PROTOCOL MSU_MSMC_008 version 1
Protein molecular weight determination using electrospray ionization
Contributed by staff of MSU Mass Spectrometry and Metabolomics Core
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Purpose: To measure the molecular weight of a purified protein for verification that the protein has the expected mass or for recognition of posttranslational modifications.

Recombinant proteins purified from heterologous expression systems are routinely used for experiments including enzyme assays and antibody production. In many cases knowing an accurate molecular weight of the protein to a much higher accuracy (often ± 0.01%) than achieved by SDS-PAGE (~±10%) can help troubleshoot problems that can arise in experiments using the protein. For example, cleavage of a tag used for purification may not be resolved by SDS-PAGE but would easily be observed in the mass obtained by LC/MS. It is not uncommon for modifications of the protein to occur in the heterologous expression system and an accurate molecular weight can often reveal what changes have been made. Also sequence differences that may arise due to cloning errors or mutations generally result in mass shifts that are detected by LC/MS.

Materials:

- Autosampler vials (BMB Stores #06718439 with low volume (250 µL) glass inserts (BMB Stores #056005203N) and PTFE-lined screw caps (BMB Stores #06718904)
- Desalting column (provided by MSMC)
    - Thermo HyperSil Gold CN 1.0 x 10 mm guard column (3µm particle size) (25803-011001)
    - Use with Thermo Uniguard Direct-Connect Guard Cartridge holder (850-00)

Sample prep:

- Purify protein by whatever means necessary (i.e., Ni-affinity chromatography or other tag-based purification). Needs to be a fairly clean sample. Too many other protein contaminants will make determining the neutral mass of the protein difficult.
- Prepare sample at a protein concentration of 1-10 µM.
    - Ideally use Bradford reagent or BCA assay or another equivalent method for determining protein concentration.
    - Protein sample can be in whatever buffer and salt concentration is necessary to keep protein stable (with some caveats – see notes below).
    - Detergents should be avoided, particularly those that are mixtures of compounds (Triton, NP-40, Tween); even relatively innocuous detergents such as octylglucoside or dodecylmaltoside may interfere with the desalting process.
- The minimum volume of protein solution needed is 50 µl in an LC vial with a limited volume insert.
- Always bring a buffer only control sample to analyze prior to the protein sample to determine background signal.

LC/MS method for desalting (typically analyzed on one of the QTof systems):

- 10 µl of protein solution is injected onto a small 1.0 x 10 mm desalting column with a cyanopropyl stationary phase.
- LC gradient:
    - Solvent A: water + 0.1% formic acid; solvent B: Acetonitrile
    - Flow rate = 0.1 ml/min
    - Initial conditions = 98% solvent A / 2% B
    - Hold at initial conditions for 5 minutes
        - Divert to waste for the first three minutes to prevent salts and buffers from contaminating the mass spectrometer ion source
    - Linear ramp to 75% B from 5 to 10 minutes
    - Hold at 75% B until 12 minutes
    - Return to 98% A / 2% B at 12.01 min
    - Hold at 98% A / 2% B until 15 min
- MS method:
    - For use with Waters Xevo G2-XS QTof
    - Capillary voltage: 3 kV
    - Sample cone: 35 V
    - Source temp: 100˚C
    - Desolvation temp: 350˚C
    - Desolvation gas flow: 600 L/hr
    - Cone gas: 40 L/hr
    - MS scan method
        - Positive ion mode electrospray
        - Sensitivity optics
        - Continuum mode data acquisition
        - Normal dynamic range
        - Scan range: m/z 200-2000; may need to extend upper m/z limit to 3000 for particularly acidic proteins
        - Scan rate: 1 scan/second
        - No collision energy
    - Method events:
        - No lockmass correction
        - Set solvent delay for first 3 minutes
        - Set flow state to waste for first 3 minutes

Data Analysis:

- For proteins, it is best to view the total ion chromatogram (TIC) rather than the base peak intensity chromatogram (BPI) which many of our systems are set to as the default
- Most proteins elute in the 8-10 minute range using this method
- Combine spectra in the time range where the protein is eluting from the column
    - May need to do a background subtraction when combining spectra
    - Figure 1 shows an example spectrum with the many different charge states of the protein

- Use the MaxEnt 1 deconvolution tool (within MassLynx-Spectrum) to determine the neutral mass of the protein
    - If there is significant baseline rise, may need to subtract the baseline prior to MaxEnt
        - Display the m/z range that encompasses most of the abundant charge states but excludes regions of the spectrum where singly-charged ions give strong signals (isotope peaks separated by 1 m/z)
        - Subtract the baseline as follows: In the spectrum window: click on the 'Process' menu and click on 'Subtract'
        - Typical settings are polynomial order 3 and % below curve 3.00 and tolerance 0.01
    - Click on the Menu item 'Process' then click on 'MaxEnt 1'; MaxEnt 1 uses an iterative algorithm that converts the various charge state signals to a zero-charge state spectrum that represents the neutral protein mass (in Da)
        - Set the output mass range to include the expected mass of your protein (you may wish to use a wide mass range at first, then narrow the range once you have observed peaks in the MaxEnt output)
        - Set output mass resolution to 0.50 Da/channel
        - Select Uniform Gaussian damage model and set width at half height to 0.5 Da
        - Minimum intensity ratios can be set to 33%
            - May need to change depending on intensity and number of different charge states observed in the spectrum
        - Set maximum number of iterations to 10
        - Click Ok to start deconvolution

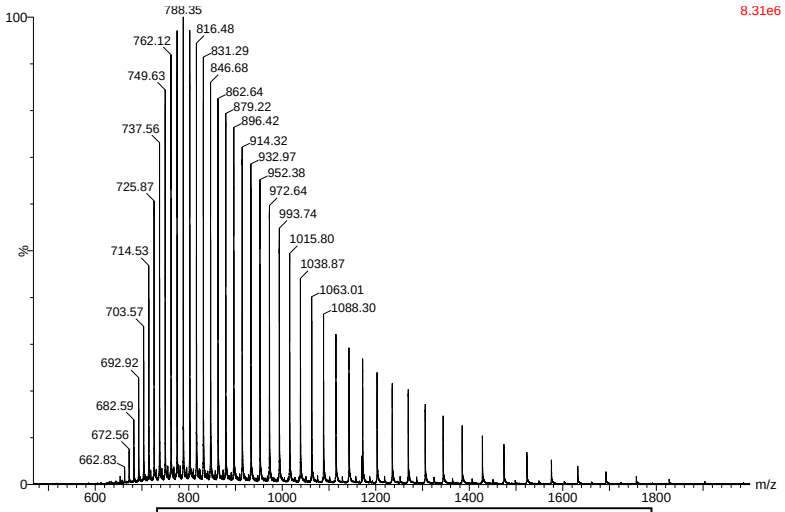

Figure 1. Example protein spectrum

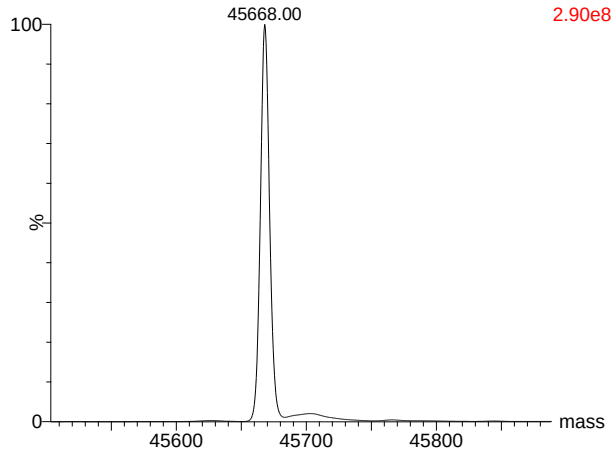

Figure 2. Zero-charge state spectrum after MaxEnt spectrum deconvolution

The resulting spectrum shows the calculated neutral mass obtained by the MaxEnt 1 algorithm. Errors are typically with 0.01% of the mass (i.e. 1 Da for a 10,000 Da protein). To check the accuracy of MaxEnt 1, you can calculate the charge states of the individual peaks in the original spectrum to verify that for the output mass obtained, you can explain the presence of each charge state. MaxEnt 1 will give artifact peaks that are ½X, 2X, 3X, etc due to incorrectly assigning charge states. However these artifacts can be eliminated by calculating the expected charge state distribution and comparing to the original spectrum.

Notes:

Common observed mass shifts for proteins:

- Loss of N-terminal methionine (-131 Da)
- Acetylation (+42)
- Phosphorylation (+80)
- Glucosylation (+162)
- Glutathionylation of GST-fusions (+305)
- Can have combinations of these modifications (i.e., loss of N-terminal Met and acetylation = -89 Da)

Sample conditions that are not compatible with MS when using the desalting column:

- Ionic detergents in the sample
    - Can tolerate single molecule non-ionic detergents like decyl- or dodecyl-maltoside
- Precipitated protein
    - Protein must be in solution

Useful math:

- To convert mg/ml or µg/µl to µM or pmol/µl:
    - µM = (mg/ml x 10^6) / protein mol wt
    - Ex. 1 mg/ml of a 30 kD protein
        - (1 mg/ml X 10^6) / 30,000 Da = 33.3 µM