STANDARD OPERATING PROCEDURE

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

SOP#: WU-SOP-MS2-01

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1. PURPOSE

The purpose of this document is to describe the mass spectrometric (MS) methods for quantitative analysis of peptides using paired internal standards and LC-MS with parallel reaction monitoring (PRM).

2. SCOPE

This procedure encompasses the optimization of methods for a TripleTOF® 5600+ mass spectrometer that are used to execute Experiments 1 and 2 for a multiplex assay of 130 synthetic peptide pairs. The method builds and optimizations are performed using the Skyline software (https://brendanx-uw1.gs.washington.edu/labkey/project/home/software/Skyline/begin.view). The *nano*-liquid chromatography methods and dual column setup are described in WU-SOP-LC1-01.

3. 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 daily monitoring of the system for meeting specifications described herein, and performing instrument evaluations as described in WU-SOP-MS1-01.

4. EQUIPMENT

Source: New Objective Digital Picoview for PV-450
Emitter tip: New Objective PicoTips® emitter silica tips (FS360-20-10N-20-C20)
LC-to-source connection: Fused silica tubing, Polymicro Technologies (1068150009)
Mass spectrometer: Sciex TripleTOF® 5600+

Micro-centrifuge: Eppendorf 5424 R
Rainin™ Pipet-lite XLS, P20, P200, P1000

5. MATERIALS

Standards, β-galactosidase tryptic digest (625 pmol) (Sciex, 4465867)
Standard peptides: QCH (WU-SOP-EXP1-1)
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

6. REAGENTS

Acetonitrile (Fluka, 34967-1L)
Formic Acid, (Fluka, 56302-50ML-F)
Water (Fluka, 39253-1L-R)

7. PROCEDURE

A. Mass Spectrometer Default Parameters (see WU-SOP-MS1-01 for optimization method)

1) Source/Gas Parameters:
    a) Curtain Gas (CUR): 25
    b) IonSpray Voltage (IS): 3200
    c) Ion Source Gas 1 (GS1): 30
    d) Ion Source Gas 2 (GS2): 0
    e) Interface Heater Temperature (IHT): 175ºC
2) Scheduled PRM Parameters:
    a) PRM detection window (sec): 600
    b) Target Scan Time (sec): 1.5
3) MS Parameters:
    a) Declustering Potential (DP): 70
    b) Collision Energy (CE): From Skyline (default ABI TTOF 5600 algorithm)
    c) Resolution Q1: Unit resolution
    d) Resolution TOF MS1: >25000
    e) Resolution TOF MS2 >15000
    f) Intensity threshold (total count): 100
    g) Settling time (ms): 0
    h) Pause between mass ranges (ms): 1.038

B. Column Conditioning

1) Run 102 min IDA method 10 times and inject 10 µL of TEN-MIX-1-100 solution (preparation described in WU-SOP-EXP1-01).

C. Assessing LC-MS Performance

1) Preparation of test solution (β-galactosidase tryptic digest working solutions)

    a) Add 625 µL of 10%AcN, 0.1%FA to vendor vial to prepare a 1 pmol/µL primary stock solution
    b) Vortex vigorously for 30 sec.
    c) Spin 30 seconds at 14,000 rcf.
    d) Dilute 1:20 with **AcN/FA-1** (described in WU-SOP-EXP1-01) in 4 mL glass vial to prepare secondary stock (50 fmol/µL).
    e) Store as 200 µL aliquots in AS vials at -20ºC.
    f) Thaw secondary stock at room temperature.
    g) Dilute the secondary stock ten-fold with the **AcN/FA-1** solutions.
    h) Transfer 200 µL to each of three autosampler vials for injection of 50fmol/10µL by the autosampler (WU-SOP-LC1-01).

2) Assessing instrument performance for LC-MS with PRM mass spectrometry

    a) LC-MS analyses with the standard β-GAL samples are opened in PeakView® to assess the total ion current trace. Files are processed in the LCMS Peak Statistics application. A report is exported for MS1 data and MS2 data to a .csv file. The .csv files are opened in Excel and the data are copied to the TTOF_performance_CURRENT Excel spreadsheet. The Excel spreadsheet plots TOFMS resolution, intensity and retention time for the 729 *m/z* precursor mass of a β-galactosidase tryptic peptide, and the 5 most intense transitions from the spectrum.

MS1 m/z	Transitions	Transition m/z
433.8792	y3	347.2037
450.6960	y5	563.2784
528.9341	y8	832.4523
542.2645	y10	1061.522
567.0551	y12	1289.633
607.8588	Parent	729.3652
671.3379		
729.3652		

    b) The benchmark specifications are given below:
        - TOFMS resolution >25,000
        - 729 intensity: ≥ 1e5
        - MS2 resolution >15,000
        - 832 intensity ≥ 500

- The optimization steps and LC-trouble shooting is initiated if instrument fails to meet specifications (WU-SOP-MS1-01).

D. Identifying Scheduling Times For Targeted Peptides

1) Targeted LC-PRM-MS method building.
   a) Load the Skyline file (WU-SOP-MS-2-MB1) containing peptide sequences, precursors, and transitions to be monitored during the LC-PRM-MS experiment.
   b) In the Skyline document under Settings/Transition Settings/Predictions, select 'ABI TTOF 5600 under 'Collision energy:' and 'ABI' under 'Declustering potential'.
   c) Export the unscheduled precursor *m/z* list as a report File/Export/Report.
   d) Import the unscheduled precursor *m/z* list as a DDA with inclusion list (DDAi) method on the TTOF 5600+ using the Analyst® TF v1.6 (http://sciex.com/products/software/analyst-software) method wizard with all other instrument parameters set as above (Part 1).

2) Building a scheduled method.
   a) Set up the autosampler and LC methods as described in WU-SOP-LC-1 and for either Experiment 1 (WU-SOP-EXP1-01) or Experiment 2 (WU-SOP-EXP2-01).
   b) Inject a (10 µl) of the QC sample containing equimolar H/L standard peptide mix (20 fmol/uL) light/heavy standard peptides in target matrix and acquire the PRM-MS data. Perform acquisition on both LC columns.
   c) Import the data files for each column into the Skyline document (3A).
   d) 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).
   e) Export the scheduled precursor *m/z* list as a report in Skyline with a column for "Precursor Mz" and "Average Measured Retention Time"averaged using above scheduled parameters. 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.

3) Scheduled method testing.
   a) Import the scheduled precursor *m/z* list as a scheduled DDAi method on the TTOF 5600+ using the Analyst® TF method wizard with all other instrument parameters set as above (Part 1).
   b) Set up the autosampler and LC methods as described in WU-SOP-LC-1.

c) Inject a (10 µl) 'HIGH' quality control sample containing equimolar H/L standard peptides (WU-SOP-EXP2-01) spiked in assay matrix and acquire the scheduled PRM-MS data. Perform acquisition on both LC columns.

4) Instrument performance evaluation with scheduled method.
   a) Import the data files into the Skyline document (Step 3A).
   b) Check the automatic peak selection/integration of all peaks.
      i. 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
      ii. 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.
   c) 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-LC-1 and re-run the column conditioning procedure.

   a) Apply criteria for peak consistency between columns.
      i. Retention time shift is < 2 min between columns.
      ii. Peak intensities between columns is <20%.

1) Final method build.
   a) Save Skyline method test file as a final method.
   b) Remove unscheduled DDAi (Step 3A) and method building scheduled DDAi data files (Step 3B) 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 averaged retention times as a report using above scheduled parameters.
   d) Import the scheduled precursor m/z list as a scheduled DDAi method on the TTOF 5600+ using the Analyst® TF method wizard with all other parameters set as above (step 1).

E. Analysis of Response Curve For Characterization of Assays.

1) LC-PRM-MS method preparation
   a) Load the Skyline file containing peptide sequences, precursors, transitions, and retention times to be monitored during the LC-PRM-MS experiment.

b) In the Skyline file under Settings/Transition Settings/Predictions, select 'ABI TTOF 5600 under 'Collision energy:' and 'ABI' under 'Declustering potential'.
c) Export the scheduled precursor *m/z* list as a report in Skyline with a column for "Precursor Mz" and "Average Measured Retention Time"averaged using above scheduled parameters. 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
d) Import the scheduled precursor *m/z* list as a scheduled DDAi method on the TTOF 5600+ using the Analyst® TF method wizard with all other instrument parameters set as above (Part 1).
e) Set up the autosampler and LC methods as described in WU SOP-LC1-01.

2) Experiment 1 or Experiment 2 .
a) Refer to WU-SOP-EXP1-01 or WU-SOP-EXP2-01 for details on samples and queues.

8. REFERENCED DOCUMENTS

A. WU-SOP-MS1-01
B. WU-SOP-LC1-01
C. WU-SOP-EXP1-01
D. WU-SOP-EXP2-01
E. Skyline manual and tutorials. https://brendanx-uw1.gs.washington.edu/labkey/wiki/home/software/Skyline/page.view?name=tutorials

9. LIST OF ABBREVIATIONS

AcN, acetonitrile
FA, formic acid
LC-MS, *nano*-LC interfaced to a high-resolution quadrupole-time-of-flight 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.