STANDARD OPERATING PROCEDURE

Title: MRM mass spectrometry for the analysis of immuno-MRM assay samples using a 5500 QTRAP

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Purpose

The purpose of this document is to describe a multiple reaction monitoring (MRM) method for quantitative analysis of peptides from immunoaffinity enriched samples (immuno-MRM samples). The method employs retention time scheduling for enhanced quantification.

Scope

This procedure includes the setup of MRM methods on a 5500 QTRAP. Liquid chromatography (LC) parameters and methods are described in a separate SOP ("Liquid Chromatography using an Eksigent NanoLC-2D, an Eksigent AS1 autosampler, two Agilent 10-port valves, a C18 trap column, and a ReproSil C18 analytical column for immuno-MRM experiments").

Responsibilities

It is the responsibility of person(s) performing this procedure to be familiar with laboratory safety procedures. The interpretation of results must be done by a person trained in the procedure and familiar with such interpretation.

Equipment

- Mass spectrometer: ABSciex 5500 QTRAP
- Ion Source: ADVANCE CaptiveSpray Source for ABSciex (Michrom Bioresources/Bruker)
- Emitter tip: CaptiveSpray tapered tip 20 µm ID (Michrom Bioresources, SS9/25000/20)
- LC-to-source connection: 75 µm ID, 360 µm OD IntegraFrit column (IF360-75-50-N-5, New Objective), packed in-house to 10.0 cm with ReproSil-Pur C18-AQ, 3 µm (Dr. Maisch GmbH, Ammerbuch, Germany)

Materials

- Water, Optima® LC/MS, suitable for UHPLC-UV (W6-4, Fisher Scientific)
- Acetic acid (ACS reagent, ≥99.7%, 242853, Sigma)
- Acetonitrile, Optima® LC/MS, suitable for UHPLC-UV (A955-4, Fisher Scientific)
- Formic acid (FA) (1.11670.1000, EMD Millipore)

Reagents

- Tryptic, light synthetic peptides were purchased at >95% purity as measured by HPLC. Cysteine residues of the peptides were carbamidomethylated. Amino acid analysis (AAA) was performed to determine peptide concentrations. Individual peptide stock aliquots were stored in 30% acetonitrile-0.1% formic acid at 100 pmol/µL at -80 °C. (Of note is that light peptides are used here instead of heavy peptides, since the response curve and repeatability experiments used light peptides as internal standards, see the SOPs titled "Peptide immunoaffinity enrichment for the characterization of immuno-MRM assays using response curves" and "Peptide immunoaffinity enrichment for the characterization of intra- and inter-day repeatability of immuno-MRM assays (Validation Samples)."
- A synthetic light peptide master mix with a 2 pmol/µL concentration per peptide was prepared from the individual light peptide stocks at 100 pmol/µL using 3% (not 30%) acetonitrile-0.1% formic acid as diluent. This master mix was aliquoted and stored the 2 pmol/µL master mix in 10 µL aliquots at -80 °C.
- A synthetic light peptide master mix sample at 2.5 fmol/µL concentration per peptide is to be prepared fresh daily from the 2 pmol/µL master mix using 3% acetonitrile-5% acetic acid (3% acetonitrile-5% acetic acid is used for the elution of immunoaffinity captured samples). The 2.5 fmol/µL concentration is based on a 10 µL injection volume to yield 25 fmol of peptide injected on column and represents a suggested concentration; the concentrations should be adjusted based on a particular set of peptides under study.

Procedure

1. Setup MRM method parameters
    a. Source/Gas Parameters:
        i. Curtain Gas (CUR): 20
        Note: No curtain gas is actually used with the CaptiveSpray source; however, since the Curtain Gas parameter in the Analyst software cannot be set to 0, an arbitrary value of 20 was used here.
        ii. IonSpray Voltage (IS): 1.3 kV
        iii. Ion Source Gas 1 (GS1): 0
        iv. Ion Source Gas 2 (GS2): 0
        v. Interface Heater Temperature (IHT): 110 °C
        vi. Collision Gas (CAD): Medium

b. Scheduled MRM Parameters
Note: These parameters were used for an 18 minute gradient, from approximately 5-50% organic phase, with the aim of obtaining at least 10 data points per peak.
   i. MRM detection window: 120 sec
   ii. Target Scan Time: 1 sec

c. MS Parameters:
   i. Declustering Potential (DP): 100 V
   ii. Entrance Potential (EP): 10 V
   iii. Collision Energy (CE): From Skyline[1], based on slope and intercept parameters of 0.0528 and -2.1786 for charge state +2, and 0.0448 and -1.2844 for charge state +3; these values had been previously optimized for this particular 5500 QTRAP instrument. For peptides for which charge state +1 was monitored, the CEs for transitions were optimized using Skyline's CE optimization feature[2].
   iv. Collision Cell Exit Potential Q1 (CXP): 10 V

d. Advanced MS Parameters:
   i. Resolution Q1: Unit
   ii. Resolution Q3: Unit
   iii. Intensity Threshold (total count): 0
   iv. Settling time: 0 ms
   v. Pause between mass ranges: 5.007 ms

2. Test the system suitability with an appropriate standard once the column is conditioned.
Note: For example, a 6 bovine protein tryptic digest equal molar mix, PTD/00006/63 (Bruker) could be used. The standard should be injected at least twice to be able to assess retention time reproducibility and whether peak shapes and intensities are acceptable based on historic data. The assessment can be done by viewing the data in the 5500 QTRAP Analyst software or by importing the data into Skyline and performing a more rigorous system suitability analysis[3].
   a. Retention time shift of <0.5 minutes (a third injection might be needed if the first two runs' retention times shift by >0.5 min; the LC system needs to be troubleshot if the retention times still shift >0.5 min after a third run).
   b. Minimal tailing or fronting (check the LC connections if necessary).
   c. No drop-out of electrospray (sparge the LC solvents or replace the emitter tip if needed).
   d. If the peak intensities are unacceptable, troubleshoot the autosampler or clean the MS.
3. Identify scheduling times for target peptides/transitions
   a. Target LC-MRM method preparation
      i. Load the Skyline file containing peptides and transitions that will be monitored during the analysis.

ii. In the Skyline file under Settings/Transition Settings/Predictions, add a new CE equation based on the slope and intercept parameters above, or select the default parameters by selecting 'ABI 5500 QTrap DQ' under 'Collision energy;' select 'Static' under 'Declustering potential'.
iii. Export the unscheduled transition list using AB SCIEX as instrument type, and by selecting Single method, ignoring proteins, Optimizing: None, Method type: Standard, and Dwell time (ms): 5.
iv. Import the unscheduled transition list into an unscheduled MRM acquisition method on the 5500 QTRAP with all other parameters set as above (step 1).

b. Timing the peptide detection
   i. Set up the autosampler and LC methods as given in the SOP "Liquid Chromatography using an Eksigent NanoLC-2D, an Eksigent AS1 autosampler, two Agilent 10-port valves, a C18 trap column, and a ReproSil C18 analytical column for immuno-MRM experiments."
   ii. Inject 10 µL of the 2.5 fmol/µL light peptide master mix sample using the unscheduled acquisition method.
   iii. Import the data files into the Skyline file.
   iv. Check the automatic integration of all peaks and adjust integrations manually if needed.
   v. Export a scheduled transition list from Skyline using the "values from a single data set" and choosing the correct data file.
c. Running a first immuno-MRM sample (see e.g. the SOP titled "Peptide immunoaffinity enrichment for the characterization of immuno-MRM assays using response curves") and adjusting the scheduling if needed
   i. Import the scheduled transition list into an MRM acquisition method on the 5500 with all parameters set as above (step 1)
   ii. Set up the autosampler and LC methods as in the SOP titled "Liquid Chromatography using an Eksigent NanoLC-2D, an Eksigent AS1 autosampler, two Agilent 10-port valves, a C18 trap column, and a ReproSil C18 analytical column for immuno-MRM experiments."
   iii. Inject one immuno-MRM sample (e.g. a 0 fmol / 10 µL plasma sample of a response curve experiment, see the SOP titled "Peptide immunoaffinity enrichment for the characterization of immuno-MRM assays using response curves").
d. Instrument performance evaluation and adjustment of scheduled retention times
   i. Import the latest data file into the Skyline file.
   ii. Check the automatic integration of all peaks.
      1. Make sure integration start and stop is identical for all transitions of a precursor (go to "Settings", and check "Integrate All" to enable this feature automatically).

2. Manually adjust the integration of peaks if necessary.

iii. Check that peak shapes are acceptable.
   1. Minimal tailing or fronting.
   2. No drop-out of electrospray (for this, no smoothing should be applied in Skyline (View/Transform/None)).
   3. No missing transitions.

iv. Export a new scheduled transition list from Skyline if needed (the retention times can shift based on whether peptides are tested in a buffer background or whether the matrix is more complex, such as in the eluted samples after an immunoaffinity capture from a plasma matrix).

4. Once the instrument is performing acceptably, run the remaining samples of the immuno-MRM experiments in the order given in the SOPs titled "Peptide immunoaffinity enrichment for the characterization of immuno-MRM assays using response curves" or "Peptide immunoaffinity enrichment for the characterization of intra- and inter-day repeatability of immuno-MRM assays (Validation Samples)." The file naming convention follows:
   a. For the response curve experiments: Date_AntibodyGroup_TypeOfExperiment_AnalyteAmount_CaptureReplicate#_InjectionReplicate#
   b. For the repeatability (validation) experiments: Date_AntibodyGroup_TypeOfExperiment_AnalyteLevel_CaptureReplicate#_InjectionReplicate#

Referenced Documents

SOP "Liquid Chromatography using an Eksigent NanoLC-2D, an Eksigent AS1 autosampler, two Agilent 10-port valves, a C18 trap column, and a ReproSil C18 analytical column for immuno-MRM experiments"

SOP "Peptide immunoaffinity enrichment for the characterization of immuno-MRM assays using response curves"

SOP "Peptide immunoaffinity enrichment for the characterization of intra- and inter-day repeatability of immuno-MRM assays (Validation Samples)"

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optimization on the measurement of peptides by selected reaction monitoring (SRM) mass spectrometry. *Anal. Chem*. 82, 10116-10124

3. Abbatiello, S. E., Mani, D. R., Schilling, B., Maclean, B., Zimmerman, L. J., Feng, X., Cusack, M. P., Sedransk, N., Hall, S. C., Addona, T., Allen, S., Dodder, N. G., Ghosh, M., Held, J. M., Hedrick, V., Inerowicz, H. D., Jackson, A., Keshishian, H., Kim, J. W., Lyssand, J. S., Riley, C. P., Rudnick, P., Sadowski, P., Shaddox, K., Smith, D., Tomazela, D., Wahlander, A., Waldemarson, S., Whitwell, C. A., You, J., Zhang, S., Kinsinger, C. R., Mesri, M., Rodriguez, H., Borchers, C. H., Buck, C., Fisher, S. J., Gibson, B. W., Liebler, D., MacCoss, M., Neubert, T. A., Paulovich, A., Regnier, F., Skates, S. J., Tempst, P., Wang, M., and Carr, S. A. (2013) Design, implementation and multisite evaluation of a system suitability protocol for the quantitative assessment of instrument performance in liquid chromatography-multiple reaction monitoring-MS (LC-MRM-MS). *Mol Cell Proteomics* 12, 2623-2639.