Label-Free High Throughput Compound Library Screening Using Multiplexed LC/MS

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Overview

Purpose: To implement multiplexed liquid chromatography and high resolution accurate mass detection for the analysis of a high throughput screening assays while improving data quality and ease of use for instrument operation.

Methods: Phosphorylated peptides were analyzed at various concentration levels in both sodium citrate and HEPES solutions to determine signal response, linearity, reproducibility, and Z' value.

Results: Several thousand sample injections were performed in multiple instrument scan modes with sample solutions dissolved various sample matrixes. Sample collection at a rate of 18 sec/cycle was demonstrated with a linear signal response across the working range of the assay. Full Scan and SIM operation at 70,000 resolution, allowed for additional spectral analysis without the need for repeat injections or sample preparation while maintaining baseline resolution from interferences in the MS spectrum.

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 radiolabel techniques. While these techniques provide for rapid sample analysis, they also contain inherent limitations, such as the occurrence of false positives, increased time for method development and costly and complex sample preparation. High resolution accurate mass LC/MS coupled with multiplexing technology provides a selective and sensitive alternative to traditional methods without the need for labeled substrates and with comparable analytical speed. Multiplexing coupled with Full Scan and SIM analysis was used to targeted and non-targeted data, providing valuable spectral information in a high throughput format with and easy to use generic MS method.

Methods

Sample Preparation

Two phosphorylated peptides were ordered in powder (catalog numbers: CREBtide 29865 and CREBtide 27018, AnaSpec™, San Jose, CA). Each peptide was reconstituted in a 50:50 ACN:Water solution at 1 mg/mL. Serial dilutions of CREBtide 29865 were prepared at concentrations of 1 nM, 5 nM, 10nM, 25 nM, 50 nM, 100 nM, and 200 nM in an 50mM Sodium Citrate solution and also in a 50 mM HEPES solution containing 50 mM MgCl2, 50mM Citric Acid, 1mM TCEP, 0.08mM EDTA, 0.01% (v/v) Tween20, and 1% (v/v) DMSO). CREBtide27018 was then added to each dilution level as an internal standard at a final concentration of 100 nM.

Liquid Chromatography and Multiplexing

Samples were injected onto a C18, 4.6 x 20 mm, 3.5 µm HPLC column. Analyte elution was accomplished using mobile phase A - water + 0.05% Trifluoroacetic acid (v/v) and mobile phase B - Acetonitrile + 0.05% Trifluoroacetic acid (v/v) with a 1-minute step gradient at a flow rate of 1.5 mL/min. The step gradient was performed at 0-10 sec, 2% B, 11-35 sec, 35%B, 36-45 sec, 98% B, and 46-58 sec, 2% B. All methods were completed using a Thermo Scientific Transcend LX-4 system with a dual injector arm with DLW (Dynamic Load and Wash). LC multiplexing was implemented with a data window of 12 seconds per LC channel.

Mass Spectrometry

A Thermo Scientific Q Exactive bench-top Orbitrap mass spectrometer was used in several different scan modes, Full Scan, SIM, and Full Scan all-ions-fragmentation (AIF). Full Scan data was collected across a mass range of 620-710 m/z at resolution 70,000 and a spectral speed of 3 Hz. A maximum inject time of 250ms for the Full Scan was applied. SIM data collection at resolution 70,000 and a spectral speed of 3 Hz was set for two center masses (642 and 677m/z) with a 6 amu isolation window applied to the quadrupole for each mass. A maximum inject time of 120ms for each SIM mass was applied with multiplexed (i.e., simultaneous) analysis of both components. Full Scan AIF was collected with Full Scan across a mass range of 620-710 m/z at resolution 70,000 and a spectral speed of 3 Hz, and alternating Full Scan AIF across a range of 150 – 2000 m/Z at resolution 17,500 and a spectral speed of 15 Hz using a Normalized Collision Energy of 35. A maximum inject time of 250ms for both the Full Scan MS and Full Scan AIF was applied.

Ion source conditions were consistent for all MS experiment scan modes with the following parameters held constant, Vaporizer temperature 350°C, capillary temperature 230°C, sheath gas 60, and AUX gas 30. Prior to experimental data collection, the instrument was calibrated in positive ion mode using ProteoMass™ LTQ/FT-Hybrid ESI Pos. Mode Cal Mix (Sigma-Aldrich, St. Louis, MO).

In all runs, multiple sample injections were collected into a single data file format using Thermo Scientific Xcalibur and Aria software to automatically control data acquisition start and stop times for each sample set. A commercially available phosphorylated peptide was analyzed at various concentration levels and over multiple replicates to determine the coefficient of variation, linear response, and lower limit of quantitation. Results for each experiment type were compared for overall performance and ease of use.

Data Analysis Software

Thermo Scientific Pinpoint software was used for exact mass calculation 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, Atlanta, GA) 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. The compound information was stored in QuickCalc software 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 easily recalled and applied to an entire data set for easy chromatographic review. QuickCalc software was also used to automatically extract and sort each of the 384 injections contained in a data file and then display individual chromatographic peaks in a table view correlated with all sample information related to each individual injection.

Results

Sample Infusion

A sample solution containing analyte and internal standard teed with mobile phase and infused to determine optimal charge state of the peptide for analysis as well as to measure the isotopic distribution of the isotopic envelope. MS spectrum in the 3+ charge state provided the most intense response and isotopic distribution correlated with the values predicted by the Pinpoint software. (**FIGURE 1**)

FIGURE 1. Exact mass calculations for CREBtide 27018 and CREBtide 29865 using Pinpoint™ software. (Left) The isotopic envelope for 27018 (Middle) and 29865 (Right) infused in neat solution at 100uM concentration

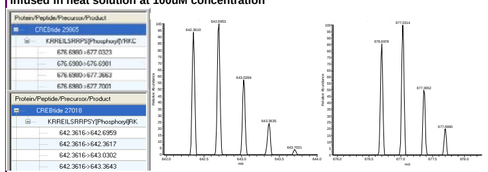
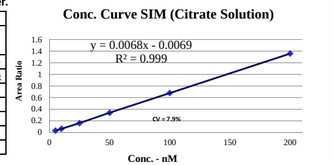
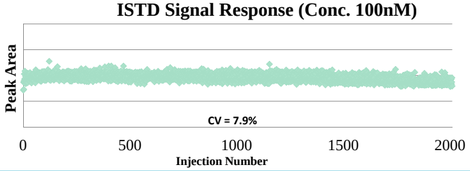


FIGURE 2. Samples in sodium citrate solution collected in SIM mode. Area ratio response from 5nM to 200nM plotted vs concentration and internal standard response plotted vs. injection number.

SIM Conc. nM	Avg. Ratio	%CV	
1	0.0125	29.7	LOD
5	0.0293	17.5	LOQ
10	0.061	12.1	
25	0.1583	6.6	
50	0.3382	5.6	
100	0.6779	3.9	
200	1.3587	4.4	





SIM Mode

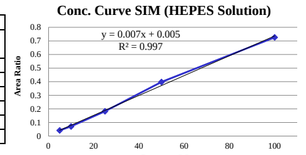
SIM mode is a targeted analysis whereby unwanted ions are selectively filtered at the quadrupole before analysis in the Orbitrap™ analyzer. However in SIM mode the quadrupole can be set to collect all ions in an isotopic envelop while filtering ion outside the range of the isotopic envelope maintaining selectivity and instrument performance.

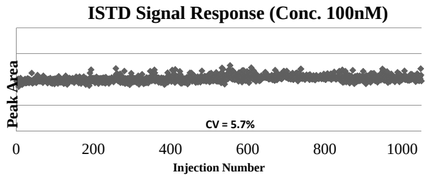
Samples in Sodium Citrate solution were analyzed at seven concentration points ranging from 1nM to 200nM with 288 replicate injections at each concentration level. The average peak area ratio was used to calculate %CV at each concentration and generate a calibration curve. The LOD and LOQ were determined to be 1nM and 5nM respectively with a linear signal response from 5nM to 200nM. Additionally signal response from the internal standard was plotted for 2016 injections and the %CV determined to be 7.9%.(**FIGURE** 2)

Samples in HEPES Buffer solution were analyzed at six concentration points ranging from 1nM to 100nM with 288 replicate injections at each concentration level. The average peak area ratio was used to calculate %CV at each concentration and generate a calibration curve. The LOD and LOQ were determined to be 1nM and 5nM respectively with a linear signal response from 5nM to 100nM. Additionally signal response from the internal standard was plotted for 2016 injections and the %CV determined to be 5.7%. (**FIGURE 3**)

FIGURE 3. Samples in HEPES solution collected in SIM mode. Area ratios from 5nM to 100nM plotted vs. concentration (middle) and internal standard response plotted vs. injection number.

SIM Conc. nM	Avg. Ratio	%CV	
1	0.0169	40.6	LOD
5	0.0413	23.9	LOQ
10	0.0706	23.8	
25	0.1821	11.2	
50	0.3976	9.9	
100	0.7259	9.3	





Full Scan

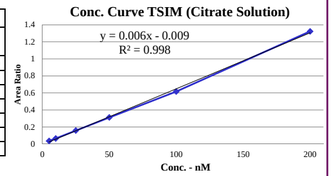
Full Scan data was collected across a mass range of 620-710 m/z allowing the collection of the entire isotopic envelope for both the analyte and internal standard in a single MS experiment as well any unexpected changes or modifications to the analyte peptide. Samples in 50mM Sodium Citrate solution were analyzed in Full Scan mode across a seven concentration points ranging from 1nM to 200nM with 50 replicate injections at each concentration level. The LOD and LOQ were determined to be 1nM and 5nM respectively with a linear signal response from 5nM to 200nM (**FIGURE 4**).

Full Scan AIF

Full Scan AIF is semi targeted analysis consisting of two alternating scan types. In the first scan, a relatively wide range of ions is permitted to pass through the quadrupole for analysis in the Orbitrap analyzer. In the second scan, data from the same range of ions is permitted to pass through the quadrupole and then directed to the high energy collision dissociation cell (HCD) for ion fragmentation rather than directly to the Orbitrap analyzer. After fragmentation in the HCD cell, the ions are then redirected to the Orbitrap analyzer for analysis. Full Scan AIF was collected across a mass range of 620-710 m/z with a resolution setting of 70,000 for the Full Scan analysis and a resolution setting of 17,500 for the MSMS analysis. Samples in 50mM Sodium Citrate solution were analyzed in Full Scan mode across a five concentration points ranging from 5nM to 200nM with 150 replicate injections at each concentration level. The LOD and LOQ were determined to be 5nM and 10nM respectively with a linear signal response from 10nM to 200nM (**FIGURE 5**).

FIGURE 4 .Samples in Sodium Citrate solution collected in Full Scan mode. Area ratios from 5nM to 200nM plotted vs. concentration (middle) and internal standard response at 100nM plotted vs. injection number. (right)

Full Scan Conc. nM	Avg. Ratio	%CV	
1	0.0124	28.3	LOD
5	0.035	12.1	LOQ
10	0.0646	7.6	
25	0.1568	6.0	
50	0.3162	4.2	
100	0.6162	4.2	
200	1.315	3.1	



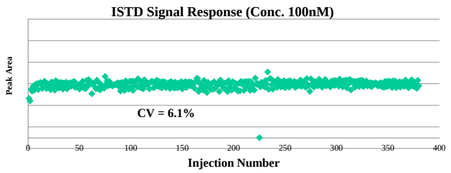
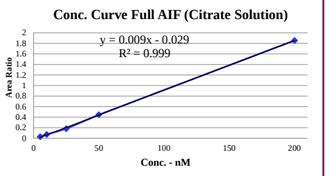
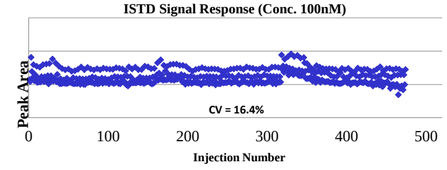


FIGURE 5. Samples in Sodium Citrate solution collected in Full Scan AIF mode. Area ratios from 5nM to 200nM plotted vs. concentration (middle) and internal standard response at 100nM plotted vs. injection number. (right)

Full AIF Conc. nM	Avg. Ratio	%CV	
5	0.0286	45.9	LOD
10	0.0706	17.8	LOQ
25	0.1821	15.6	
50	0.3976	13.7	
100	0.7259	11.7	





Z' Calculations

A common statistical measure to demonstrate the reliability and robustness of an assay is Z'. The Z' calculation incorporates the standard deviation and mean across the dynamic range for the 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 robust assay with a Z' = 1 describing a perfect assay. Samples analyzed in Full Scan Mode in Sodium Citrate solution were determine to produce a Z' = .85. Samples analyzed in SIM Mode in Sodium Citrate solution were also determine to produce a Z' = .85. Samples analyzed in SIM Mode in HEPES solution were determine to produce a Z' = .66. Additional variability can be seen when components such as detergent and reducing agents are added to the sample matrix but separation by LC enable a robust assay of large sample sets.

Although the fragment data was not utilized for confirmation in this study, the data collected in Full Scan AIF mode demonstrates a suitable number of Full Scan mode data points across the LC peak for accurate quantitation while also collecting MSMS data from 150-2000m/z. (**FIGURE 6**)

Resolution

Without the specificity provided by MS/MS, it is important to confirm that the mass resolution of the instrument method provides adequate separation from interferences in the sample matrix. In both the Sodium Citrate solution and HEPES solution, resolution at 70,000 provided base line separation in the MS spectrum for the most intense peptide isotopes and isolation from isobaric interferences at a mass accuracy of less than 5ppm for all injections. (**FIGURE 7**)

FIGURE 6. Alternating Full Scan and Full Scan AIF. Alternating scan mode provides an avg. of 7-11 scan per mode across 3 second peak. Full Scan XIC Analyte and ISTD (a and b). Full Scan AIF TIC (c). Base Peak for AIF Scan (d and e).

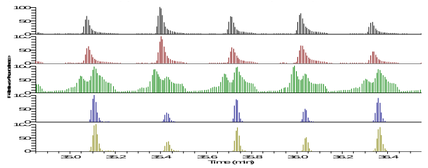
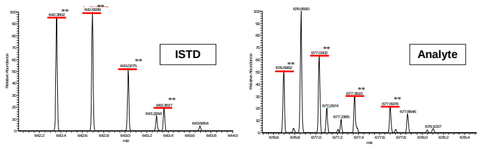


FIGURE 7. Full Scan MS Spectra for analyte and internal standard at 70,000 resolution in HEPES solution at LOQ for analyte.

****** **Denotes ions of interest, all other ions result from impurities in sample matrix.**



Conclusion

- Liquid chromatography with high resolution mass spec analysis provided a robust and reproducible proof of concept method for high throughput screening analysis of phosphorylated peptides.
- Full scan data collection across a wide mass range enabled the acquisition of spectral information for the entire isotopic charge envelope without sacrificing data quality.
- Statistical analysis of a large injection set resulted in Z' values ranging from 0.66 to 0.85 indicating method robustness the analysis.
- Multiplexing using a four-channel LC system provided rapid injection cycle times of 18 seconds, on average, while proving separation from detergents and other interferences containing in sample matrixes.
- Mass accuracy of better than 5 ppm was maintained throughout all injections using external recalibration.
- Low sensitivity and linear dynamic range was achieved with various instrument scan modes enabling non targeted Full Scan operation as well as more selective experimentation in SIM mode.

References

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2. Roddy,T.P.; Horvath,C.R.; Stout,S.J. *Anal. Chem.* **2007,** 79, 8207-8213.