	7.93	10.40	10.02	6.60
Z' Value	0.77					

Figure 6. Calibration curve (top) and injection reproducibility table (bottom) for modified CREBtide 29865 with internal standard in ACN/water solution

The sample set analyzed in HEPES buffer solution did not yield the expected results. The spectral peaks expected for CREBtide 29865 were not detected at any concentration level in any of the sample solutions containing HEPES buffer, while CREBtide 27018 was present at the expected levels. Although no structures or modifications were confirmed, it was speculated that, due to the presence of an additional cystine in CREBtide 29865, an unexpected modification of the peptide occurred in the buffer solution at ambient temperature. Pinpoint software was used to predict possible modifications to the peptide along with the corresponding exact mass information, and the previously collected data files were reexamined. When possible modifications were compared to the data files, an additional oxidation and hydroxylation provided a reasonable explanation for the observed mass shift (Figure 7).

The predicted modification of CREBtide 29865 was present in all samples above the 50 nM concentration calibration point. In over 3,000 sample injections, the predicted modification was present with an identical chromatographic retention time and peak shape to that of the internal standard. Furthermore, the area ratio of the analyte/ISTD showed a linear response across the remaining points in the calibration curve.

Another statistical measure to demonstrate the reliability and robustness of an assay is Z′. The Z′ calculation incorporates the standard deviation and mean, as well the dynamic range for a set of samples analyzed providing an additional measure of quality not provided by the individual components alone. Any assay with a Z′ greater than 0.5 is considered to be an excellent assay with a Z′ = 1 describing a perfect assay. The samples analyzed in the neat solution produced a Z′ = 0.77, which indicates a high quality assay. Due to the unexpected modification of the peptide, a meaningful Z′ value for the sample set collected in HEPES buffer could not be calculated, although the reproducibility for the remaining samples was calculated for each of the three remaining calibration levels (Figure 8).



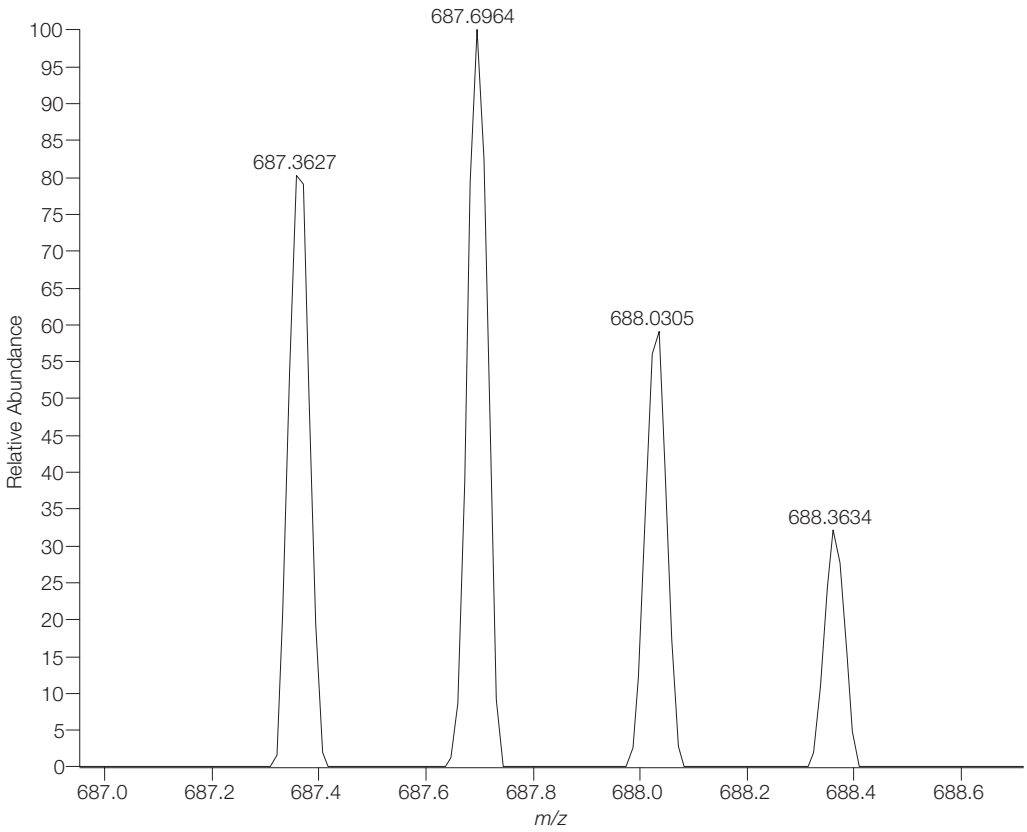
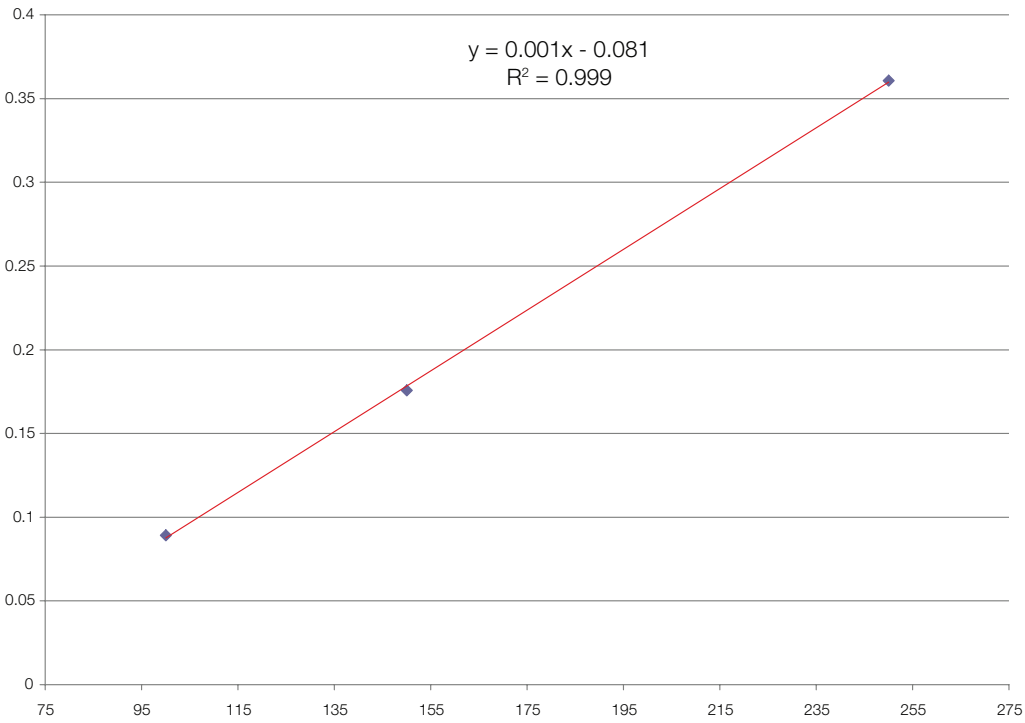


Figure 7. CREBtide 29865 modification prediction in Pinpoint software, underlined in red (top), and observed MS spectra in collected data files (bottom)



	100nM	150nM	250nM
# of Injections	384	768	1535
%CV	19.96	13.48	8.26

Figure 8. Calibration curve (top) and injection reproducibility table (bottom) for modified CREBtide 29865 with internal standard HEPES buffer

The slope of the curve for the samples analyzed in buffer solutions is significantly less than that of the samples analyzed in ACN/water. This may indicate that the predicted modifications either did not affect 100% of the peptide present in the sample or that additional modifications may have possibly occurred that did not ionize or may be outside of the experimental *m/z* scan range. As a result of the full scan MS experimental data collection during the initial acquisition, all supplemental analysis was completed through re-evaluation of existing data files without the need for additional sample preparation or injections.

Conclusion

Multiplexing using a Transcend TLX-4 system with four LC channels, coupled with HRAM analysis, provided a robust and reproducible proof-of-concept method for high throughput screening analysis of phosphorylated peptides. Rapid injection cycle times of 17 seconds, on average, were achieved while maintaining chromatographic integrity. The collection of full scan data across a wide mass range enabled the acquisition of spectral information for the entire isotopic charge envelope for each peptide at multiple charge states, as well as information regarding unexpected behavior. A mass accuracy window of less than 3 ppm was maintained throughout a large number of sample injections without the need for recalibration. In addition, the statistical analysis of a large injection set resulted in a Z′ value of 0.77, which indicated this is an excellent assay for screening applications.

References

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