



PRG-2010: Tackling Unforeseen Problems in Otherwise 'Straight-Forward' Proteomics Analyses

D.B. Friedman¹, T.M. Andacht², M.K. Bunger³, A.S. Chien⁴, D.H. Hawke⁵, J. Krijgsveld⁶, R.L. Moritz⁷, R.E. Settlege⁸, C.W. Turck⁹

¹Vanderbilt Univ., Nashville TN; ²Centers for Disease Control and Prevention, Atlanta, GA, ³RTI International, Research Triangle Park, NC, ⁴Stanford University, Stanford, CA, ⁵UT MD Anderson Cancer Center, Houston, TX, ⁶EMBL Heidelberg, Germany, ⁷Institute for Systems Biology, Seattle, WA, ⁸Virginia Bioinformatics Institute, Blacksburg, VA; ⁹Max Planck Institute of Psychiatry, Munich, Germany

ABRF Proteomics Research Group

ABRF Proteomics Research Group

Introduction

An experiment frequently performed in proteomic laboratories today is the analysis of protein complexes isolated by co-immunoprecipitation or affinity enrichment. Such protein complexes are usually isolated from a cell lysate, and analysis of these complexes often includes many challenges such as sample reproducibility and the presence of non-specific binding partners. The Proteomics Research Group (PRG) of the Association of Biomolecular Resource Facilities (ABRF) developed the 2010 study to assess the abilities of and the approaches used by laboratories to identify and characterize components of a protein complex. Additional challenges were included in the study design, including ¹⁵N-labeled proteins, an altered expression construct, and an unanticipated contaminant, modeling as close as possible to a realistic sample.

Objectives

The primary goals of this study are to document the approaches used by the scientific community, and how successfully they are used to address the following questions:

- Identification of proteins in a relatively simple mixture
- Identification of a low-abundant constituent
- Identification of ¹⁵N-labeled proteins
- Identification of proteins with differently processed N-termini

These questions were formulated in the context of a mock protein pull down where unlabeled and ¹⁵N-labeled proteins had been combined individually.

3 samples – 3 challenges

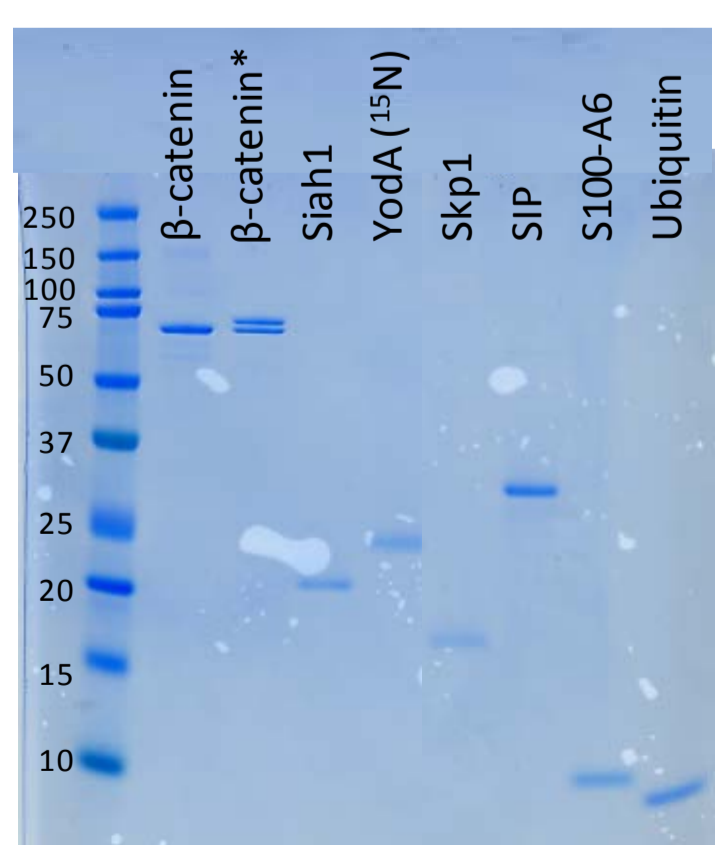
The sample	The challenge
An ACTIVE protein complex for ubiquitination of β -catenin	Can participants identify all 6 proteins? Do they identify the 2 different forms of β -catenin?
An INACTIVE protein complex containing two ¹⁵ N-labeled proteins	Can participants identify the ¹⁵ N-labeled proteins, one of which is a bacterial contaminant (YodA)?
An ACTIVE protein complex containing two ¹⁵ N-labeled proteins	Can participants identify the ¹⁵ N-labeled protein that has restored biological activity (Siah1)?

Sample Preparation

Proteins used in this study were all expressed in *E. coli* as His6-fusions with or without additional fusions of either glutathione S-transferase or maltose binding protein. Proteins were then enriched by Ni-NTA metal affinity chromatography (preceded by MBP or GST cleavage if necessary), followed by Source Q chromatography. Expression of ¹⁵N-labeled proteins was carried out in minimal medium supplemented with ¹⁵NH₄Cl as the sole nitrogen source.

Extensive sample testing

Before samples were shipped, they were extensively tested. Gel electrophoresis (right) and mass spectrometry of individual components confirmed protein identity and labeling status. The doublet of β -catenin was shown to be due to N-terminal variation.

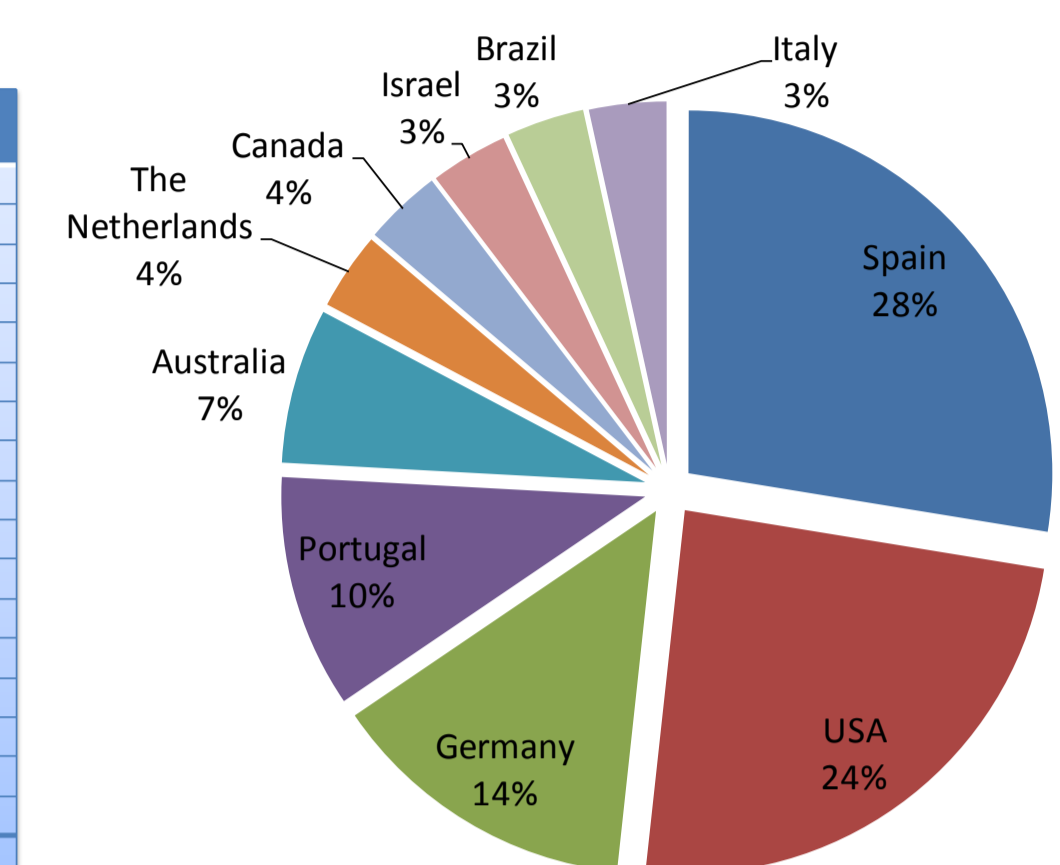


Acknowledgements

We are indebted to Yoana Dimitrova and Walter Chazin (Vanderbilt) for providing samples. We thank Sarah Stuart and Salisha Hill (Vanderbilt), Maurizio Splendore (Stanford), Xinxin Zhang (RTI International) and Bih-Fang Pan (MD Anderson) for sample preparation and testing. This study is dedicated to Lidia Dimitrova.

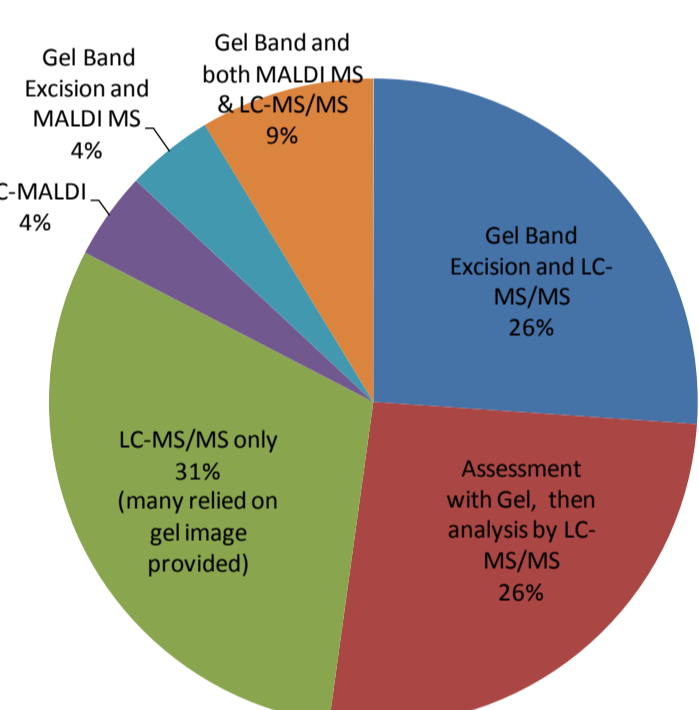
Sample requests and study participation

Request Country	Sample requests	ABRF members	Responders	ABRF members
USA	40	26	7	5
Spain	17	0	8	0
Germany	8	2	4	0
Canada	7	0	1	0
Australia	4	1	2	1
Portugal	4	0	3	0
The Netherlands	2	1	1	0
Switzerland	2	1	0	0
Italy	2	1	1	0
Denmark	2	1	0	0
UK	1	1	0	0
Turkey	1	0	0	0
Singapore	1	1	0	0
Japan	1	1	0	0
Israel	1	1	1	0
Brazil	1	0	1	0
Argentina	1	1	0	0
Total	95	38	29	6

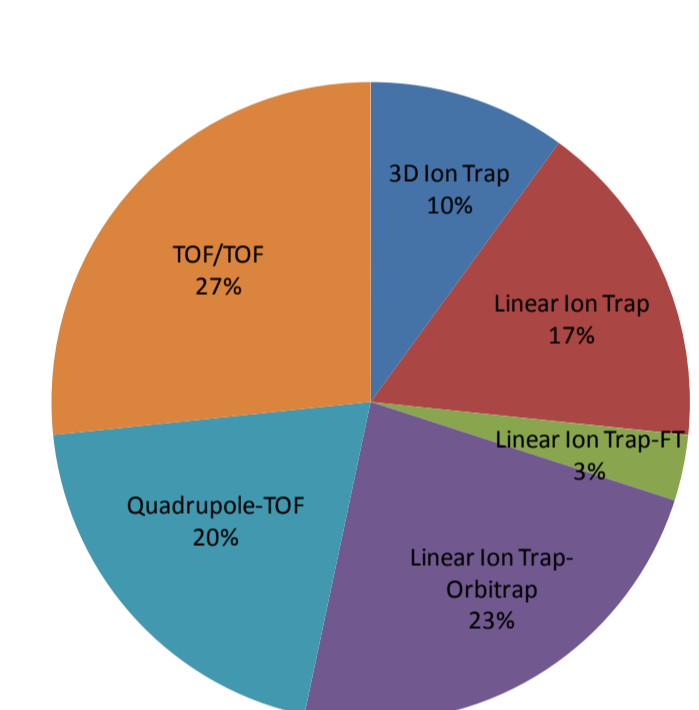


- Samples were distributed to 95 labs in 17 countries
- 47 labs returned results, 29 of these had data
- Among the participants, a minority (6/29) are a member of the ABRF

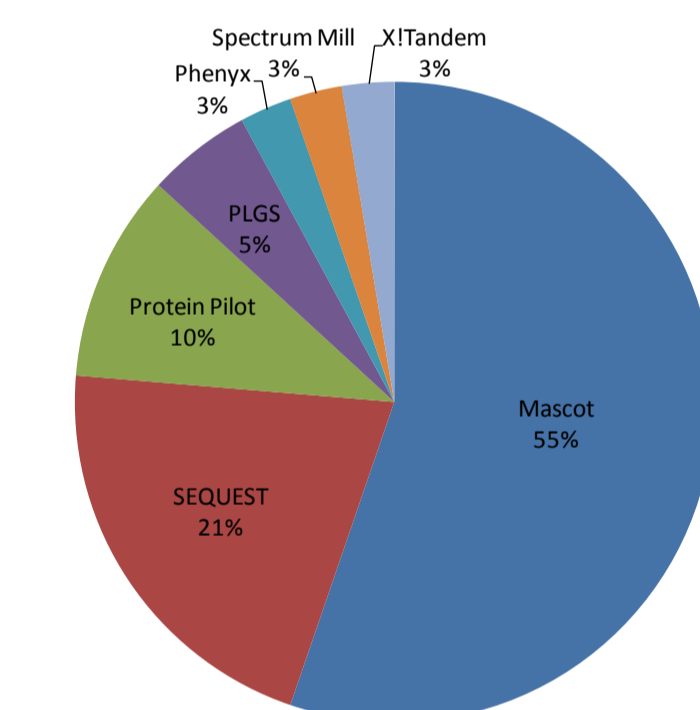
Experimental approach



Use of mass spectrometry

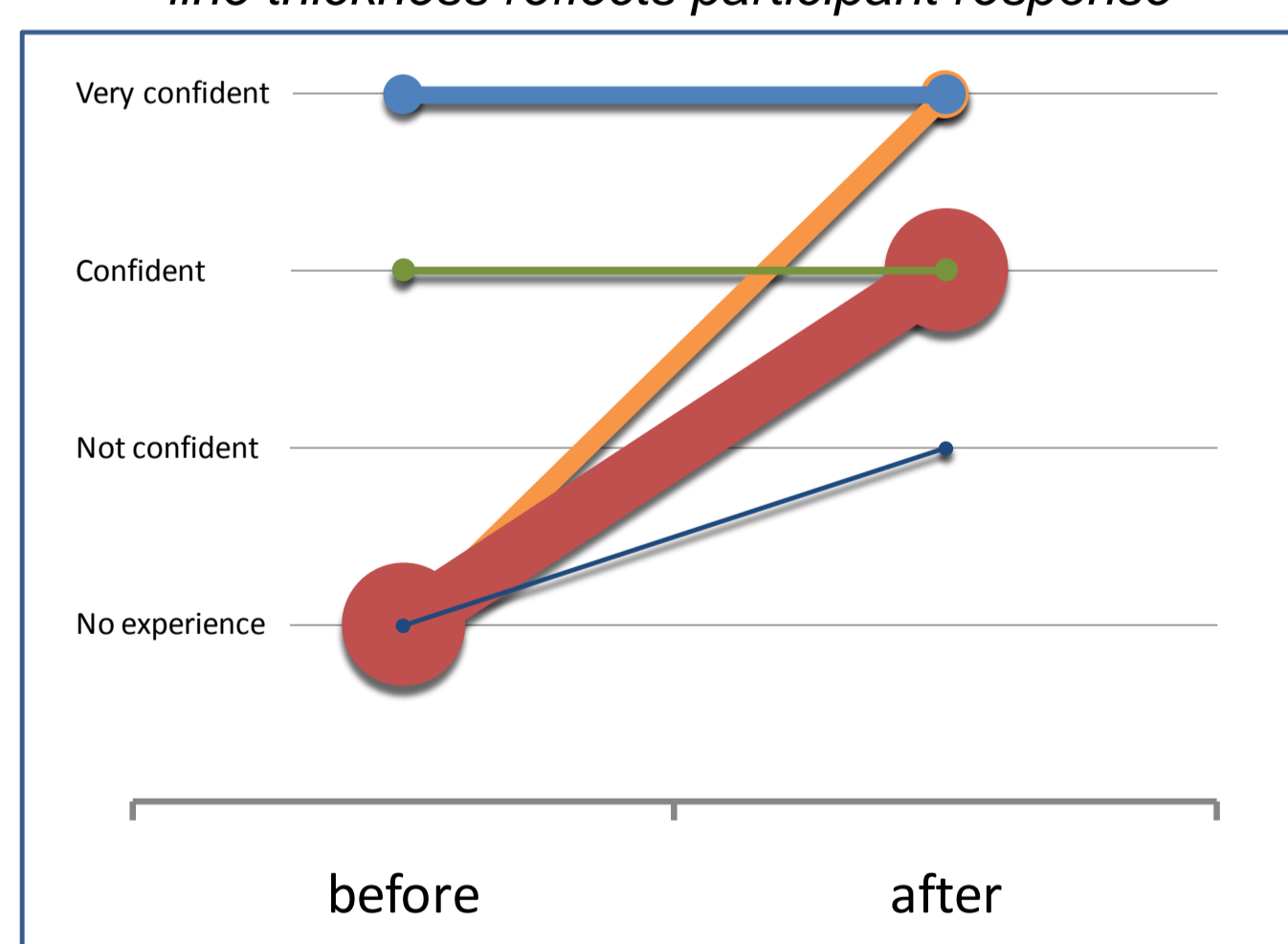


Use of software



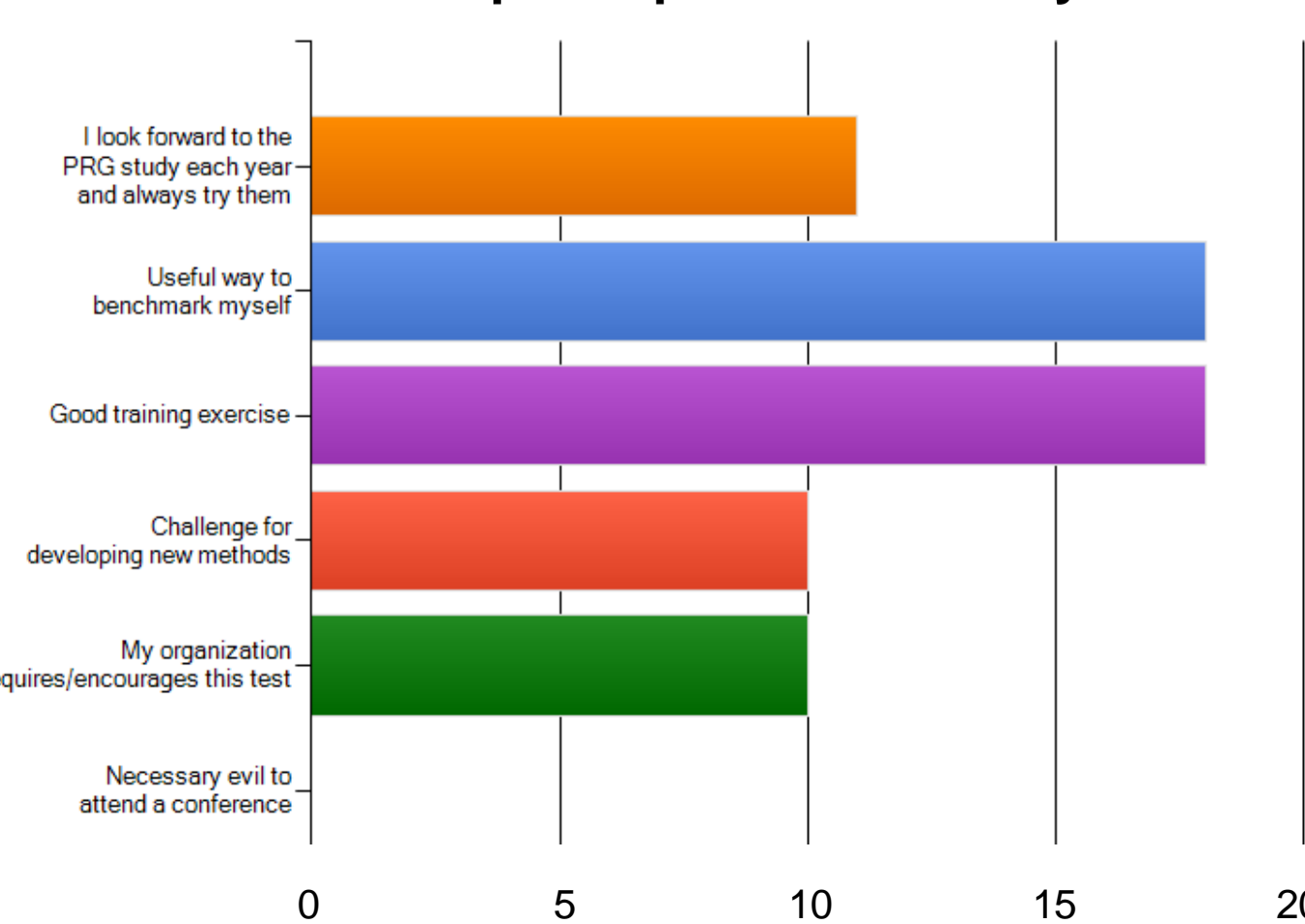
The learning curve

Participants' confidence in the identification of ¹⁵N-labeled proteins before and after the study - line thickness reflects participant response -



The fun

Motivation to participate in the study



A	Sample 1		# IDs Tube 1		Sample 2		# IDs Tube 2		Sample 3		# IDs Tube 3		DOUBLET	YodA	S100A6
	participant	1	2	3	4	5	6	7	8	9	10				
20091			2	6			6				6		✓	✓	✓
12358	2			6			6	3			6		✓	✓	✓
71415				6			6				6		✗	✓	✓
73648				6			6				6		✓	✓	✓
34621				6		3	6				6		✓	✓	✓
72327				6			5				6		✓	✓	✓
18018	2			5			5	2			6		✓	✓	✓
97319	2	2	2	5		2	5	2	2	2	5		✗	✓	✗
26402				4			4				5		✗	✓	✗
14125				5			4				5		✓	✗	✗
v11297				5			4			5	5		✓	✗	✗
20032				5	5		4			6	4		✓	✓	✓
71613				5			4				5		✓	✓	✗
36918				5	5		4			2	2	5	✓	✗	✗
27479	2			3		2	2			2	3		✓	✗	✗
20139	2			2	6	2	5	4			6		✗	✗	✗
30603				4	5		3				5		✗	✗	✗
28475				2	5		4			2	5		✗	✗	✗
12727				5			4				5		✗	✗	✗
29850				5			2	4			5		✗	✗	✗
29754	2			4			1			2	1		✗	✗	✗
15973				4	2		4				4		✗	✗	✗
40385				3			1				1		✗	✗	✗
27774				10	0		1				2		✗	✗	✗
46012							2						✗	✗	✗

Legend: bCAT (14N in #1, 15N in #2,3); S100-A6; Siah1 (14N in #1, absent in #2, 15N in #3); 15N-YodA (tube #2 only); SIP; SKP1; Ub; contaminants (n); 15N-contaminants (n); Siah1 incorrectly identified; 6: reported both expected 15N-proteins

Results

Table A

Results that were returned by PRG participants were scored for their ability to address the challenges in each of the 3 samples. Initial ranking was based on the ability to identify all 6 proteins in sample 1. Next, participants were scored for the following:

- **Doublet:** succeed ✓ or fail ✗ to recognize the appearance of β -catenin in 2 different forms
- **YodA:** succeed ✓ or fail ✗ to identify the ¹⁵N-labeled contaminant YodA
- **S100A6:** succeed ✓ or fail ✗ to identify the low-abundant component S100-6A. Reporting another S100 protein was scored with ⚠

Table B

Respondents used various combinations of gel- and LC-based protein and peptide separation, coupled to either MALDI- or ESI-based mass spectrometry. Although few participants got all the answers right, this indicates that there is no single road to success.

Participants rated this study from very easy to very hard, but this appeared to be a weak measure for completing the study successfully. Almost all labs successfully identified ¹⁵N-labeled proteins. Some of them had difficulty setting up database searching or did not succeed finding the contaminant, reflective of the different levels of difficulty built into the study.

Experience counts, but even labs that became operational only recently were very successful.

B	Participant	DOUBLET	YodA	S100A6	Difficulty	Experience	Time to complete study	General approach	Mass spectrometer used
20091	✓	✓	✓	Moderate	<6 months	2-4 days	Gel + LC-MS	Quadrupole-TOF	
12358	✓	✓	✗	Moderate	3-5 years	5-7 days	Gel + ESI-MS	3D Ion Trap	
71415	✗	✓	✓	Moderate	3-5 years	2-4 days	Gel + LC-MS	Quadrupole-TOF	
73648	✓	✓	✓	Moderate	5-10 years	2-4 days	LC-MS	Linear Ion Trap-Orbitrap	
34621	✓	✓	✓	Moderate	3-5 years	5-7 days	LC-MS	Quadrupole-TOF	
72327	✓	✓	✓	Moderate	<6 months	2-4 days	LC-MS	Quadrupole-TOF	
18018	✓	✓	✗	Moderate	>10 years	5-7 days	Gel + LC-MS	Linear Ion Trap	
97319	✗	✓	✗	Moderate	1-3 years	5-7 days	Gel + LC-MS	Linear Ion Trap	
26402	✗	✗	✗	Moderate	5-10 years	2-4 days	Gel + MALDI MS + ESI-MS	3D Ion Trap + TOF/TOF	
14125-2	✗	✗	✗	Moderate	5-10 years	2-4 days	Gel + ESI-MS	Linear Ion Trap-Orbitrap	
v11297	✓	✗	✗	Moderate	5-10 years	2-4 days	LC-MS	Quadrupole-TOF	
20032	✓	✗	✗	Very easy	>10 years	2-4 days	Gel + LC-MS	Linear Ion Trap-Orbitrap	
71613	✓	✗	✗	Hard	5-10 years	8-10 days	Gel + LC-MALDI	TOF/TOF	
36918	✓	✓	✗	Moderate	1-3 years	5-7 days	Gel + ESI-MS	Linear Ion Trap-Orbitrap	
27479	✓	✗	✗	Moderate	3-5 years	8-10 days	Gel + MALDI MS + ESI-MS	TOF/TOF	
20139	✗	✗	✗	Moderate	3-5 years	2-4 days	Gel + ESI-MS	Linear Ion Trap-FT	
30603	✗	✗	✗	Hard	3-5 years	>2 weeks	Gel + LC-MALDI	TOF/TOF	
28475	✗	✗	✗		5-10 years	2-4 days	LC-MS	Linear Ion Trap-Orbitrap	
12727	✗	✗	✗	Moderate	5-10 years	2-4 days	LC-MALDI	TOF/TOF	
29850	✗	✗	✗	Easy	5-10 years	2-4 days	LC-MS	Linear Ion Trap	
29754	✗	✗	✗	Moderate	5-10 years	11-14 days	Gel + ESI-MS	3D Ion Trap	
15973	✗	✗	✗	Easy	3-5 years	1 day	LC-MS	Linear Ion Trap-Orbitrap	
40385	✗	✗	✗	Moderate	3-5 years	2-4 days	Gel + ESI-MS	Linear Ion Trap	
27774	✗	✗	✗	Hard	First time	5-7 days	Gel + LC-MS	Linear Ion Trap	
46012	✗	✗	✗		5-10 years	2-4 days	Gel + MALDI MS	TOF/TOF	

Gel + LC-MS: Gel band excision and LC-MS/MS. Gel + ESI-MS: Assessment with gel, then analysis by LC-MS/MS