STANDARD OPERATING PROCEDURE

Title: Optimizing Mass Spectrometer Performance for Experiments 1 and 2

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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.

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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)

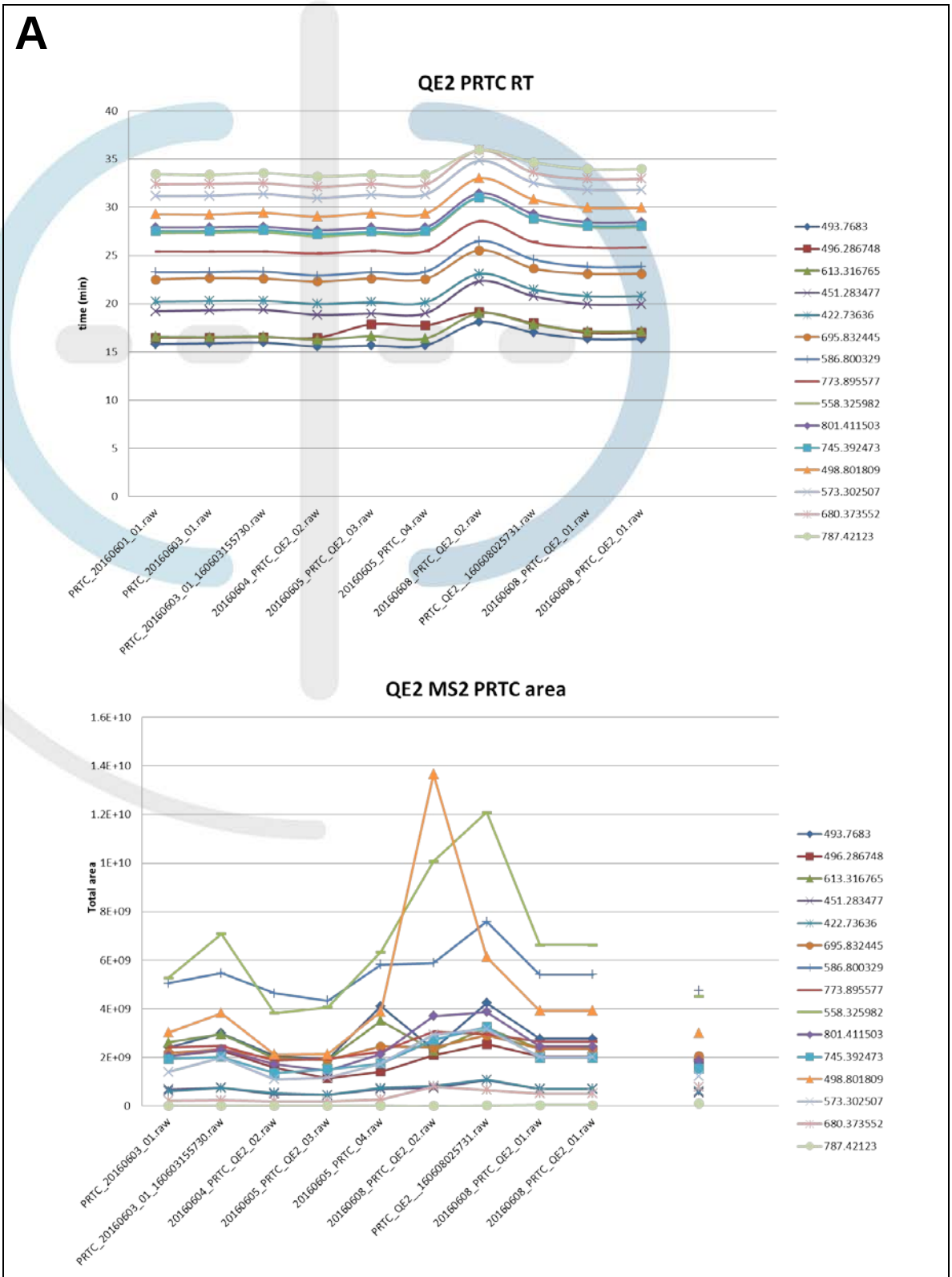

Figure 1. PRTC Peptide Data to Assess Instrument Performance. A) Retention times for the PRTC benchmark peptides over a five day period ; **B**) Peak area sum for all y ion transitions for each benchmark peptide.