STANDARD OPERATING PROCEDURE

Title: Optimizing Mass Spectrometer Performance for Experiments 1 and 2

SOP#: WU-SOP-MS3-01

Version #: 1

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Purpose

The purpose of this document is to describe the procedures for calibrating , optimizing and benchmarking the performance of the high-resolution hybrid triple-quadrupole mass spectrometer that is used to generate scheduled full scan MS2 data for CPTAC Experiments 1 and 2 (https://assays.cancer.gov/guidance-document/) . The system is an EASY-nanoLC™ coupled to a Q-Exactive™ mass spectrometer (ThermoFisher).

Scope

This procedure encompasses the i) preparation of benchmark solutions; ii) spectral acquisition of benchmark peptides iii) optimization procedure; and iv) assessment of instrument performance after calibration and tuning.

Responsibilities

It is the responsibility of person(s) performing this procedure to be familiar with laboratory safety procedures and the user manual for the instrument. 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 these procedures when the system has failed to meet specification requirements that are set by the vendor and the laboratory. Figure 1 shows the monitoring of the LC-MS during the acquisition of data for Experments 1 and 2.

Equipment

- Source: EASY Spray™ Source (ThermoFisher) (EASY-Spray Series Ion Source User Guide)
- LC to Source Connection: Nano Viper TM Tubing (ThermoFisher)
- Q Exactive™ Hybrid Quadrupole Orbitrap™ Mass Spectrometer (ThermoFisher)
- EASY-Spray™ column (PepMap, C18, 2µm particles, 50 cm x 75 µm ID) (ThermoFisher)
  NOTE: Additional details of instrument setup and operation is available at the ThermoFisher Scientific Custmer Manual website.

Materials

- Syringe (Hamilton 8175) (250 µL)
- PEEK Tubing, red, 1/16" x 0.005" (IDEX part# 00301-22912)
- Graduated cylinders (10 mL and 100 mL)
- Glass media bottle (Pyrex).

Reagents

- Peptide Retention Time Calibration Mix (500 fmol/µL) (Pierce # 88320)
- Pierce Hela tryptic digest (part#88328)
- 0.1% Formic Acid in Water (Honeywell Burdick & Jackson, LC452-2.5)
- 0.1% Formic Acid in Acetonitrile (Honeywell Burdick & Jackson, LC441-2.5)
- LTQ Velos ESI Positive Ion Calibration Solution (Pierce, 88323)
- LTQ Velos ESI Negative Ion Calibration Solution (Pierce, 88324)
- Methanol (SIGMA, 34860)
- House DI water
- Nitric Acid (Fluka, 84385-500mL)

Solutions

- Peptide Retention Time Calibration Stock Solution (50 fmol/µl)
- HeLa Standard (100 ng/µl HeLa digest)

Procedure

1. Preparation of Benchmark Solutions for Instrument Performance
    a. Preparation of Peptide Retention Time Calibration Stock Solution (50 fmol/µl)
        i. Prepare a 50 fmol/µl PRTC stock by adding 450 µL of 0.1% Formic Acid in Water to the 50 µL 500 fmol/µL PRTC stock vial

ii. Vortex for 15-30 s and spin down to collect solution at the bottom of the vial.
iii. Pipette into 20 µL aliquots into autosampler vials and freeze at -20°C

b. Preparation Of HeLa Standard (100 ng/µl HeLa digest)
i. Add 200 µ l PRTC Stock to the 20 µg HeLa vial
ii. Vortex for 30-60 seconds.
iii. aliquot into 20 µL aliquots into autosampler vials and freeze at -20°C

2. Preparation of Instrument for Analysis of Benchmark Solutions
a. Alignment of EASY Spray Source™

This procedure assumes that the instrument is clean and ready to run

b. Checking column emitter alignment
i. Turn the instrument into standby in the tune window before starting this procedure.
ii. Use the emitter positioning tool to set x, y, and z positions for column emitter alignment and proximity to the ion transfer tube.
1. Center the tip of the emitter positioning tool on the ion transfer tube.
2. Adjustments in the x direction (side to side) can be made by turning the left-side hex socket-head screw on the housing of the EASY-spray™ source.
3. Adjustments in the y direction (up and down) can be made by turning the bottom hex socket-head screw.
4. Adjustments in the z direction (front to back) can be made by turning the Z-axis positioning control knob located below the column holder. The emitter positioning tool should touch the ion transfer tube.
iii. Remove the emitter positioning tool and replace it with the column.

c. Checking spray stability
i. Turn instrument into standby in the tune window.
ii. Start flow on the LC system at 300nL/min with 20%B. a bubble should form at the emitter tip and the backpressure should stabilize (between 550-650 bar depending on age of the column).
iii. Load the correct tune file for nanoflow and turn instrument ON. The bubble at the end of the emitter tip should start getting smaller and should disappear over time. If pulsing/ bubble formation persists, adjust the spray current (typically around 2-2.5 kV)
iv. Check TIC variation in the tune window under instrument, current scan (top right). The variation should be <10% to be considered stable.

3. Preparing to Run the Q Exactive
a. Tuning the instrument optimizes source parameters with regards to ionization technique and LC flow only. All other optics have been optimized at the factory and only need to be calibrated after venting and cleaning, after

installation of new parts, or if instrument evaluation fails. Follow instructions in the Operating manual.

b. Three parameters that are used for optimization:
    i. Spray voltage (2-2.5kV)
    ii. Capillary temperature (250-350°C)
    iii. S-lens RF level (50-60)
c. Adjust parameters to maximize signal as described in the Q-Exactive™. manual.

4. Assessment of Instrument Performance using Benchmark Solutions
    a. Setup MS Properties for PRTC (see Table 1)
    b. Import inclusion list into MS DIA method (see example Table 2)
    c. Preparation of test solution (PRTC working solutions)
        i. Aliquot 5 µL of PRTC solution into 500 µL Eppendorf tubes. Freeze at -80C.
        ii. Add 495 µL of 10% AcN/0.1%FA to the Eppendorf tube to make a 50 fmol/µL PRTC solution.
        iii. Vortex vigorously for 30 sec.
        iv. Spin 30 seconds at 14,000 rcf.
        v. Store as 20 µL aliquots in AS vials at -20ºC.
        vi. Inject 2µL on column (WU-SOP-LC2-01).
    d. Begin data acquisition
    e. Assessing LC-MS Performance During Acquisition of Data for Experiments 1 and 2
        i. LC-MS analyses with the standard PRTC samples are imported into Skyline to assess intensity, peak shape, retention time and mass error for the 15 precursor masses, and the y-series transitions from each precursor spectrum.
        ii. The benchmark specifications are given below:
            - TIC: 1e9 or better
            - MS1 intensity: +/- 20% intensity compared to reference at outset
            - MS2 INTENSITY: +/- 20% intensity compared to reference at outset
            - Retention times: within 0.3 minutes of the average retention time
            - The optimization steps and LC-trouble shooting is initiated if instrument fails to meet specifications (WU-SOP-MS3-01).
            - See Figure 1 for Example Data
    f. LC-MS of Complex Protein Digest (Hela)
        i. Setup MS Properties for Hela (see Table 1)
        ii. Repeat as for PRTC above

iii. Begin data acquisition

5. Procedure for Mass Calibration – Positive Mode
    a. Attach the HESI-II source and set parameters according to Table 3
    b. Infuse the standard at 3 µl/min
    c. Check
        i. all masses are present
        ii. Intensity is at least $1e^8$
        iii. TIC variations < 10%
        iv. IT < 5 ms
    d. Start calibration. At the end of the procedure a dialog window will appear with status of the calibration. All successful calibrations are automatically saved in the master.cal file.
6. Procedure To Clean ion transfer tube
   Note: cleaning past the S-lens is performed by Unity Lab services.

    a. Turn the MS off by clicking the On/Standby button in the tune software window to shut off all high voltages and sheath and auxiliary gas.
    b. Remove the EASY-spray™ source. Wait for the front end to cool down before proceeding.
    c. Remove the ion transfer tube using the specialized tool.
    d. Sonicate ion transfer tube in water, followed by 30% nitric acid, then DI water for 20 min each. Rinse with 100% methanol (LC-MS grade) and dry by blowing off with nitrogen. Check if ion transfer tube is clean using a microscope.
    e. Install ion transfer tube (make sure the o-ring on the ion source interface is seated correctly before installing the ion transfer tube).
    f. Install HESI-II source and follow mass calibration instructions (see above)
    g. Remove HESI-II source and install EASY spray™ source.

7. Procedure To clean the S-lens
    a. Turn the MS off by clicking the On/Standby button in the tune software window to shut off all high voltages and sheath and auxiliary gas.
    b. Place electronics service switch, located on the power panel, in Service Mode position
    c. Put the main power circuit breaker switch of the MS in the Off position.
    d. Remove the nano source. Wait for the front end to cool down before proceeding.
    e. Remove the ion source interface using the lever tool. Pull the assembly straight out.
    f. Remove the S-lens from the interface cage. Remove the exit lens by loosening the two thumb screws.

g. Clean the exit lens and S-lens plates with a 6000 grit MICRO-MESH fastened to a small spatula that has been moistened in 1% Alconox solution. Use a swab for the circular holes in the plates of the S-lens.
h. Rinse well with warm tap water, followed by rinsing with DI water. Sonicate for 20 minutes in LC-MS grade methanol. Dry with nitrogen.
i. Reassemble the exit lens, S-lens and interface cage. Insert interface assembly and push until you hear a click. Install ion transfer tube (make sure the o-ring on the ion source interface is seated correctly before installing the ion transfer tube).
j. Open the tune window on the computer before starting up the mass spectrometer.
k. Place main power circuit breaker switch to the ON position. The forepump and turbomolecular pumps should start automatically. Allow mass spectrometer to pump down for 5 min.
l. Turn electronics service switch to Operation Mode.
m. Monitor the pump speed in the tune window to make sure they reach operating speed.
n. Start the Bakeout procedure.
    i. Put the system in off condition in the Tune software window.
    ii. Click the Vaccuum/Bakeout task panel
    iii. Set desired Bakeout length (minimum 12 h)
    iv. Click on Bakeout and confirm
    v. After Bakeout the system needs 3-6 hours to cool down and stabilize before instrument calibration can be started.

Referenced Documents

- "Overview of assay characterization for the CPTAC assay portal", CPTAC Assay Development Working Group (https://assays.cancer.gov/)
- WU-SOP-MS4-01- Mass Spectrometry Using Parallel Reaction Monitoring for Experiments 1 and 2.
- Exactive™ Series Operating Manual, ThermoFisher Scientific
- EASY-Spray Series Ion Source, ThermoFisher Scientific
- 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
- Q.S., quantum satis
- MS, mass spectrometer

- PRM, parallel reaction monitoring mass spectrometry
- PRTC, Peptide Retention Time Calibration
- TIC, Total Ion Chromatogram

Table 1. Instrument Parameters		
	PRTC	**HeLa**
Global Settings		
User Role	Advanced	Advanced
Use lock masses	off	off
Chromatography peak width (seconds)	15 s	15 s
Gradient Time (minutes)	36 min	133 min
General		
Run Time (minutes)	0 to 36 min	0 to 133 min
Polarity	positive	positive
In-source CID	0.0 eV	0.0 eV
Default Charge	2	2
Inclusion	Yes	N/A
Exclusion	N/A	N/A
Tags	N/A	N/A
Full MS		
Microscans	1	1
Resolution	70,000	70,000
AGC target	3.00E+06	1.00E+06
Maximum IT	50 ms	60 ms
Scan range (m/z)	350 to 2000 m/z	375 to 1500 m/z
Spectrum data type	profile	profile
dd-MS2 / dd-SIM DIA		
Microscans	1	1
Resolution	17,500	17,500
AGC target	2.00E+05	1.00E+05
Maximum IT	auto	60 ms
Loop count	15	10
MSX count	1	1
TopN		10
MSX isochronous Its	on	
Isolation window: 2.0 m/z	2.0 m/z	2.0 m/z
Isolation offset	0.0 m/z	0.0 m/z
scan range:	N/A	200-2000 m/z
Fixed first mass	N/A	100 m/z
NCE / stepped	27	27
Spectrum data type: profile	profile	profile

Table 1. Instrument Parameters (cont'd)		
dd Settings		
Underfill ratio	N/A	2%
Minimum AGC target	N/A	2.00E+03
Intensity threshold	N/A	3.30E+04
Apex trigger	N/A	N/A
Charge exclusion unassigned	N/A	1
Peptide match	N/A	preferred
Exclude isotope	N/A	on
Dynamic Exclusion (seconds)	N/A	20s

Table 2. Retention Times for Pierce Calibration Peptides

m/z	CS [z]	Peptide
493.77	2	SSAAPPPPPR
613.32	2	GISNEGQNASIK
496.29	2	HVLTSIGEK
451.28	2	DIPVPKPK
422.74	2	IGDYAGIK
695.83	2	TASEFDSAIAQDK
586.80	2	SAAGAFGPELSR
773.90	2	ELGQSGVDTYLQTK
558.33	2	GLILVGGYGTR
801.41	2	GILFVGSGVSGGEEGAR
745.39	2	SFANQPLEVVYSK
498.81	2	LTILEELR
573.30	2	NGFILDGFPR
680.37	2	ELASGLSFPVGFK
787.42	2	LSSEAPALFQFDLK

Table 3. Mass Calibration Parameters	
Sheath gas flow rate	10
AUX gas flow rate	0
Sweep gas flow rate	0
Spray Voltage (kv)	3.5
Spray current	(actual 0.8)
Capillary temp (oC)	300
S-lens RF level	50.0
Heater temp (°C)	0 or 'off' (actual 47)

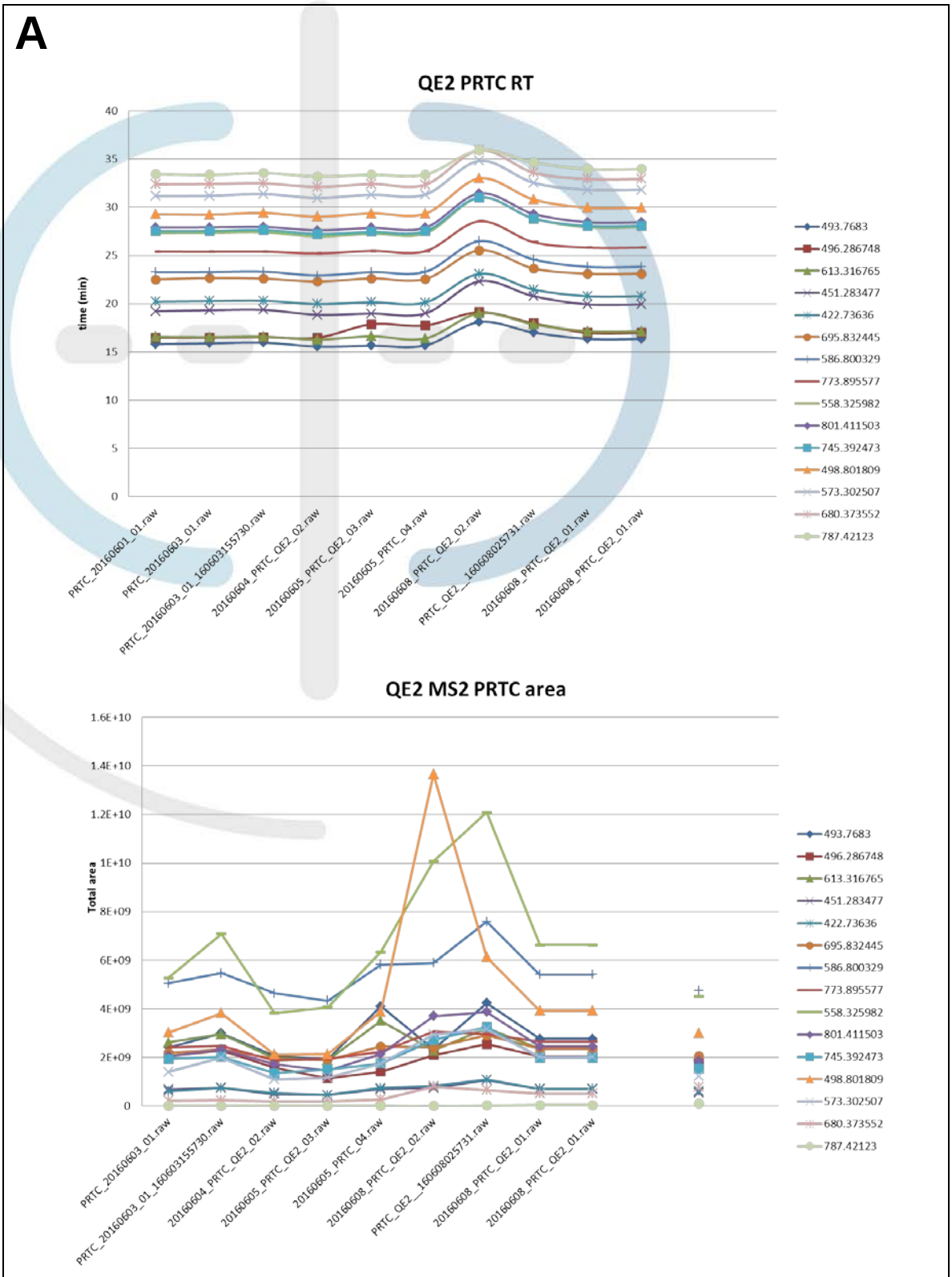

Figure 1. PRTC Peptide Data to Assess Instrument Performance. A) Retention times for the PRTC benchmark peptides over a five day period ; **B**) Peak area sum for all y ion transitions for each benchmark peptide.

STANDARD OPERATING PROCEDURE

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

SOP#: WU-SOP-MS4-01

Version #: 2

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Reviewer: Sherri Davies PhD.
WU-PCC Director: R. Reid Townsend, MD, PhD.

Date: 06/30/2016

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.