PROTOCOL: GCP MS Analysis (MSA)

Purpose

Analyze and relatively quantify post-translational modifications on core histone H3 with a fully-targeted mass spectrometry assay.

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This protocol should be started day before or day of the beginning of mass spec time
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Preparation

1. Thaw enough “Equimix” {MSA-M04} for the assay. If not enough Equimix is available, see protocol “GCP Mastermix and Equimix Formulation”
2. Thaw enough “2x Mastermix” {MSA-M03} for the assay on ice. See procedure to determine amount needed. If not enough 2x Mastermix is available see protocol “GCP Mastermix and Equimix Formulation”
3. Prepare MSA-MIX01 if not enough available
4. Thaw dried eluate rack from “GCP Final Desalt” at room temperature

Materials

- Micronic Vials Rack {MSA-M01} [SOURCE NEEDED]
- Micronic PTFEE Pre-Slit Vial Caps {MSA-M02} [SOURCE NEEDED]
- 2x Mastermix {MSA-M03}
- Equimix {MSA-M04}
- Acetonitrile (ACN) {MSA-M05} [EMD Millipore, AX0156-1]
- Formic Acid (FA) {MSA-M06} [Sigma-Aldrich, S6302-50ML-F]
- HPLC-grade Water {MSA-M07} [JT Baker, 4218-03]
- 40 mL Amber Vial, Solid Screw Top, PTFE Liner {MSA-M87} [SUPLECO, 27182]
- Axygen foil seal {MSA-M09} [Axygen, PCR-AS-200]
- Matrix tube caps (not pre-slit) {MSA-M10} [Matrix Technology Corp, 4463]

Assets

- Bench-top vortex {MSA-A01}
- Bench-top mini centrifuge {MSA-A02}
- 12 channel multichannel pipette {MSA-A03}
- Proxeon Easy nLC Autosampler {MSA-A04}
- Thermo Q Exactive, Q Exactive +, or Q Exactive HF mass spectrometer {MSA-05}

Reagent Mixes

ID	Name	Step	Composition	Stock Volume	Use
MSA-MIX0	3%ACN/ 5% FA (%)	MSA	3% {MSA-M04} and 5%	40 mL	final resuspension and dilution of peptides. Helps

1			{MSA-M05} in {MSA-M06}		ionize peptides for better transfer into the mass spec
MSA-MIX02	Dilution Mastermix	MSA	{MSA-M03} at a 1.25x concentration in {MSA-MIX01}	As much as needed	Addition of heavy synthetic peptides for internal standards, and dilute sample to right concentration for MS analysis

Reagent Mix Preparation

MIX01-3% ACN/ 5% FA:

1. In a 40 mL amber vial {MSA-M07} add the following reagents:
   - ❒ 36.8 mL HPLC-grade Water {MSA-M06}
   - ❒ 1.2 mL ACN {MSA-M04}
   - ❒ 2 mL FA {MSA-M05}

   Invert 7 times to mix

Procedure

Sample Preparation

1. Add 10 uL of MSA-MIX01 to each sample in dried sample rack from "SepPak Desalt"
   a. Use 12 channel multichannel pipette {MSA-A03} to deliver reagent
2. Place an axygen foil seal {MSA-M09} on plate and vortex. Spin plate down. Repeat.
3. Make Dilution Mastermix {MSA-MIX02}:
   a. For a full plate make enough Dilution Mastermix {MSA-MIX02} for 110 samples:
      - ❒ 5 uL "2x MasterMix" {MSA-MSA03} x _____ samples = _____ uL 2x MasterMix
      - ❒ 3 uL ⅜ {MSA-MIX01} x _____ samples = _____ uL ⅜ {MSA-MIX01}

      Add reagents to 1.5 mL microcentrifuge tube. Vortex and spin down.
5. Add 8 uL Dilution Mastermix (MSA-MIX02) to each vial in a Micronic Vial Rack labeled with initials, date, study name, and "diluted 1:5 for MS analysis" {MSA-M01}
6. Add 2 uL sample to vials containing Dilution Mastermix in "mass spec" vial rack. Place a foil seal on the rack and spin down to mix. Cap vials with the Micronic Vial pre-slit caps {MSA-M02}. Store rack at 4℃ until it can be loaded onto the Proxeon Easy nLC 1000 autosampler {MSA-A05}. When the rack is ready to be loaded onto the deck, spin it down,
7. Cap vials containing remainder of resuspended sample with Matrix tube caps (not pre-slit) {MSA-M10} and store at -80℃.
8. **During data acquisition rack needs to be spun down every 2 days.**

NB: Samples can be prepared while scheduling runs occur on the mass spec simultaneously

Xcalibur Sequence Generation

NB: Instrument systems use Xcalibur software to coordinate running of samples. An Xcalibur sequence dictates the data file name, where the data shall be deposited, LC/MS method to use, and position of the sample on the autosampler deck. You can also attach a study name, unique sample identifier, and comment to each file. Generating the sequence is very important in making sure the correct data is associated with thae correct sample, and that the correct instrument methods are used in the assay

1) Create new study folder in "Xcalibur and Metadata Sheets" Folder inside the "Combined" folder with same naming format as prior studies. Copy/paste the document "UNIFIED UPDATED-Metadata and Xcalibur Sequence Template". Rename with the study plate.
2) Fill in "Sample Details" tab with the sample information dictated in each tab. "Additional Comment" column can include experiment-specific information
3) In "Acquisition Details" tab, fill in information in the top half of the fields. Fields in grey are automatically populated from the information provided
4) In "For Metadata Import" tab, drag equations in first row down to automatically populate the fields. Make sure information is seen for all samples. Value under "Provenance Code" column will always be "GR1"
5) In "For Xcalibur Sequence" tab, drag down equations so information is seen for all samples. Value under "Inj Vol" column will always be "1", and value under "Dil Factor" will always be "5"
6) Look over "For Xcalibur sequence" tab to make sure sequence is accurate and complete. Save this tab as a separate CSV file in the study folder. Make sure file has study name included.
7) On the instrument's computer open Xcalibur and click on "Sequence View" in the tool bar:

8) Click "File"→"Import Sequence"→"Browse"
9) Navigate to CSV file of sequence in your study folder. Once the file path is loaded, make sure all the boxes are checked and click "OK"
10) Your sequence should now be loaded into Xcalibur. If some columns are missing, you can make them visible by clicking "Column Arrangement" in the tool bar:

Scheduling the Targeted Mass Spec Method for Data Acquisition

NB: To increase sensitivity and guarantee that all modifications in the assay can be detected in each sample, a targeting MS method is first employed to determine the retention times of each peptide. The retention times are used to configure "windows" of time where the instrument scans only for the peptides falling within that time frame.

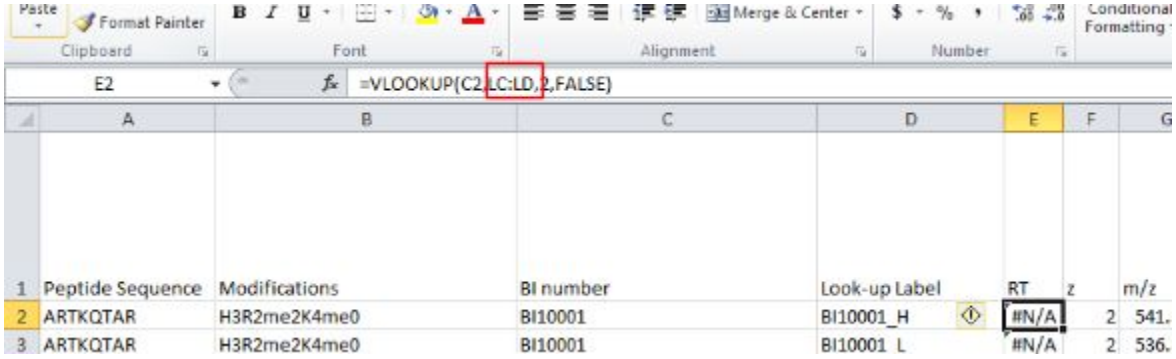
1) Place an HPLC vial of Equimix {MSA-M03} on the autosampler deck.
2) Run MS Method “H3_for_Scheduling_DIAway”, with two technical repeats of the Equimix sample:
    a) Parameters to use in Xcalibur sequence:
        i) Filename: Header used in study samples, “Equimix_for_scheduling”, technical repeat number
        ii) Path: same folder destination as samples
        iii) Inj Vol: 1
        iv) Comment: “Equimix for scheduling”
3) Open Skyline file “H3_only_template_annotations_Histone2013lib”. This file is found in: Histone-MRM\Skyline\SkylineDocuments\Current_Templates. File and save with date, name of study, and that that runs are for scheduling the assay. Save file in the “Rescheduling runs” Folder, in its own subfolder titled with the date
4) Once runs have finished collecting, import into Skyline file just created. QC peaks to make sure correct boundaries have been drawn.
5) Save QC’ed file and export report “RT Results for Scheduling” into folder containing the Skyline file. If your Skyline program does not have that report listed, you can load the template found in: Histone-MRM\Skyline\Shared Reports
6) Open recently created report in Excel. You should see columns labeled “Protein Name”, “Averaged Measured Retention Time”, and “Precursor Mz”
7) Using the text-to-columns function, separate the BI number from the PTM name in the column “Protein Name” label the column just containing BI numbers “BI number”
8) Insert a new column between “BI number” and “Average Measured Retention Time”. Label this column “Light/Heavy”. Fill column down with alternating L/H values

NB: Adding the L/H value next to the BI number columns denotes whether that row is the light or heavy version of the monitored peptide (should see two rows for every BI number, this is why). You can check to make sure your Light/Heavy label is correct by looking at the Precursor Mz values. Light versions have a lesser value than the heavy version of the peptide

9) Sort all columns by the ascending order of the values in “Light/Heavy”. Your sheet should now look like this:

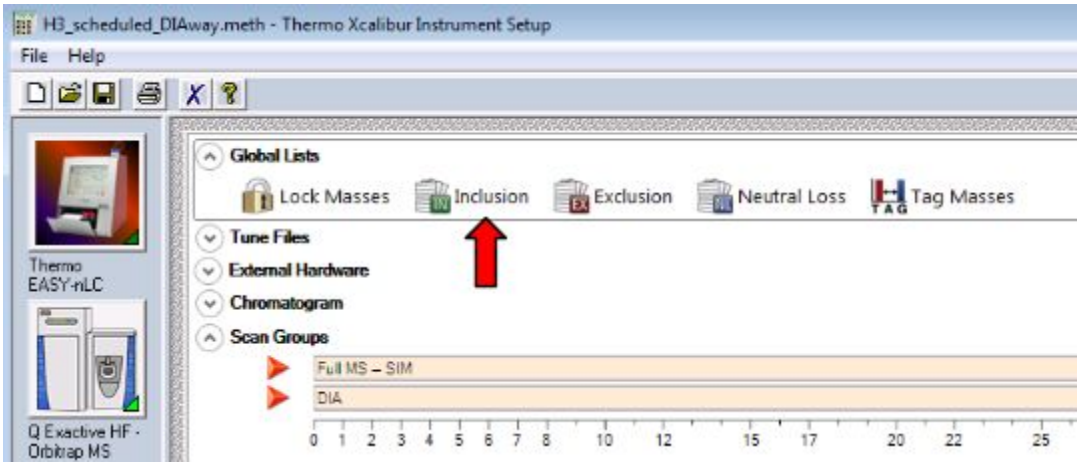
Protein Name	BI Number	Light/Heavy	Average Measured Retention Time	Precursor Mz
H3K4me0	BI10003	H	20.21	413.7365
H3K4me1	BI10004	H	24.25	420.7443
H3K4me2	BI10005	H	7.23	399.739
H3K4me3	BI10006	H	6.85	406.7468
H3K4ac1	BI10007	H	17.48	406.7286
H3K9me0K14ac0	BI10008	H	26.47	540.3078
H3K9me1K14ac0	BI10009	H	29.74	547.3156
H3K9me2K14ac0	BI10010	H	18	526.3103
H3K9me3K14ac0	BI10011	H	17.81	533.3182
H3K9ac1K14ac0	BI10012	H	24.37	533.3
H3K9me0K14ac1	BI10013	H	24.67	533.3
H3K9me1K14ac1	BI10014	H	27.98	540.3078
H3K9me2K14ac1	BI10015	H	16.23	519.3025
H3K9me3K14ac1	BI10016	H	16.04	526.3103
H3K9ac1K14ac1	BI10017	H	22.52	526.2921
H3K9me0S10ph1K14ac0	BI10018	H	28.02	580.291
H3K9me1S10ph1K14ac0	BI10019	H	31.17	587.2988
H3K9me2S10ph1K14ac0	BI10020	H	20.34	566.2935
H3K9me3S10ph1K14ac0	BI10021	H	20.03	573.3013
H3K9ac1S10ph1K14ac0	BI10022	H	25.78	573.2831
H3K9me0S10ph1K14ac1	BI10023	H	26.03	573.2831
H3K9me1S10ph1K14ac1	BI10024	H	29.35	580.291
H3K9me2S10ph1K14ac1	BI10025	H	18.54	559.2857
H3K9me3S10ph1K14ac1	BI10026	H	18.13	566.2935
H3K9ac1S10ph1K14ac1	BI10027	H	23.91	566.2753
H3K18ac0K23ac0	BI10028	H	37.97	582.8524

10) Your sheet should have separated all of peptides into H and then L, with the BI numbers in ascending order. Open up Excel file “Master rescheduling sheet Rev Aug2014 - H3, non-H3, R10K0, R10K8-USE THIS FOR SCHEDULING” found in the “Rescheduling runs” folder
11) Scroll to the end of the “RT mz z and ce of all peptides” tab. Copy/paste the “BI Number” and “Average Measured Retention Time” column from your scheduling report for just the first iteration of BI numbers (or all BI Numbers with an “H” label). Look to the left in of this file for examples. Label the tops of your columns with the gradient/column used and the date of scheduling. Look to the left in this tab for examples.
12) To make sure your scheduling agrees with past runs, subtract your retention times from the previous study. All peptides should differ by a consistent value (-1 min, 2 min, etc). If there are peptides with significantly different values, check the skyline for correct boundary picking.
13) Scroll to the very beginning of the tab. Change the locations in cell E2’s VLOOKUP equation, to the locations of your two columns:



14) Perpetuate changes down the column. All the values should change to your RT’s
15) Move to tab “Full list for Xcalibur”. This tab is the inclusion list layout for peptides monitored in this assay, with scheduled time windows based on the retention times provided in the first tab. Copy values up to and including row 98, and “Paste as Values” into the next tab “Copy for text only”
16) Save file and exit out. At the instrument’s computer, open up this file, and the instrument file “H3_scheduled_DIAway”. You can open this file by clicking on it from your sequence in Xcalibur.

17) Once the method file is open, Click the Mass Spectrometer Icon →"Global Lists"→"Inclusion":



18) In the inclusion list window, paste values from the "Copy for text only" tab from rows 2-98, columns B-K. Do not paste in the column headers. Nothing but the values in columns "Start [min]" and "End [min]" should change. There should also be the same number of rows before and after pasting in the new values.
19) Click "Done" and then press the "Save" icon. You have now just updated the inclusion list for this MS method. Exit out of the MS method file. You can now begin data collection

Monitoring your Data Collection

The following should be completed **every day** while you have samples running on an instrument:

1) Twice a day, the most recently completed run should be imported into Skyline. Use the file generated for scheduling, complete with equimix runs. Save As a new file in the study folder, with the study's name and that it is the working document. Example file name:
    a) LINCS_Y1Q1_Plate13_GCP_working
2) In Skyline check for the following aspects of the run: retention time, peak shape, peak width, intensity.
    a) Retention time should waver only about 0.3 minutes from run to run. If your runs gradually deviate a minute from the equimix retention times, reschedule the acquisition windows using the most recent run. Acquisition windows fall roughly 1.5 min before and after the scheduled retention time. If you do not monitor the RT's of your runs consistently you run the risk of missing data. If you see that your runs vary significantly (0.5 min or more between consecutive runs) you may have a leak in the HPLC system. It would be prudent to pause acquisition and check for leaks. As a column ages, you may observe peptides retaining later. Make sure they won't retain so late as to not elute.
    b) Peak shape and width should be relatively the same from run to run. Some peptides have specific characteristics. For example, H3K4me2 and H3K4me3 are normally very wide, jaggy, and elute early. If you are not familiar with the peak characteristics compare your data to historically curated files. If peak shape/width changes significantly over time it can either be column or LC-related. For example, mark H3K27me3K36me2 tails to the left. If you see the mark suddenly tail to the right you should stop acquisition and change columns to prevent further degradation of peak shape for samples yet to be analyzed. When checking for shape you should also compare peak width over time. If peaks become significantly wider (say a peak has been normally 0.2 min, and is now 0.4 min wide) you should stop data acquisition and change columns. Peaks can become so wide that they fall out of acquisition windows
    c) Intensities may gradually increase due to plate evaporation. While acquiring data, spin down and check plate every day or two to make sure wells yet to be run have sufficient volume (at

least 3-4 uL). If wells have less volume/look dry, add several uL 3/5 (MSA-MIX01). Record how much volume is added. If there is a sudden drop in peptide intensity, that proceeds for more than 2 samples stop acquisition. If only 1 sample has a sudden drop in intensity, try rerunning the sample. All equimix peptides should have intensities in the 5e3 to mide9 range. If peptides fall below 300x10^3 do not acquire data until equimix intensities are back up to par. If you have any confusion as to what a good equimix run should look like, refer back to curated historical data.