SOP_001_NU_1_6_Top_Down_Standard _v1_PMT_RF

Rafael Melani and Paul Thomas

❖ Reagent and Materials List

Item	Part Number	Vendor
Ubiquitin	U6253	Sigma Aldrich
Trypsinogen	T1143	Sigma Aldrich
Myoglobin	M5696	Sigma Aldrich
Carbonic Anhydrase	C2624	Sigma Aldrich
Optima Grade Water	W6-4	Fisher Scientific
Optima Grade Acetonitrile	A955-4	Fisher Scientific
MS-Grade Formic Acid	PI-28905	Fisher Scientific
1.5 mL Protein LoBind Microcentrifuge Tubes	13-698-794	Fisher Scientific

❖ Important Notes

- Use 1.5 mL Eppendorf LoBind microcentrifuge tubes for protein stock preparation, top-down (TD) standard preparation, and long-term aliquot storage. In our experience, these tubes have shown the lowest degree of plasticizer leaching and/or protein binding during use and storage.
- Approximate final protein amounts (loaded on-column): 0.1 pmol ubiquitin, 0.5 pmol trypsinogen, 1 pmol myoglobin, and 0.6 pmol carbonic anhydrase. Superoxide dismutase (SOD1) is present as a contaminant in carbonic anhydrase.
- A TD standard prepared in this way should be stable for up to three days at 4 °C (before significant protein oxidation becomes evident).

❖ Recipe

- Prepare 2 mg/mL stocks of each protein standard in Optima H_2O. (Aliquots can be stored at -80 °C.)
- Prepare the following (volumes shown from respective stock solutions):

Protein	Volume (µL)	Stock Concentration (pmol/µL)	Amount Loaded on Column (pmol, 1X)
Carbonic Anhydrase	40	25.7	0.64
Myoglobin	40	43.9	1.09
Trypsinogen	25	19.6	0.49
Ubiquitin	2.5	5.5	0.14
Total	**107.5**		

- Divide final mixture into 2.5 uL aliquots and store at -80 °C.

❖ Preparation

- Dilute one aliquot of TD STD in 100x vol. of Buffer A (95% Optima H_2O, 5 % Optima Acetonitrile, 0.2% MS-grade formic acid), where 1x vol. is the intended injection volume (e.g. **600 µL Buffer A** for an intended injection volume of **6 µL**). This will ensure that the correct amount of each TD standard protein is present in each injection.
- Mix thoroughly by pipetting, then transfer to a clean autosampler vial. The standard is now ready for use.

❖ Column Parameters

- Self-packed PLRP-S columns

  - Packing Material: PLRP-S resin, 1000 Å pore size, 5 µm particle size (obtained from Agilent Technologies)
  - Trap column: 2 cm bed length, 150 µm I.D.
  - Analytical column: 20 cm bed length, 75 µm I.D.
  - Nanospray Emitter: 15 µm PicoTip emitter, packed with 2 mm PLRP-S resin (P/N FS360-50-15-N-20-C12, New Objective)

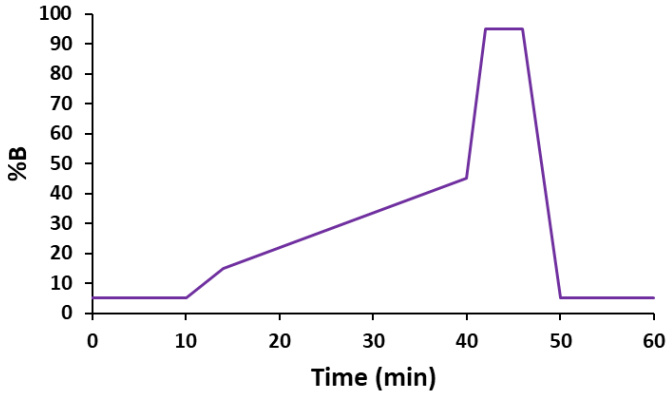
❖ LC Parameters

- **Solvent A:** 95% Optima H_2O, 5% Optima Acetonitrile, 0.2% MS-grade formic acid
- **Solvent B:** 5% Optima H_2O, 95% Optima Acetonitrile, 0.2% MS-grade formic acid

- Self-packed PLRP-S columns

  - Trapping configuration: 3 µL/min flow rate (10 min. trap cycle, 55 °C)
  - Analytical configuration: 0.3 µL/min flow rate (48 min. analytical gradient, 55 °C)
  - Gradient Parameters:

Time (min.)	% B	Curve
0.0	5.0	
10.0	5.0	0% in 10 min.
14.0	15.0	10% in 4 min.
40.0	45.0	30% in 26 min.
42.0	95.0	50% in 2 min.
46.0	95.0	0% in 4 min.
50.0	5.0	90% in 4 min.
60.0	5.0	0% in 10 min.



❖ MS Parameters

- **Instrument Tuning and Method Parameters:**

  Positive and profile mode, RF Lens 30%, with 15.0 V source CID. “Intact Protein” on if available, “Low Pressure” selected if available, Default Charge State 15, “Advanced Peak Determination” on

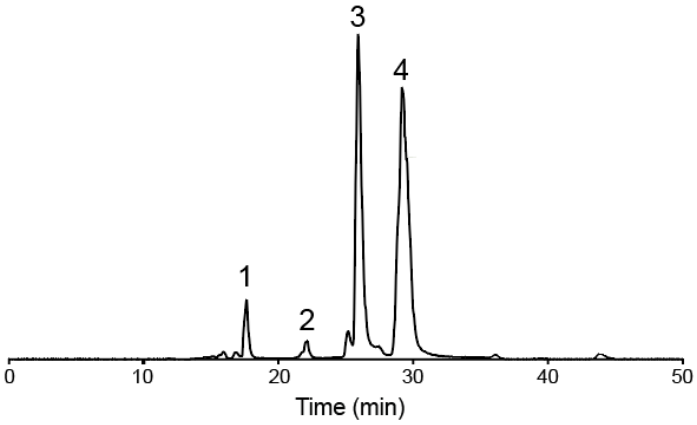
Experiment 1		
Master Scan: FTMS1	Scan Range (*m/z*)	600.00 – 2000.00
(120k RP)	Microscans	1 (2 if no low-pressure mode)
Full Scan	Max Inject Time (ms)	100.00
Normal mass range	MS1 AGC Target	1.20e +06 (300%)
Scan Event 1: ddMSNScan	Activation type	HCD
Scan range	Define *m/z* range	350-2000
(60k RP)		
Quadrupole isolation ON	Isolation Width (*m/z*)	3.0
Top N 3 sec, dd	Normalized Collision Energy	28.0
Isolation offset OFF	Microscans	1 (2 if no low-pressure mode)
Supplemental activation OFF	Max Inject Time (ms)	600.00
Charge Filter: $5 \leq z \leq 30$	MS2 AGC Target	5.00e+05 (1000%)
Experiment 2 (Eclipse Only)		
Master Scan: ITMS1	Scan Range (*m/z*)	600.00- 2000.00
Full Scan	Microscans	20
Rapid scan rate	Max Inject Time (ms)	10.00
Normal mass range	MS1 AGC Target	3.00e +04

- **Dynamic Exclusion Settings (MS2):**

Repeat Count	1
Exclusion Duration (s)	60
Exclusion Mass Width (High/Low, m/z)	0.50
Exclude Isotopes	True

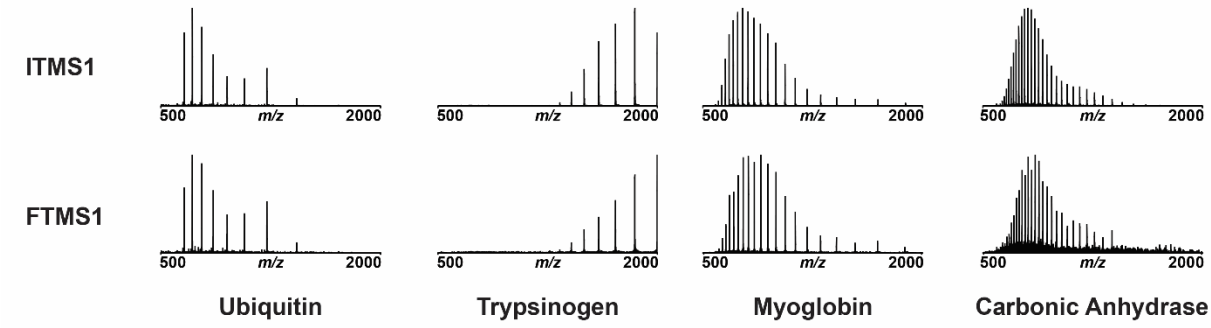
❖ Data Interpretation and Analysis

◆ Example Chromatogram:

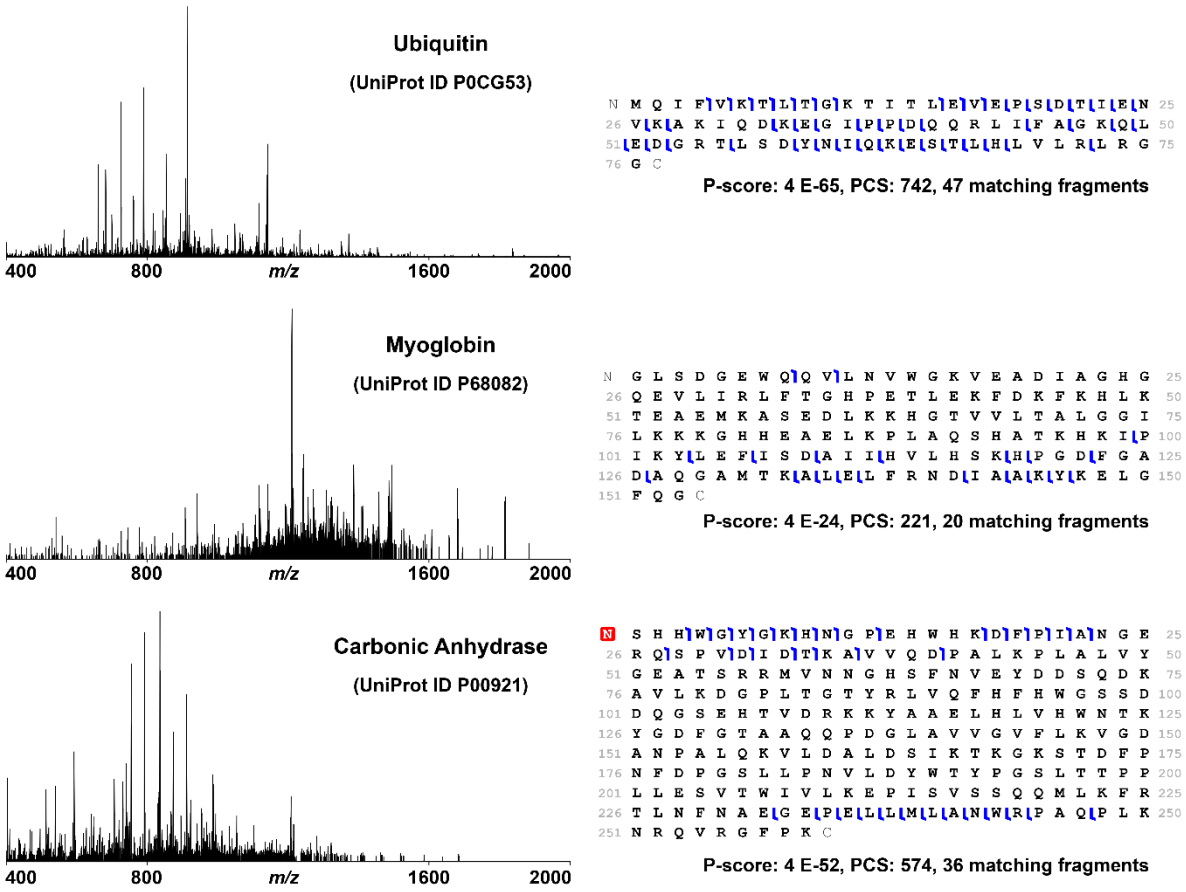


Example Chromatograms: Typical base peak chromatogram of the NRTDP TD standard on column described above, showing four separate eluted protein peaks (**1.** Ubiquitin, **2.** Trypsinogen, **3.** Myoglobin, **4.** Carbonic Anhydrase). Superoxide dismutase, a characteristic contaminant of carbonic anhydrase, is not present in these batches of TD standard. The elution order and relative height ratio of all other protein peaks should remain consistent.

◆ Example IT and FT MS1 spectra



Example IT (Eclipse) and FT MS1 spectra: Averaged ITMS1 and FTMS1 spectra for each of the four peaks in the above chromatogram, showing the characteristic isotopic peak distributions for each protein. Note the fidelity of the FTMS1 spectra to those acquired in the ion trap; this serves as an indicator of optimal ion transmission and FT performance for the Eclipse.



Example FT MS2 spectra: Single-scan fragmentation spectra for ubiquitin (**top**), myoglobin (**middle**), and carbonic anhydrase (**bottom**) from the dataset shown above. The fragment ion masses from each of the above spectra were deconvoluted using the Xtract algorithm (Thermo) and searched against the respective protein sequences using ProSight Lite. Typical P-scores for ubiquitin should be below E -50, while P-scores for myoglobin and carbonic anhydrase should be below E -25.

- **Data Analysis Methods:**

  - **ProSight Lite:** The software is available for free download at http://prosightlite.northwestern.edu/. A detailed protocol for the analysis of the NRTDP Top-Down Standard with Xtract and ProSight Lite can be found at https://link.springer.com/content/pdf/10.1007%2F978-1-4939-6783-4_18.pdf
  - **ProSight PC 4.0:** A "Standards" search database for high-throughput data analysis of the NRTDP Top-Down Standard with ProSight PC 4.0 is available for download here: http://proteinaceous.net/database-warehouse/
  - **NRTDP TDPortal:** A custom workflow for high-throughput analysis of the NRTDP Top Down Standard is available on the TDPortal Quest-based, high-performance computing environment available through NRTDP and Northwestern University. User accounts can be requested at http://nrtdp.northwestern.edu/tdportal-request/. A detailed protocol for data analysis on TDPortal by external users (**NRTDP SOP_004**) can be found at http://nrtdp.northwestern.edu/wp-content/uploads/2017/01/ExternalUserJan10.pdf

❖ **Longitudinal Data Tracking**

The NRTDP recommends including the following metrics into longitudinal tracking of LC and MS performance:

- **Peak Intensity:** IT and FT MS1 peak intensity of ubiquitin, myoglobin, and carbonic anhydrase
- **Peak Area (log):** Chromatographic peak area (and retention time) of ubiquitin, myoglobin, and carbonic anhydrase
- **FWHM:** Full width at half maximum of ubiquitin, myoglobin, and carbonic anhydrase peaks
- **Injection Time:** MS1 and MS2 injection times for ubiquitin, myoglobin, and carbonic anhydrase
- **P-score (-log):** ubiquitin, myoglobin, and carbonic anhydrase obtained by low- or high-throughput data analysis

Paying close attention to these parameters over time can help identify LC or MS issues before they become significant, thus reducing loss of important sample data.

The NRTDP further recommends running at least three injections of the Top Down Standard before and after running experimental samples, as well as at least one injection of Top Down Standard every twenty-four hours. Evaluation of these standards on the fly will not only help detect LC or MS issues, but also provide confirmation that optimal performance is maintained when consistency and reproducibility are crucial.

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