October 15th, 2015

Dear Colleagues,

Please find enclosed the ABRF 2016 PSRG samples that you requested from the ABRF Protein Sequence Research Group. This is the 28th study in an annual series designed to assist laboratories in evaluating their abilities to obtain and interpret protein sequencing data. Thank you for your interest and participation!

C-terminal identification of proteins is a desired technology, especially when coupled with N-terminal identification experiments. For purposes of structural studies of large proteins, it provides identity of fragments generated by limited proteolysis. For Core laboratories, C-terminal identification technology should be reproducible, easy to perform, sensitive, and robust.

The method for C-terminal identification presented this year utilizes heavy-labeled, O18 water. When proteins are enzymatically-digested in the presence of O18 water, all specifically-cleaved internal peptides will exchange the O18 tag except for the peptide originating from the carboxy-terminus of the protein. The goal for this year's study is to test the abilities of participating core laboratories to a) successfully digest the provided protein in presence of 50% (v/v) O18 labeled water, b) identify the pairs of internal peptides with and without O18 tag, c) identify C-terminal most peptide with no O18 tag d) obtain MS/MS data to confirm C-terminal amino acid information.

The PSRG will provide two soluble, known proteins to participating laboratories to carry out (i) individual analysis of each protein in the absence and (ii) in the presence of O18 water (1). Participants will be encouraged to carry out protein in-gel or solution digestion, peptide clean-up, LCMS/MS and database searches for peptide identification using in-house optimized protocols; PSRG will also provide recommendations for all of the steps above as well as a detailed outline of O18 digestion and C-terminal peptide identification. At the end of the study, the participants will be asked to report the C-terminal sequence as determined by bottom-up mass spectrometry, to the PSRG.

Study restrictions: Participants must use bottom-up mass spectrometry. Analysis may include the use of bioinformatics tools to derive the C- terminal sequence.

Participating laboratories will receive an aliquot of purified myoglobin and protocols for O18 labeling: **(A)** in-solution labeling with SDS-PAGE or cut-off filter cleanup, or **(B)** SDS-PAGE separation and in-gel labeling. Participants may choose which workflow to perform and will follow the sample preparation with trypsin digestion and mass spectrometry using the MS system and operating parameters of their choice.

(A) Workflow "In-solution digestion":

1) Solution digestion of protein with trypsin in presence of 50%, v/v, O18 water
2) Desalting of peptides
3) MS analysis
4) Data analysis for identification of O18 tagged peptides
5) Conclusive identification of C-terminal peptide

(B) Workflow "in-gel digestion"

1) SDS-PAGE, excision of bands in-gel trypsin digestion (2) in presence of 50%, v/v, O18 water
2) Desalting of Peptides
3) MS analysis
4) Data analysis for identification of O18 tagged peptides
5) Conclusive identification of C-terminal peptide

Materials supplied in this study:

Protein	Catalog Number	Quantity
Myoglobin	Sigma M1882	100 ug
Beta Lactoglobulin	Sigma L3908-250MG	100 ug
O18 water (99% labeled)	Sigma 487090	100 uL

Data submission:

Please provide data and analysis results directly to Dr. Sara McGrath (sara.mcgrath@fda.hhs.gov). You will receive an identification number via email from Dr. McGrath, who will act as the anonymizer and is the only person who will know the relation between your email and your identification number. In order to ensure anonymity, Dr. McGrath will remove all identifying marks prior to forwarding your data to the PSRG committee for analysis. The sequencing results will be presented at the ABRF 2016 meeting February 20-23rd, Ft. Lauderdale, Florida, and subsequently posted on the ABRF website, and will also help guide future studies and tutorial sessions.

Please include in your email to Dr. McGrath Power Point files with representative mass spectra containing the digested labeled fragments, and report the C-terminal sequence as determined by bottom-up mass spectrometry. You may also include relevant spectra, SDS-PAGE images, database search results, or chromatographic data supporting your analysis and sequence calls. **Please include your laboratory identification number and Workflow type in the title of the supporting document.** If alternative methods are used, PSRG requests a brief description of the protocol used and literature references as appropriate. Recommendations for successful analytical and bioinformatics methods are included in the Appendix.

In addition to submission of data, the PSRG is requesting that each participant report findings and protocol information used in the study on Survey Monkey. Your participation will be recorded using the identification number assigned to you and will be used to determine aggregate information for all participants.

The results survey can be accessed here:
https://www.surveymonkey.com/s/X9NC8GC

If you are unable to open the link, if your sample arrived damaged, or if you have questions about the study, please contact Dr. Sara McGrath (sara.mcgrath@fda.hhs.gov).

***** Equipment failures and "no data obtained" analyses are as important to us as data from "successful" runs. Please send us your results and fill out the online survey, regardless of your success.**

*****The deadline for receiving data for inclusion in the study is December 31st, 2015.**

Thank you for your valuable participation in this year's study!
The Protein Sequencing Research Group:

Robert English	–	Shimadzu Scientific Instruments
Sara McGrath	–	FDA Center for Food Safety and Applied Nutrition
Greg Cavey	–	Launch MI Lab, Southwest Michigan Innovation Center
Hediye Erdjument-Bromage	–	Memorial Sloan-Kettering Cancer Center
Xuemei Luo	–	University of Texas Medical Branch
David Wood	_	St. Louis University
Brian Field	_	Shimadzu Scientific Instruments

Appendix

Sample Preparation:

1. Record date sample is received and store sample in -20°C freezer.
2. Each sample contains 100 ug dried protein
3. Solubilize the Beta-Lactoglobulin and Myoglobin with 588 uL DI H2O to make a 10 pmol/uL solution.
4. Dilute the stock solution to make a 1 pmol/uL solution by adding 10 uL of myoglobin solution to 90 uL of 100mM Ammonium Bicarbonate.

Tips for Success:

1. Initially, carry out in-gel or solution digest of about 5 picomoles of each protein in regular (O16 water) and verify that you are recovering the C-terminal peptides and good sequence coverage (>80%) of the two proteins before beginning work with O18 water.

2. To carry out companion digests with O18 water:
    a. prepare Trypsin (Modified, Sequencing grade or Gold, Mass Spectrometry grade, both available from Promega or other vendors) to have a final composition of 50:50 O16:O18 water
    b. Prepare a 0.2 M Ammonium bicarbonate solution with trypsin stock, and combine 25 uL with 25 uL* of O18 water to have final concentration of 0.1 M ammonium Bicarbonate in 50% O18 water.

    * May use more or less volume depending on the SOP digestion conditions.

3. Visually inspect the final mass spectra and confirm the O16/O18 tagged pairs of peptide masses (MS1). C-terminal tryptic peptide should be a singlet, with O16 water only.

4. Database searches should include the following modifications:
    - **Fixed**: Carbomidomethylation (or choice of alkylation) of Cysteines if the proteins are reduced and alkylated
    - **Variable**:
        - Oxidation of Methionines,
        - Protein N-terminal Acetylation,
        - Deamidation of Asparagine and Glutamine
        - O18 incorporation at C-terminal of peptides

5. **Use mass accuracy settings that is appropriate for the equipment you are utilizing**
    a. For data obtained using Orbitrap/QE mass spectrometers, mass accuracy requirement of 10ppm and 0.8-0.3daltons were set for peptide and fragment ions, respectively.

Sample Analysis and Data Analysis

Samples are to be analyzed by MS/MS mass spectrometry typically used for protein identification. LC-MS with vented column/trap loading has been used to successfully remove excess reagent and identify proteins. MALDI-TOF users may wish to perform the digest using FASP as described in Reference (3), or use manual desalting if the digestion was performed in solution.

Data analysis is performed similar to protein identification analysis with trypsin as the proteolytic enzyme, carbamidomethyl Cys set as a fixed modification, pyro-Glu and Met oxidation, deamidation of Asparagine (N) and glutamine (Q) as variable modifications, and maximum number of missed cleavages should be set to 2.

Software for data analysis such as GPM, Mascot, PLGS, etc., must allow for custom modifications and preferably to the) O18 labeling of C-terminal amino acid of a peptide.

During visual inspection, participants should look for experimental massses (m/z) at pairs of peptide masses with O16 and O18. At z=+1, the delta mass for O18 incorporation is + 2 Daltons. At z=2, delta mass is +1 dalton. The Calculated, C-terminal peptide for Myoglobin and BLG are: 650.3071 and 1657.777 (no cys alkylation; 1714.798 with Carbomidomethyl Cys

Participants are requested to complete the table below, provide MS/MS spectra of the C-terminal trypsin peptide, and an output file or screen capture from software used to make the C-terminal identification. In addition to data on the C-terminal trypsin peptide, participants are asked to provide a list of additional peptides that show evidence internal, newly formed peptide identities with and without O18 tags, a result of trypsin digestion. Please fill out the accompanying analysis survey accessible by the link on Page 2.

Results for Identifying the N-Terminal Peptide of Dimethyl Labeled Proteins

Myoglobin / BLG starting amount	Amount of sample analyzed by MS (fmol)	C-terminal labeling digest		Positive Identification of O16/O18 tagged internal peptides		Positive ID of protein C-terminal peptide
		In solution	In-Gel	In solution	In-Gel	
5 pmol						
1 pmol						
0.3 pmol						
Lower (specify)						

References:

1.Samir Julka, Demetrius Dielman, Scott A. Young. Detection of C-terminal peptide of proteins using isotope coding strategies.2008, Journal of Chromatography B, 874: 101-110.

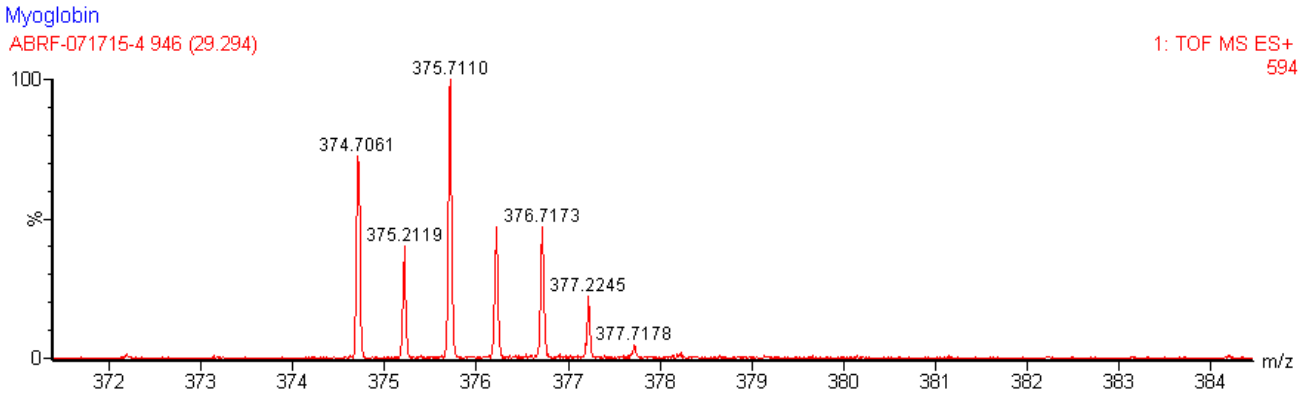
2.Shevchenko A, Tomas H, Havlis J, Olsen J & Mann M; *In-gel digestion for mass spectrometric characterization of proteins and proteomes*.(2006) Nature Protocols, 1(6), 2856-2860.

3.Jacek R Wiśniewski, Alexandre Zougman, Nagarjuna Nagaraj & Matthias Mann. Universal sample preparation method for proteome analysis (2009) *Nature Methods* 6, 359-362.

Example Data: Myoglobin

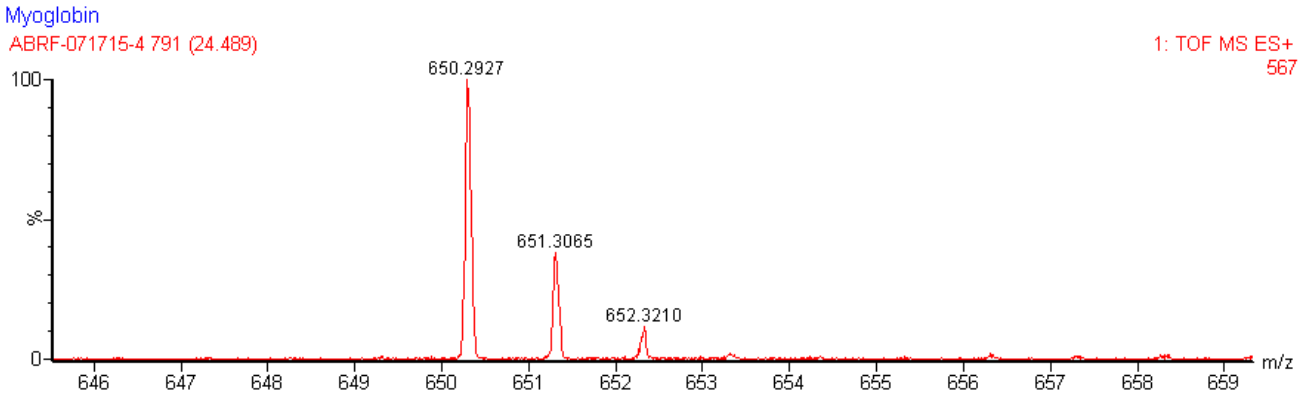
AMTKALELFRNDIAAKYKELGFQG:

solution digest in presence of 50% O18



AMTKALELFRNDIAAKYKELGFQG

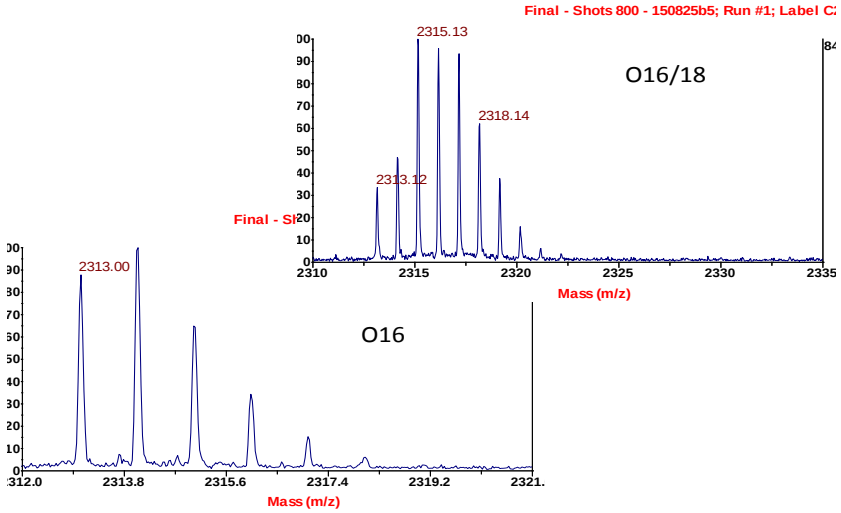
solution digest in presence of 50% O18



Example Data: B-Lactoglobulin

solution digest in presence of 50% O18

VYVEELKPTPEGDLEILLQK(57-76)



LSFNPTQLEEQCHI(165-178 C-terminal): NO REDUCTION and ALKYLATION of Cys176!!)