STANDARD OPERATING PROCEDURE

Title: Mass Spectrometry Using Parallel Reaction Monitoring for Experiments 1 and 2

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Purpose

The purpose of this document is to describe the mass spectrometric methods for quantitative analysis of peptides in a complex matrix using stable isotope dilution mass spectrometry as described in Experiments 1 and 2 (https://assays.cancer.gov/guidance-document/).

Paired, high purity internal standards for surrogate peptides for 214 kinases were analyzed by LC-MS with parallel reaction monitoring (PRM) on a triple quadrupole-Orbitrap™ mass spectrometer (ThermoFisher Q-Exactive™). The PRM-MS methods were adapted from Peterson et al. (2012).

Scope

This procedure encompasses the optimization of a *nano*-LC-MS method for the scheduled acquisition of full scan MS2 spectra for 420 high purity H/L peptide pairs from 210 kinases in a single LC-MS analysis. The method (PRM, parallel reaction monitoring) to acquire the data for reverse response curves and assess measurement repeatability for each peptide using a Q-Exactive™ hybrid quadrupole mass spectrometer is detailed. The kinases and surrogate peptides are given in WU-SOP-EXP1-02. The configuration and optimization of the PRM method were performed with Skyline software (https://brendanx-uw1.gs.washington.edu/labkey/project/home/software/Skyline/begin.view). The procedures for calibrating and determining the performance of the system using standard benchmark peptides and a standard complex peptide mixture are described in WU-SOP-MS3-01. The gradient methods for both Experiments 1 and 2 are described in WU-SOP-LC2-01.

Responsibilities

It is the responsibility of person(s) performing this procedure to be familiar with laboratory safety procedures and the user manual for the instruments. The interpretation of results must be done by a person with expertise in mass spectrometry and familiar with such interpretation. It is the responsibility of the primary instrument operator to perform the instrument evaluations described in WU-SOP-MS3-01 during the acquisition of data for Experiments 1 and 2.

Equipment

- Source: EASY-Spray™ source (ThermoFisher)
- LC-to-source connection: nano Viper™ tubing (ThermoFisher)
- Q-Exactive triple quadrupole-Orbitrap™ mass spectrometer
- EASY-nLC™ 1000 (Thermo Scientific, LC120)
- EASY-Spray Column: 75 µm x 50 cm PepMap™ RSLC C18, 2 µm, 100 Å (Thermo Scientific, ES803)
- Injection loop: 20 µL PEEKsil™, 100 µm (Thermo Scientific, LC472)
- Micro-centrifuge: Eppendorf 5424 R
- Rainin™ Pipet-lite XLS, P20, P200, P1000.

Materials

- Autosampler vials: Sun-Sri (200 046)
- Autosampler vial caps: Sun-Sri (501 382)
- Clear vials (4 mL, National Scientific, B7990-2)
- Axygen® MAXYmum™ recovery tips: P200 and P20: T-200-C-L-STK; P1000: T-1000-C-L-R.

Reagents

- Acetonitrile (Fluka, 34967-1L)
- Formic Acid, (Fluka, 56302-50ML-F)
- Pierce Retention Time Calibration Mixture, ThermoFisher (88321)
- Pierce HeLa Protein Digest Standard, ThermoFisher (88328)
- Water (Fluka, 39253-1L-R).

Solutions

- TEN-MIX-1-100 solution (preparation described in WU-SOP-EXP1-02 and WU-SOP-EXP2-02)

Procedure

1. Identifying Scheduling Times for Targeted Peptides
   (NOTE: The assessment of LC-MS system performance is documented in WU-SOP-MS3-01)
   a. Place vial containing equimolar solution of all standard peptides into autosampler (solution preparation in WU-SOP-EXP1-02).
   b. Begin data acquisition with optimized instrument according to WU-SOP-MS3-01.

a. Building a scheduled method.
   i. Set up the autosampler and LC methods as described in WU-SOP-LC2-01 for either Experiment 1 (WU-SOP-EXP1-02) or Experiment 2 (WU-SOP-EXP2-02).
   ii. Inject 2.5 µl of the QC sample containing equimolar H/L standard peptide mix (200 fmol/2.5 µL) light/heavy standard peptides in target matrix and acquire the DDA data. The preparation of the equimolar sample is described in WU-SOP-EXP2-01.
   iii. Convert and search file and build library of the .dat files in Skyline document.
   iv. Load raw data file into skyline document.
   v. Manually check the automatic peak selection/integration of all peaks. Adjust if necessary. See Skyline tutorial, "Targeted Method Refinement" for reference. https://brendanx-uw1.gs.washington.edu/labkey/wiki/home/software/Skyline/page.view?name=tutorial_method_refine).
   vi. Export an isolation list for the PRM targets under File/Export/Isolation List. Set instrument as Q-Exactive, single method and for Method Type, select Scheduled. See Skyline tutorial, "Custom & Live Reports" for reference. https://brendanx-uw1.gs.washington.edu/labkey/wiki/home/software/Skyline/page.view?name=tutorial_custom_reports.

b. Scheduled method testing.
   i. Import the scheduled precursor m/z list as an inclusion list under global settings in the DIA method on the QExactive instrument with parameters set as above (steps 1 and 2).
   ii. Set up the autosampler and LC methods as described in WU-SOP-LC2-01.
   iii. Inject a (2.5 µl) 'HIGH' quality control sample containing equimolar H/L standard peptides (WU-SOP-EXP2-02) spiked in assay matrix and acquire the scheduled PRM-MS data.

c. Instrument performance evaluation with scheduled method.
   i. Import the .raw files into the Skyline document.
   ii. Check the automatic peak selection/integration of all peaks.

1. Manually adjust integration of peaks, if necessary. See Skyline tutorial, "Targeted Method Refinement" for reference. https://brendanx-uw1.gs.washington.edu/labkey/wiki/home/software/Skyline/page.view?name=tutorial_method_refine
2. Check integration by determining if start and stop are identical for all transitions of a precursor (go to "Settings", and check box "Integrate All" to enable this feature automatically.

d. Assess peak shape.
   i. No tailing or fronting.
   ii. No missing data (e.g. drop-out of electrospray).
   iii. If the peaks are unacceptable, troubleshoot the LC system using the benchmark procedure described above and in WU-SOP-LC2-01 and re-run the column conditioning procedure.

e. Apply criteria for peak consistency.
   i. Retention time shift is < 0.4 min between injections.
   ii. Peak intensities between injections is <20% variation

2. Final Method Build

a. Save Skyline method test file as a final method.
b. Remove unscheduled DDAi (Step 5A) and method building scheduled DDAi .raw files (Step 5B) from the document (Edit>Manage Results). Save the document. The resulting Skyline document should now contain the most recent scheduled method testing data files with current retention times.
c. Export the scheduled precursor m/z list with retention times as an isolation list using above parameters.
d. Import the isolation list into PRM method on the QExactive instrument with all other parameters set as above (steps 1 and 2).

10. Analysis of Response Curve for Characterization of Assays

a. LC-PRM-MS method preparation
   i. Load the Skyline file containing peptide sequences, precursors, transitions, and retention times to be monitored during the LC-PRM-MS experiment.
   ii. Export the scheduled precursor m/z list as an isolation list (File-Export-Isolation list) and select data files for RT setting. Set the RT window to 3 minutes. See Skyline tutorial, "Custom & Live Reports" for reference. https://brendanx-uw1.gs.washington.edu/labkey/wiki/home/software/Skyline/page.view?name=tutorial_custom_reports

iii. Import the scheduled precursor m/z list as an inclusion list in the Xcalibur Mass spectrometer method on the QExactive instrument with all other instrument parameters set as above (steps 1 and 2).
iv. Set up the autosampler and LC methods as described in WU SOP-LC2-01.

b. Experiment 1 or Experiment 2.
i. Refer to WU-SOP-EXP1-02 or WU-SOP-EXP2-02 for details on samples and queues.

Table 1. PRM Method Parameters	
Source/Gas Parameters:	
IonSpray Voltage (IS)	1800
S-lens	60
Interface Heater Temperature (IHT)	275ºC
Scheduled PRM Parameters	
PRM detection window (sec)	180
Target Scan Time (sec)	
Full MS-SIM Parameters	
Resolution	70,000
AGC target: 2e5	3.00E+06
Maximum IT	60ms
Spectrum data type	profile
MS2 Parameters (DIA)	
Collision Energy (CE)	27
Isolation window	2.0 m/z
Resolution MS2	17,500
AGC target	2.00E+05
Maximum IT	60 ms
Spectrum data type	profile
Loop count	30

Referenced Documents

- WU-SOP-MS3-01
- WU-SOP-LC2-01
- WU-SOP-EXP1-02
- WU-SOP-EXP2-02
- Skyline manual and tutorials. https://brendanx-uw1.gs.washington.edu/labkey/wiki/home/software/Skyline/page.view?name=tutorials
- Peterson AC, Russell JD, Bailey DJ, Westphall MS, Coon JJ. Parallel reaction monitoring for high resolution and high mass accuracy quantitative, targeted proteomics. Molecular & cellular proteomics : MCP. 2012;11(11):1475-88. Epub 2012/08/07. doi: 10.1074/mcp.O112.020131. PubMed PMID: 22865924; PMCID: Pmc3494192.
- ThermoFisher Scientific customer manuals website

Abbreviations

- AcN, acetonitrile
- FA, formic acid

- LC-MS, nano-LC interfaced to a high-resolution quadrupole-orbitrap mass spectrometer as described in WU-SOP-LC-1 and WU-SOP-MS-1
- H or heavy, stable isotopically labeled synthetic peptide
- L or light, natural abundance synthetic peptide
- β-GAL, standard tryptic digest of β-galactosidase
- Q.S., quantum satis
- PDX, patient-derived xenografts
- PRM, parallel reaction monitoring mass spectrometry
- PS, primary stock solution; prepared by direct dilution and transfer from the vendor vials.
- HSS, secondary stocks of the heavy primary peptide stock solution.
- LSS, secondary stocks of the light primary peptide stock solution.