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| STANDARD OPERATING PROCEDURE |
| |  |  | | --- | --- | | **Title: LC-MRM Mass Spectrometry Analysis of Peptide Surrogate Markers of Protein Expression on TSQ Quantiva and Data Processing in Skyline** | | |  |  | | **Version #: 1** | **Author: Koomen Lab on behalf of the RAS Immuno-MRM Working Group** | | **Date: 5/30/2018** |  | |

# Purpose

The purpose of this document is to describe the liquid chromatography – multiple reaction monitoring mass spectrometry (LC-MRM) method used for quantification of protein expression by targeting unmodified surrogate peptides.

# Scope

This procedure may be used set up an LC-MRM method to run digested protein samples containing desalted peptides. Parameters are specific to a Dionex RSLCnano LC system using the trap and elute mode interfaced with a Thermo TSQ Quantiva triple quadrupole mass spectrometer.

# 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

* LC System: Dionex Ultimate 3000, RSLCnano System
* Autosampler: Dionex Ultimate 3000, RS Autosampler (Nano)
* Mass Spectrometer: Thermo TSQ Quantiva with NanoFlex source

# Materials

* Injection loop: NanoViper sample loop, 20 µL, FS/PEEK sheathed (Thermo, 6826.2420)
* Trap column: Acclaim Pepmap™ 100, 100 µm x 2 cm, C18, 5 µm, 100A (Thermo, 164564)
* Analytical column: Acclaim Pepmap™ RSLC, 75 µm x 25 cm, C18, 2 µm, 100A (Thermo, 164941)
* Spray tip: precut SilicaTip emitters (New Objective, FS360-25-10-N-20-CT)
* Autosampler vials, 250 µL (Thermo, 160133)
* epT.I.P.S. reloads (Eppendorf)
* Pipettes

# Reagents

* Water, HPLC grade (Burdick & Jackson, AH365-4)
* Acetonitrile, HPLC grade (Burdick & Jackson, AH015-4)
* Methanol, HPLC grade (Burdick & Jackson, AH230-4)
* Formic Acid, Optima LC-MS Grade, ≥99.5% (Fisher, A117-50)
* Pierce Retention Time Calibration Mixture (Thermo, 88321)

# Solutions

All solvents must be degassed.

* Loading Solvent: 1.5% acetonitrile, 0.1% formic acid
* Mobile phase A: 1.5% acetonitrile, 0.1% formic acid
* Mobile phase B: 90% acetonitrile, 0.1% formic acid
* Wash solvent
  + Prepare 40% acetonitrile, 50% methanol in HPLC water.
  + Place in an autosampler vial in the autosampler
* Sample buffer: 2% acetonitrile, 0.1% formic acid
* PRTC Sample
  + Dilute the pierce retention time calibration mix to 50 fmol/ µL (10x) to use in each sample for retention time calibration and instrument QC.
  + Dilute to 5 fmol/ µL
  + Place 5 fmol/ µL solution in a fresh autosampler vial in the autosampler
* Blank/A solvent wash
  + Place sample buffer into a fresh autosampler vial and put in the autosampler

# Procedure

**LC-MRM Method**

1. All samples are formulated in aqueous 2% acetonitrile, 0.1% formic acid with 5 fmol/ µL PRTC standards added to assess instrument performance.
2. The autosampler uses the microliter pickup mode and sample injections are 5 µL each. Sample injection volume must be less than 1/3 of the sample loop (~6.67 µL).
3. Set the column compartment to 30 oC.
4. Prepare a fresh transfer vial with fresh mobile phase A (aqueous 2% acetonitrile, 0.1% formic acid).

The following LC method is used:

1. Load samples isocratically at 2% B with a flow rate of 2.4 µL/min for 7.5 minutes.
2. Switch the trap column in line with the analytical column at 7.5 minutes, and run the gradient specified in the table below.

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| --- | --- | --- |
| **Time (min)** | **% B** | **% A** |
|
| 0 | 2 | 98 |
| 6 | 2 | 98 |
| 8 | 10 | 90 |
| 50 | 28 | 72 |
| 55 | 50 | 50 |
| 56.5 | 92 | 8 |
| 62.5 | 95 | 5 |
| 63.5 | 2 | 98 |
| 80 | 2 | 98 |

1. Wash the system with two acetonitrile/methanol wash injections.
2. Equilibrate the sample loop with an injection of a background matrix digest sample and two mobile phase A solvent wash injections.
3. Assess system suitability with two PRTC QC injections prior to running all samples. Retention times must be stable (within 1 minute of each other) and peak shapes should have at most minimal tailing or fronting. Peak widths should be < 30-40s and be consistent. Peak intensity should be within 25% of historical average.
4. Examine pressure trace to assess LC system. Pressure should not be too high and should not have abrupt changes except when valve switching and no signifincant changes in pressure, except during the high mobile phase B solvent wash and return to high mobile phase A equilibration.
5. If system suitability is not acceptable, perform routine instrument maintenance.
6. If retention times have changed, export a new transition list from Skyline to adjust the expected times for peak detection.
7. Prepare the method with scheduled windows using Skyline and export the transition list.
8. Add transition list to the LC-MRM method using Xcalibur.
9. Use the following MS parameters in the LC-MRM method:

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| --- | --- |
| MS Resolution | Q1: 0.4, Q3: 0.7 |
| MS Spray Voltage | 2200 V |
| Transfer Tube Temp | Transfer tube 275° C |
| CID Gas | 2 mTorr |
| Dwell time | 14-24 ms (varied) |

1. Blank injections are inserted between every sample to reduce carryover, particularly for hydrophobic or other peptides bound strongly to the LC column packing material.
2. Prepare a list of names using the following conventions:
   1. Date\_Project\_Experiment\_Sample\_Replicate
   2. Experiment 1 Example: 020718\_RAS-Unmod\_F\_1
   3. Experiment 2 Example: 020718\_RAS-Unmod\_E2\_Med\_D3\_R1
3. Inject a matrix blank at the beginning and end of each calibration curve replicate sequence to generate a blank measurement and for carryover assessment.
4. A high organic wash and A solvent wash are both injected after the carryover blank following high concentration samples (such as the high curve concentration and high sample for repeatability.
5. Randomize the sample order of the repeatability experiment to simulate real samples with varying concentrations of endogenous analytes.

**Skyline Data Processing**

1. Raw data is imported into the Skyline (Reference 1) document containing all monitored peptides for data processing.
2. Annotations are specified by CPTAC assay portal instructions.
3. Make sure peaks are not transformed.
4. Pull down menu 🡪 View 🡪 Transform. Select either none or interpolated.
5. After all annotations are completed as specified, add the Peak Areas – Replicate Comparison View. (F7)
6. Prepare the peak areas view settings to facilitate easy data review.
   1. Right click 🡪 Transitions 🡪 Single
   2. Right click 🡪 Normalize To 🡪 Light
   3. Right click 🡪Group By 🡪SampleGroup
   4. Right click 🡪 CV Values
7. CV values for data should be less than 20%.
   1. If the CV value is higher than 20%, investigate the nature of the variation (*e.g.* poor signal or interference with light or SIS peptide).
   2. Click on the bar graph to cycle through each file within each group.
   3. Make sure the window selected the entire peptide peak, adjust as necessary or narrow the selection if there are interfering transitions.
8. Force integration of blank values. (Drag while clicking the mouse under the peak, or select a transition and click the RT number.
9. Click on the precursor to see individual fragment CVs. If one stands out as a poor performer across the board, fix or remove.
10. Check the ratios for each fragment. If there is a transition that has a vastly different ratio than the others, fix or remove.
11. Save as with new file name. The document is ready to be uploaded and shared.

# Referenced Documents

MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, Kern R, Tabb DL, Liebler DC, MacCoss MJ. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics* **2010**, *26*, 966-8. PMID: 20147306 PMCID:[PMC2844992](https://www-ncbi-nlm-nih-gov.ezproxy.lib.usf.edu/pmc/articles/PMC2844992/)