LC/MS Top-Down Proteomics Standard Operation Protocol
Ge Lab, University of Wisconsin Madison

Materials	Part Number	Vendor
Carbonic Anhydrase	PS-121-1	Protea
ESI-L Low Concentration Tuning Mix	G1969-85000	Agilent
MS-Grade Formic Acid	94318	Fluka
Myoglobin	M1882	Sigma Aldrich
HPLC Grade Reagent Alcohol (Ethanol)	A955	Fisher Sci
HPLC Grade Water	W5	Fisher Sci
Ribonuclease A	R6513	Sigma Aldrich
Trypsinogen	T1143	Sigma Aldrich
Ubiquitin	U6253	Sigma Aldrich

Instruments

- LC:
  - nanoAcquity UPLC system (Waters Corporation)
  - nanoAdvance HPLC system (Bruker Daltonics)
- MS:
  - maXis II ETD QTOF (Bruker Daltonics)
  - impact II QTOF (Bruker Daltonics)
  - solariX 12T FTICR (Bruker Daltonics)

Note

- Final Standard Protein Mixture Amount Loaded: Carbonic Anhydrase 1.7 pmol, Myoglobin 0.44 pmol, Ribonuclease A 0.36 pmol, Trypsinogen 0.65 pmol, Ubiquitin 0.12 pmol.
- Calibration: offline at least once a day; or online using an automated calibrant loop injection/lockmass
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Standard Protein Mixture Preparation

- Prepare 10 mg/mL stocks of each protein standards in HPLC H_2O (1 mg individual standard dissolved in 100 μL H_2O).
- Dilute the stocks of Myoglobin, Ribonuclease A to 1.0 mg/mL, Ubiquitin to 0.10 mg/mL in H_2O.
- Prepare 100x standard protein mixture according to the table below:

Standard	Volume (μL)	Stock concentration (mg/mL)	100x mixture concentration (μg/mL)
Carbonic Anhydrase	10	10	1000
Myoglobin	15	1.0	150
Ribonuclease A	10	1.0	100
Trypsinogen	3.0	10	300
Ubiquitin	20	0.10	20
Water	42	-	-

- Divide 100x standard protein mixture into 2 µL aliquots and store at -80 °C.
- Dilute 1 aliquot of top down standard in 100x volume of Mobile Phase A (100% HPLC H_2O, 0.1% MS-grade formic acid). 2 µL aliquots to final volume of 200 µL.
- Mix thoroughly by vortex, then transfer to a new autosampler vial with pre-slit cap.
- Final concentration of each protein for injection: Carbonic Anhydrase 0.34 µM, Myoglobin 0.089 µM, Ribonuclease A 0.073 µM, Trypsinogen 0.13 µM, Ubiquitin 0.023 µM.

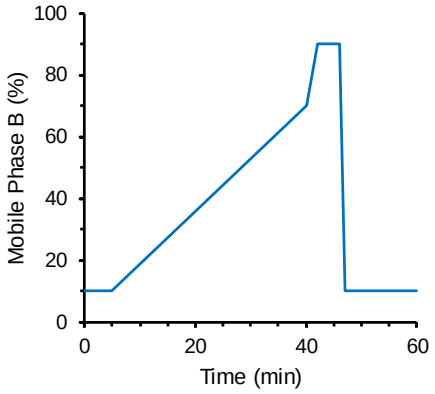
Column Parameters

- **Self-packed**
  - **Packing Material**: PLRP-S, 1000 Å pore size, 5 or 10 µm particle size (Agilent Technologies)
  - **Capillary Column**: 20 cm bed length, I.D.: 500, 250, 150 µm
- **Commercial available**
  - **Custom Column**: 20 cm bed length, I.D.: 500, 250, 150 µm; PLRP-S material, 1000 Å pore size, 5 or 10 µm particle size (nanoLCMS Solutions)

LC Parameters

- Mobile Phase A: 100% HPLC H_2O, 0.1% MS-grade formic acid
- Mobile Phase B: 50% HPLC Acetonitrile, 50% HPLC Ethanol, 0.1% MS-grade formic acid
- Configuration: Direct injection (no trap column used)
- Injection Volume: 5 µL
- Flow Rate: 12, 4, and 2 µL/min for 500, 250, and 150 µm I.D. column, respectively
- Gradient Parameters:

Time (min)	Mobile Phase B (%)
0.0	10.0
5.0	10.0
40.0	70.0
42.0	90.0
46.0	90.0
47.0	10.0
60.00	10.0



MS Parameters

- maXis II and impact II QTOFs
  - Positive ion mode
  - Calibration: Tuning Mix (External)

Ion Source	Apollo electrospray	
	End Plate Offset	500 V
	Capillary Voltage	4500 V
	Nebulizer	0.5 Bar
	Dry Gas	4 L/min
	Dry Temperature	220 °C

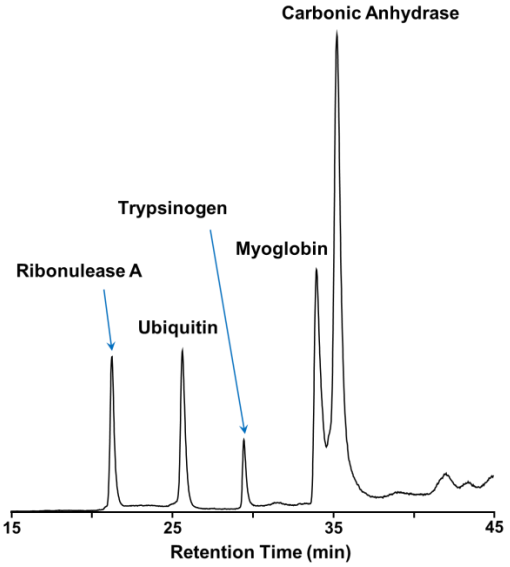
Ion Transfer	Funnel RF	400 V
	Octopole RF	600 V
	In-source CID Energy	10-40 V
	Quadrupole Energy	4 V
	Low Mass Cutoff (LMCO)	*m/z* 500
	Transfer Time (collision cell to TOF)	110 µs
	Pre Pulse Storage	10 µs
MS1	Scan Range	*m/z* 500.00 - 2500.00
	Scan rate	1 Hz
	Collision Cell Energy	8 V
	Collision RF	2500 V
MS2	Scan Range	*m/z* 200.00 - 2000.00
	Scan rate	1 Hz
	Collision RF	1500 V
Automatic MS/MS	Activation Type	CID
Data-Dependent Analysis	Precursor Ion Number	3
Active Exclusion	Exclude After	3 spectra
	Release After	1 min
	Reconsider if	5.0-fold intensity increase
Isolation Window	*m/z* 300	4
	m/z 2000	8
Targeted ETD (maXis II)	Activation Type	ETD
Isolation Window	Precursor ions ~*m/z* 1000	5
Most Abundant Charge State	Analyte accumulation	800 ms
Reagent	2,5-Hexanedione	*m/z* 114 (-)
	Reagent accumulation	8 ms

- solariX 12T FTMS:
    - Positive ion mode
    - Calibration: Tuning Mix

Ion Source	Apollo electrospray	
	End Plate Offset	800 V
	Capillary Voltage	4500 V
	Nebulizer	0.4 Bar
	Dry Gas	4 L/min
	Dry Temperature	220 °C
Ion Transfer	In-source CID Energy	10-40V
	Funnel RF	150Vpp
	Octopole 5MHz	350 Vpp
	Quadrupole LMCO	*m/z* 500.00
	Collision Voltage	6 V
	Collision RF 2MHz	2000 Vpp
	Transfer Time (collision cell to FT)	1000 µs
	Transfer RF 4MHz	350 Vpp
Detection	Sweep Excitation Power	30%
	Sweep Step Time	15 µs
	Transient length	1M
	Processing	Full Sine
MS1	Scan Range	*m/z* 500.00 - 2500.00
	Scan rate	1.4s per scan
	Accumulation Time	80 ms
MS2	Scan Range	*m/z* 200.00 - 2000.00
	Scan rate	1.4s per scan
	Accumulation Time	100 ms
Automatic MS/MS	Activation Type	CID
Data-Dependent Analysis	Precursor Ion Number	3
Active Exclusion	Exclude After	3 spectra
	Release After	1 min
	Preferred Charge State	5-Maximum (30)
	Isolation Window	10

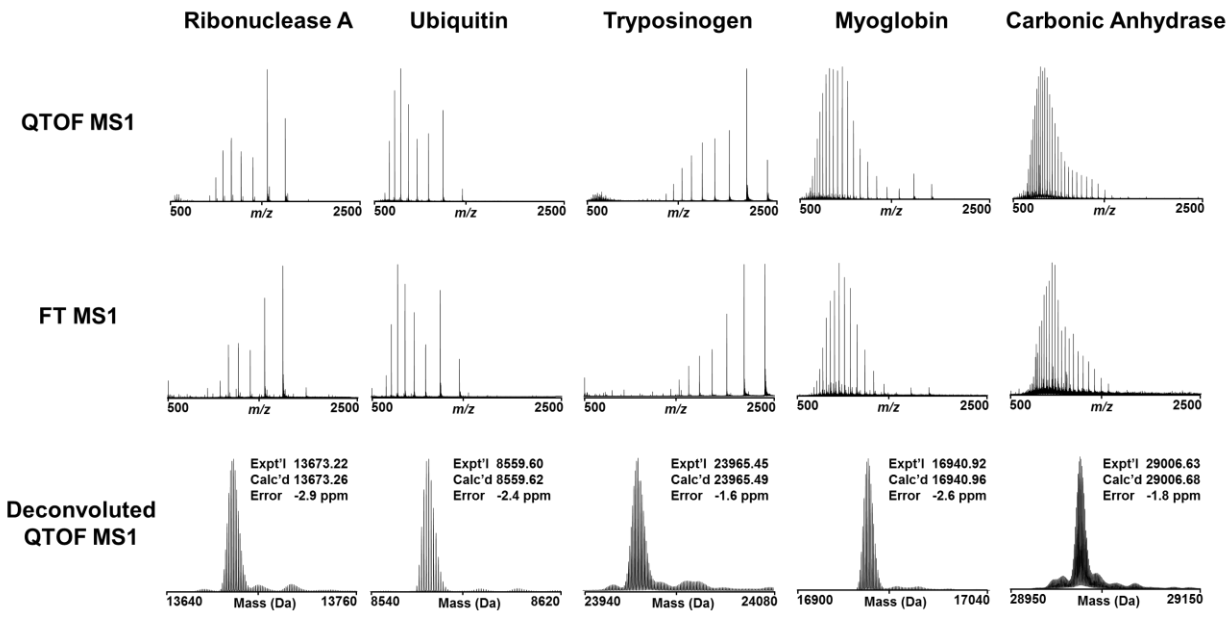
Data Interpretation Analysis

- **Example Chromatogram:**



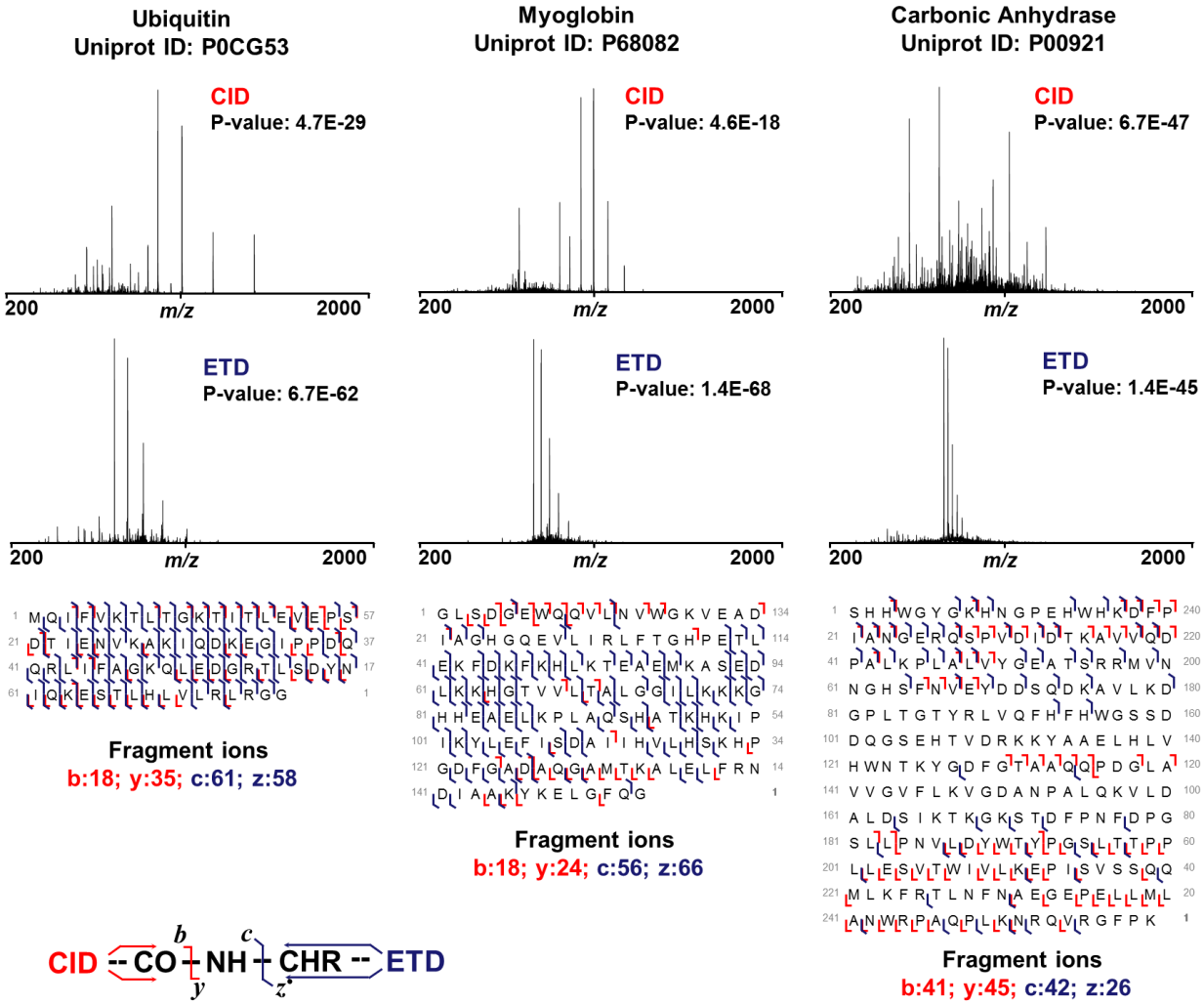
Example Chromatogram: Typical total ion chromatogram of the TD standard, showing five separate eluted protein peaks. The elution order should remain consistent. The example shown was obtained on impact II QOF, using the LC and MS1 parameters described above.

Example QTOF and FT MS1 spectra:



Example QTOF and FT MS1 spectra: Averaged QTOF (impact II) and FT MS1 spectra for each of the five peaks in the above chromatogram, showing the characteristic charge distributions for each protein. The deconvoluted spectra should show isotopic resolution for all five proteins with the monoisotopic masses within 5 ppm compared to the calculated values.

- **Example QTOF MS2 spectra:**



Example QTOF MS2 spectra: Example averaged fragmentation spectra for ubiquitin (**left**), myoglobin (**middle**), and carbonic anhydrase (**right**) from maXis II. MS/MS spectra were exported from Data Analysis 4.3 (Bruker) and processed by MASH Suite Pro (available for free request at http://crb.wisc.edu/yinglab/software.html).