STANDARDISATION AND GUIDELINES

The ABRF Proteomics Research Group Studies: Educational exercises for qualitative and quantitative proteomic analyses

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Resource (core) facilities have played an ever-increasing role in furnishing the scientific community with specialized instrumentation and expertise for proteomics experiments in a cost-effective manner. The Proteomics Research Group (PRG) of the Association of Biomolecular Resource Facilities (ABRF) has sponsored a number of research studies designed to enable participants to try new techniques and assess their capabilities relative to other laboratories analyzing the same samples. Presented here are results from three PRG studies representing different samples that are typically analyzed in a core facility, ranging from simple protein identification to targeted analyses, and include intentional challenges to reflect realistic studies. The PRG2008 study compares different strategies for the qualitative characterization of proteins, particularly the utility of complementary methods for characterizing truncated protein forms. The use of different approaches for determining quantitative differences for several target proteins in human plasma was the focus of the PRG2009 study. The PRG2010 study explored different methods for determining specific constituents while identifying unforeseen problems that could account for unanticipated results associated with the different samples, and included ^{15}N-labeled proteins as an additional challenge. These studies provide a valuable educational resource to research laboratories and core facilities, as well as a mechanism for establishing good laboratory practices.

Keywords:
Identification / Serum biomarkers / Shotgun proteomics / Technology

1 Introduction

The primary goal of the Proteomics Research Group (PRG) of the Association of Biomolecular Resource Facilities (ABRF) is to sponsor research studies that permit assessment of current proteomics techniques and capabilities. Through the promotion of broad participation across a variety of core facilities and research laboratories, the PRG provides these studies to enable investigators to assess laboratory capabilities using controlled test samples designed to mimic real-life proteomics experiments. Due to the widespread popularity of these studies combined with an increasing breadth in the

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Abbreviations: ABRF, Association of Biomolecular Resource Facilities; **hCG,** β human chorionic gonadotropin; **MRM,** multiple reaction monitoring; **PRG,** Proteomics Research Group; **PSA,** prostate-specific antigen; **SRM,** selected reaction monitoring

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field of proteomics, the ABRF has recently expanded the PRGs to include the sub-disciplines of standards (sPRG), informatics (iPRG), and glycomics (gPRG).

The ABRF RG studies are world-renowned for their utility in benchmarking, establishing good laboratory practices, for aiding investigators in gaining experience with and testing new technologies, and providing information about the relative strengths and limitations of various technologies [1]. The studies are also useful as a way for participants to assess their expertise level relative to other facilities. The utility of the ABRF PRG studies has also recently been noted by the *ProteoRed* consortium in Spain, where a network of core laboratories has been using the PRG study samples for benchmarking and inter-laboratory assessment [2].

Over the past 10 years, the PRG has organized studies that have dealt with various aspects of proteomic analysis (http://www.abrf.org/prg). In this manuscript, we report on the three most recent studies. Although the PRG2008 and PRG2010 studies were designed to compare different strategies for characterizing qualitative differences between protein samples, the PRG2009 study addressed methods for detecting known biomarkers in the complex background of human plasma.

All of the studies were presented to the participants as straightforward analyses that are typical of the projects submitted to a core facility for proteomic analysis. In view of the fact that experience with a given technique often plays a critical role in a successful proteomics analysis, the PRG studies intentionally have different levels of challenges. Thus, it is anticipated that not every participant will necessarily obtain the best answer for every aspect of the study. In this way, the PRG studies provide an effective mechanism for promoting good laboratory practices, and the experience gained by the participants and the information that becomes shared are the true measures of success of these studies. These results provide a cross-sectional view of current methodologies as well as a vehicle for sharing information regarding experimental protocols and education for the proteomics community.

2 Materials and methods

2.1 PRG2008

The extracellular region of the receptor for advanced glycation end products, designated as soluble RAGE (sRAGE, consisting of V, C1, and C2 domains), and two truncated variants of the protein (VC1 and V) were overexpressed in *Escherichia coli* as described [3]. Stock solutions were prepared in 25 mM ammonium bicarbonate and transferred to polypropylene vials for 5 and 10 μg of total protein in tubes "A" and "B," respectively (Fig. 1A and B, and Table 1). Samples were then dried by vacuum centrifugation and packaged for shipping. Participants were advised that the samples were soluble in 25–50 mM ammonium bicarbonate, with or without 20% ACN, as well as in 0.1% formic acid.

2.2 PRG2009

The sample consisted of human plasma (Analytical Biological Services, Wilmington, DE, USA), prostate-specific antigen (PSA; BIOTREND Chemikalien GmbH, Koeln, Germany), human chorionic gonadotropin (βhCG; Sigma-Aldrich, St. Louis, MO, USA), and glycogen phosphorylase (GP-α/β; Sigma-Aldrich). Stock solutions for each protein were prepared at a concentration of 1 mg/mL in 50 mM Tris-HCl, pH 8. Study samples were prepared by adding appropriate volumes of stock solutions into human plasma (Table 2) and were then dried by vacuum centrifugation and packaged for shipping.

2.3 PRG2010

Proteins used in this study were expressed in *E. coli* as His_6-tagged proteins as described in [4] (Fig. 1C). ^{15}N-labeled proteins were generated in minimal medium supplemented with $^{15}NH_4Cl$ as the sole nitrogen source. Stock solutions were prepared and mixed as described in Section 3, with each vial containing approximately 3 μg of total protein (Table 3). The samples were dried in a vacuum centrifuge and packaged for shipping.

Guidelines for ABRF RG study preparation and testing can be found in the RG Handook available at http://www.abrf.org. For each study, after preparation, samples were comprehensively tested by PRG member laboratories to ensure the feasibility of the study. Verification was also made that the proteins could be maintained for several days (either in solution or dry, as appropriate) at room temperature without any detectable deterioration. Samples for North American locations were stored an additional 2 days at room temperature prior to shipping to approximate conditions that might be experienced by samples sent to international participants. Care was taken to ensure the anonymity of the participants throughout the study and in the presentation and publication of the results. Technology vendors were eligible for participation, and their results are denoted by a "v" appended to the identifier number.

3 Results

3.1 PRG2008: Qualitative proteomics study – identifying differences in primary structure

Two samples were prepared for this study (Fig. 1A and B). In tube "A" was a protein corresponding to the soluble V+C1+C2 domains of the receptor for advanced glycation

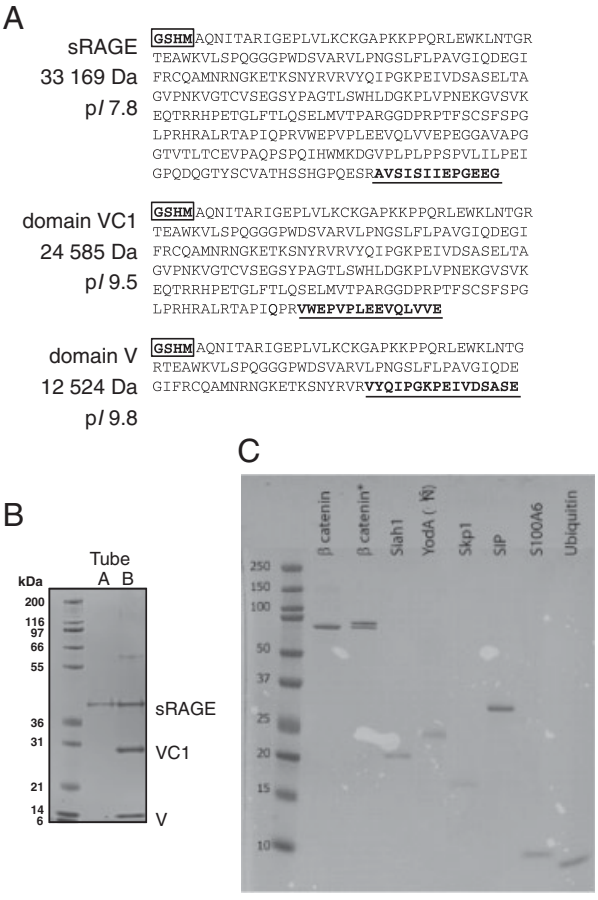


Figure 1. Expressed and purified proteins used in the PRG2008 and 2010 studies. (A) Expression constructs for soluble regions of the receptor for advanced glycation end products (sRAGE). Sequences in the His-tag included during expression in *E. coli* and remained after thrombin cleavage are boxed. The C-terminal tryptic peptide for each of the fragments used is underlined. (B) 1-D SDS-PAGE of sRAGE fragments included in the study samples, stained with Coomassie blue. (C) Individual proteins expressed for the PRG2010 study (Section 2). SDS-PAGE 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.

Table 1. PRG2008 sample composition

Protein	MW (Da)	Quantity	
		Tube A	Tube B
sRAGE	33 169	5 mg (151 pmol)	5 mg (151 pmol)
VC1	24 585		3 mg (122 pmol)
V	12 524		2 mg (160 pmol)

end products (sRAGE). Tube "B" contained the same sRAGE construct plus two variants: sRAGE V domain and V+C1 domains. Proteins were present at approximately equimolar ratios (122–160 pmol each). The participants

Table 2. PRG2009 sample composition

Tube	PSA	βhCG	GP-*a*	GP-*b*	GPA-(*b*/*a*)
	(fmol/μL)	(fmol/μL)	(fmol/μL)	(fmol/μL)	ratio
A and E	125	375	62.5	12.5	0.2
B and F	25	1250	25	50	2
C and D	250	25	12.5	62.5	5

Table 3. PRG2010 sample composition

Tube 1	Tube 2	Tube 3
β-catenin (doublet)	^{15}N β-catenin	^{15}N β-catenin
Siah1	^{15}N YodA (*E. coli*)	^{15}N Siah1
SIP	SIP	SIP
SKP1	SKP1	SKP1
S100-A6	S100-A6	S100-A6
Ubiquitin	Ubiquitin	Ubiquitin

were specifically asked to identify the major protein(s) in each sample and to report any qualitative differences in protein composition found between samples A and B. Participants were not told that truncated protein forms were present or that the actual *C*-termini would not be revealed through a standard peptide-based MS database analysis using strict enzyme cleavage rules. It was anticipated that the results of the analyses would permit assessment of the capabilities of various proteomics technologies to ascertain the differences in protein forms and would also facilitate assessment of potential complementation between different techniques. Samples were requested by 114 laboratories, and results were reported back anonymously via an online survey by 57 participants. Additional experimental details and results were provided by some participants and are publicly available on the PRG website.

As shown in Fig. 2, most of the study participants used peptide-based MS/MS (denoted as "B" for bottom-up) for protein identification, and in many cases this was in conjunction with other techniques that included 1-D or 2-D SDS-PAGE, Edman degradation, and intact protein MS. Twenty-one of the 57 responding participants provided at least some information about the truncated forms present in sample B (Fig. 2), of whom eight reported finding all truncation termini. The majority of participants completed the study within a week's time.

For the eight cases where all protein forms and unique truncation C-terminal endpoints were found and correctly assigned, six employed 1-D SDS-PAGE prior to in-gel digestion and HPLC-ESI-tandem MS, but similar success was also achieved by three groups who used intact mass determination by MS (with one group also using 1-D SDS-PAGE as well as Edman degradation). Among the 26 participants who only reported finding the full-length construct and not any of the truncated forms present in

sample B, 13 employed only peptide-based MS methods, suggesting that complementary approaches are often necessary to obtain a more complete result for proteomics analyses. There was no additional distinction between LC-MS/MS and MALDI-TOF or TOF/TOF instrument configurations with respect to ability to identify the truncations and their unique C-termini. None of the participants reported the use of electron capture dissociation (ECD) or electron transfer dissociation (ETD) to sequence intact proteins.

In addition to the requested information captured by the online survey, 33 of the 57 participants also provided detailed narratives about the methods used. These written descriptions are a valuable resource and provide insight into how groups approached the problem. For example, group ♯23300 applied a peptide-based MS/MS approach and reported only the full length construct in both samples A and B. The group then obtained intact molecular mass data by MS and discovered the presence of truncated forms in sample B, allowing them to re-interrogate the original peptide-MS analysis to extract information that accurately described the unique C-termini in sample B. Overall, 28 respondents reported that the study was of ''moderate'' difficulty, with 24 reporting that the study was ''difficult,'' and there were three responses each of ''easy'' and ''very easy.''

3.2 PRG2010: Tackling unforeseen problems in otherwise straightforward proteomics analyses

Like its 2008 predecessor, the PRG2010 study was presented as a general laboratory challenge typical for a wide cross-section of core proteomics facilities. Although the PRG2008 study had the added challenge of unanticipated truncation products, the PRG2010 study included an unanticipated contaminating protein in a ^{15}N-labeled background. Information was provided for the PRG2010 study about the expected contents of the sample in a manner that is similar to a real-life sample submission. However, the substitution of the contaminant in place of a known component was not disclosed to the participants to mimic an actual sample submission more effectively. Samples were requested by 96 laboratories, and results were reported back anonymously via online survey by 47 participants (25 of which contained information on the results reported herein).

The PRG2010 study was comprised of three consecutive sample submissions to a core facility of an ubiquitination complex that was isolated using expressed Siah1 E3 ligase and β-catenin in a fashion that co-purified associated proteins. These proteins collectively produce a complex exhibiting in vitro ubiquitination activity [4] (Fig. 3A, Table 3). The first sample corresponded to an isolated complex with high ubiquitination activity, with the goal to find four additional exogenously added bacterially expressed proteins. The second was a sample prepared for NMR structural studies, wherein Siah1 and β-catenin were ^{15}N-labeled, but the submitted complex was unable to carry out the in vitro ubiquitination reaction. The third sample contained new preparations from a repeat of the ^{15}N-experiment that now exhibited full in vitro activity. As demonstrated by PRG members, separation of the proteins by 1-D SDS-PAGE analysis (Fig. 3A) was able to reveal several anomalies that might explain the loss of activity in the second submission, such as a high molecular weight doublet that is only seen in tube 1 or an apparent mobility-shift in the protein components observed in tube 2. The main tasks of the study were to

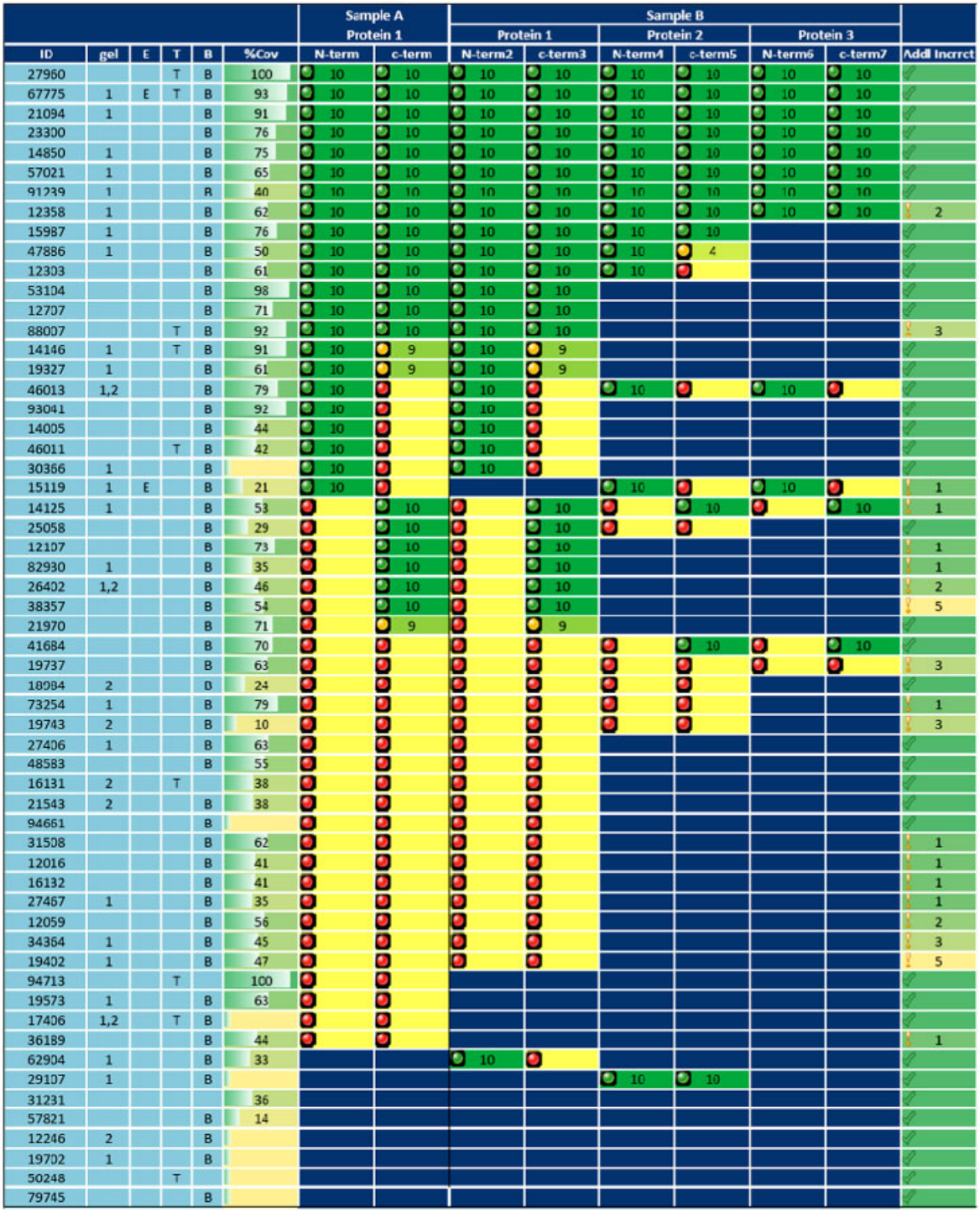
(i) Identify the contents of tube 1.
(ii) Identify what is different in tube 2 that might explain the loss of activity in the in vitro ubiquitination assay.
(iii) Identify what is different in tube 3 that might explain the restoration of the in vitro activity.
(iv) Define the nature of the unusual gel doublet that is present only in tube 1.

Participants were informed that the samples contained protein complexes that were either active or inactive when used in an in vitro ubiquitination assay, but that β-catenin should not be ubiquitinated in these samples. Participants were also told that some samples contained proteins grown in ^{15}N medium, and recommendations for conducting a database search for ^{15}N-labeled proteins were provided (http://www.abrf.org/ResearchGroups/Proteomics/Studies/PRG2010supplementaryinformation.pdf). The expressed proteins used for this study were derived from an actual in vitro ubiquitination assay [4].

The study was designed to offer different levels of challenge. The contents of tube 1 were typical for a sample submitted for identification of unknown proteins in a defined mixture. In addition to Siah1 and β-catenin whose sequences were provided, this sample also contained human CYBP, SKP1, UBIZ and S100A6. Preliminary testing by the PRG indicated that the identification of S100A6 may be challenging for some participants due to the relatively low number of peptides/spectral counts observed for a typical LC-MS/MS experiment and database search. Indeed, only a third of the participants were able to identify S100A6, whereas most were able to identify the other protein components (Fig. 3B).

The second sample (tube 2) provided the greatest challenge because it contained ^{15}N-labeled proteins and there was a contaminant (*E. coli* ^{15}N-YodA instead of ^{15}N-human Siah1). This situation mimics the original analysis that this study is based on [4], wherein ^{15}N-YodA was originally expressed and IMAC-enriched instead of the expected ^{15}N-HIS$_6$-Siah1 (data not shown). Siah1 binds zinc, so Siah1 expression from the ^{15}N medium presumably resulted in the expression of bacterial YodA, which is produced in response to zinc starvation and also can be enriched via IMAC strategies [5].

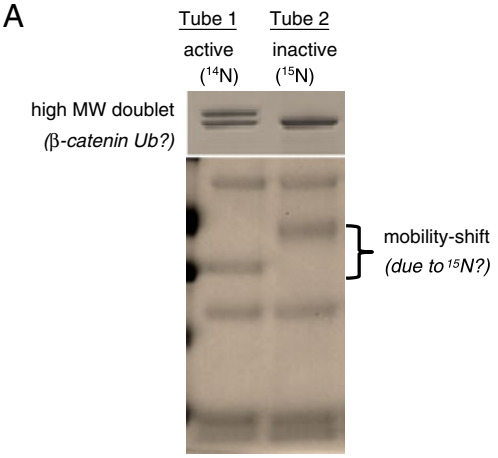
Searching MS/MS data from ^{15}N-labeled peptides is not always straightforward, with only some algorithms able to easily accommodate searching with labeled amino acid



						Sample A Protein 1		Sample B Protein 1		Sample B Protein 2		Sample B Protein 3		
ID	gel	E	T	B	%Cov	N-term	c-term	N-term2	c-term3	N-term4	c-term5	N-term6	c-term7	Addl Incrrct
27960			T	B	100	10	10	10	10	10	10	10	10	
67775	1	E	T	B	93	10	10	10	10	10	10	10	10	
21094	1			B	91	10	10	10	10	10	10	10	10	
23300				B	76	10	10	10	10	10	10	10	10	
14850	1			B	75	10	10	10	10	10	10	10	10	
57021	1			B	65	10	10	10	10	10	10	10	10	
91239	1			B	40	10	10	10	10	10	10	10	10	
12358	1			B	62	10	10	10	10	10	10	10	10	2
15987	1			B	76	10	10	10	10	10	10			
47886	1			B	50	10	10	10	10	10	4			
12303				B	61	10	10	10	10	10				
53104				B	98	10	10	10	10					
12707				B	71	10	10	10	10					
88007			T	B	92	10	10	10	10					3
14146	1		T	B	91	10	9	10	9					
19327	1			B	61	10	9	10	9					
46013	1,2			B	79	10		10		10		10		
93041				B	92	10		10						
14005				B	44	10		10						
46011			T	B	42	10		10						
30366	1			B		10		10						
15119	1	E		B	21	10				10		10		1
14125	1			B	53		10		10		10		10	1
25058				B	29		10		10					
12107				B	73		10		10					1
82930	1			B	35		10		10					1
26402	1,2			B	46		10		10					2
38357				B	54		10		10					5
21970				B	71		9		9					
41684				B	70						10		10	
19737				B	63									3
18984	2			B	24									
73254	1			B	79									1
19743	2			B	10									3
27406	1			B	63									
48583				B	55									
16131	2		T		38									
21543	2			B	38									
94661				B										
31508				B	62									1
12016				B	41									1
16132				B	41									1
27467	1			B	35									1
12059				B	56									2
34364	1			B	45									3
19402	1			B	47									5
94713			T		100									
19573	1			B	63									
17406	1,2		T	B										
36189				B	44									1
62904	1			B	33			10						
29107	1			B						10	10			
31231					36									
57821				B	14									
12246	2			B										
19702	1			B										
50248			T											
79745				B										

Figure 2. PRG2008 results. In addition to reporting on percent coverage, participants were asked to return specific information on the N- and C-termini. The number of correct residues for the first ten amino acids of each terminus is indicated, with highlighting transitioning from green to yellow to reflect relative success (green having all ten residues reported). Dark blue indicates that no protein sequence was returned. The right-hand column shows the number of proteins reported that were not correct. Key: ID = participant identifier; Gel 1 = 1-D-SDS-PAGE; Gel 2 = 2-D-SDS-PAGE; E = Edman sequencing; T = analysis of intact proteins; B = peptide-based analysis; %Cov = % sequence coverage reported by participant.

A



B

participant	Tube 1	# IDs Tube 1	Tube 2	# IDs Tube 2	Tube 3	# IDs Tube 3	DOUBLET	YodA	S100A6	Difficulty	General Approach	Mass Spectrometer Used
20091	2	6		6		6	✓	✓	✓	Moderate	Gel + LC-MC	Quadrupole-TOF
12358	2	6		6	3	6	✓	✓	!	Moderate	Gel + ESI-MS	3D Ion Trap
71415		6		6		6	✗	✓	✓	Moderate	Gel + LC-MS	Quadrupole-TOF
73648		6		6		6		✓	✓	Moderate	LC-MS	Linear Ion Trap-Orbitrap
34621		6	3	6	2	6		✓	✓	Moderate	LC-MS	Quadrupole-TOF
72327		6		5		6		✓	!	Moderate	LC-MS	Quadrupole-TOF
18018	2	5		5	2	6	✓	✓	!	Moderate	Gel + LC-MS	Linear Ion Trap
97319	2 2 2 2 2	5	2 2 2 2	5	2 2 2 2	5	✗	✓	✗	Moderate	Gel + LC-MS	Linear Ion Trap
26402		4		4		5	✗	✓	✗	Moderate	Gel + MALDI MS + ESI-MS	3D Ion Trap + TOF/TOF
14125		5	3	4		5	✓	✗	✗	Moderate	Gel + ESI-MS	Linear Ion Tra
v11297		5		4	5	5	✓	✗	✗	Moderate	LC-MS	Quadrupole-TOF
20032	5	5	4	5	6	4	✓	✗	!	Vy easy	Gel + LC-MS	Linear Ion Trap-Orbitrap
71613		5		4		5	✓	✗	✗	Hard	Gel + LC-MALDS	TOF/TOF
36918	5	5	2 2	4	2 2	5	✓	✗	✗	Moderate	Gel + ESI-MS	Linear Ion Trap-Orbitrap
27479	2	3	2	2	2	3	✓	✗	✗	Moderate	Gel + MALDI MS + ESI-MS	TOF/TOF
20139	2 2	6	2	5	4	6	✗	✗	!	Moderate	Gel + ESI-MS	Linear Ion Trap-FT
30603	4	5		3		5	✗	✗	!	Hard	Gel + ESI-MSI	TOF/TOF
28475	2	5	2	4	2	5		✗	✗		LC-MS	Linear Ion Trap-Orbitrap
12727		5	3	4	2	5		✗	✗	Moderate	LC-MALDI	TOF/TOF
29850		5	2	4	2	5	✗	✗	✗	Easy	LC-MS	Linear Ion Trap
29754	2 2	**4**		**1**		**1**	✗	✗	✗	Moderate	Gel+ESI-MS	3D Ion Trap
15973		4	2 3	4		4	✗	✗	!	Easy	LC-MS	Linear Ion Trap-Orbitrap
40385		3		1		1		✗	✗	Moderate	Gel + ESI-MS	Linear Ion Trap
27774	10	0		1		2		✗	✗	Hard	Gel + LC-MS	Linear Ion Trap
46012				2							Gel + MALDI MS	TOF/TOF

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

Figure 3. PRG2010 results. (A) representative SDS-PAGE gel resolving the components present in tubes 1 and 2 (colloidal Coomassie blue staining). Potential anomalies that might explain the loss of ubiquitination activity in tube 2 are indicated. (B) The identification of each component is demarked by a colored box: red = β-catenin (^{14}N-labeled in tube 1 and ^{15}N-labeled in tubes 2 and 3), pink = S100-A6, green = Siah1 (^{14}N-labeled in tube 1, absent in tube 2, and ^{15}N-labeled in tube 3), orange = ^{15}N-labeled *E. coli* YodA (tube 2 only), light blue = SIP, purple = SKP1, dark blue = ubiquitin. Grey and black boxes reflect the reporting of contaminants that were not intentionally introduced into the samples, and a green box outlined in red reflects the incorrect assignment of Siah1 in tube 2 (participants were not told that ^{15}N-Siah1 was replaced with ^{15}N-labeled YodA). Numbers within each colored cell indicate the number of separate entries for the indicated protein, or number of contaminants. A yellow field indicates that both ^{15}N-labeled components (either β-catenin and YodA in tube 2, or β-catenin and Siah1 in tube 3) were identified. Results were empirically scored for relative success in the three main challenges, which were ranked based on the ability to identify all six proteins in tube 1, followed by correct assessment of the doublet in tube 1, identification of ^{15}N-labeled YodA in tube 2, and ability to detect S100-A6. Relative success was scored using a green checkmark to indicate correct assessment, an orange exclamation point for partial success, a red X for incorrect assessment, and a blank field for no data returned.

masses. However, since Siah1 was an expected component of this sample and the sequence was provided, most respondents were able to correctly calculate the expected ^{15}N-labeled peptide masses and determine that Siah1 was missing from tube 2 (whereas the ^{14}N-labeled Siah1 peptides were clearly detected in tube 1), and this result provided a reasonable explanation for the loss of activity. Although the majority of respondents reported being able to analyze data from the ^{15}N-labeled proteins (either ''with ease'' or with our assistance), only nine were able to determine the presence of ^{15}N-YodA in tube 2 (Fig. 3B).

An additional challenge was determining the difference between the two species present in the high molecular weight doublet observed in 1-D SDS-PAGE for tube 1 (both bands were derived from β-catenin). This challenge was similar to that of the PRG2008 study, which involved careful examination of the MS data and comparison with the provided amino acid sequence for the in vitro expressed protein. In this case, two forms of β-catenin were present; one was the expressed variant, which was missing the N-terminal 133 amino acids and contained a fusion-specific four amino acid linker, and the other was the full-length form. These differences could be determined by detection of peptides that were specific for the two versions (GGILHAVVNLINYWDDELATR (fusion) and HAVVNLINYWDDELATR (full length)), as well as two peptides found in the N-terminal region that was not present in the expressed variant. Seventeen of the respondents reported that both doublet bands correspond to β-catenin, but only nine correctly determined the difference between the two proteins.

The third sample (tube 3) represented a new preparation with ^{15}N-labeled β-catenin and ^{15}N-labeled Siah1 that was reported to be active for the in vitro ubiquitination assay. In this case, ^{15}N-Siah1 was properly expressed and IMAC-purified because of supplementation of exogenous $ZnCl_2$ to the growth medium (data not shown). The experimental tasks required for the sample in tube 3 were less challenging than for tube 2, with more participants now being able to identify ^{15}N-labeled Siah1 but still having similar difficulty in identifying S100A6.

The majority of participants rated the overall study difficulty as ''moderate,'' with only three rating it as ''easy'' or ''very easy.'' Although the participants selecting ''easy''/''very easy'' did not report on the more challenging aspects of the study, including the presence of S100A6, ^{15}N-YodA or the explanation of the high MW doublet. Overall, the ability to provide data on all of the challenges presented was not associated with experience with a particular technique (ranging from 3–5 years to greater than 10 years experience).

3.3 PRG2009: Relative protein quantification in a clinical matrix

More focused in scope than the PRG2008 and PRG2010 studies, the PRG2009 study was designed to test a laboratory's ability to find and quantify three proteins that were spiked at different levels into the same mixtures of human plasma proteins. This type of task is typically encountered in biomarker projects and also reflects the increasingly common request for proteomics laboratories to determine quantitative differences among samples in clinical matrices such as urine, plasma, or CSF. The experiment often begins with analysis of a small set of pilot samples (e.g. experimental versus control) where putatively differentially expressed protein species are identified. Additional sample sets are subsequently examined for a more detailed evaluation of these proteins to determine if the initial observation of quantitative differences holds within a larger sample cohort. The major challenges associated with this type of analysis are detection and accurate quantification in very complex matrices.

The target proteins used in this study were introduced into samples of human plasma at levels ranging from 2.5 fmol/μL to 1.25 pmol/μL. Three different mixtures were used, and samples were presented as duplicates, resulting in six different samples for which the contents of each was blind to the participants (Table 2). Two of the proteins were chosen as representative biomarkers of human disease or diagnosis (PSA and βhCG). The third protein was rabbit glycogen phosphorylase (GP) that was present at the same total quantity but with different ratios of two isoforms that differed by the presence or absence of an N-terminal phosphorylation event. Samples were requested by 49 laboratories, and results were reported back by 27 participants.

Six tubes were distributed to participants along with information about the study, including disclosure that there were two blinded duplicate sets, the sequences of the target proteins, and examples of typical tandem mass spectra of tryptic peptides derived from the proteins used in the sample (http://www.abrf.org/ResearchGroups/Proteomics/Studies/PRG2009-SampleLetter_final.pdf). The participants were asked to report the following:

(i) Relative quantities of the four specified proteins among the six samples.
(ii) Proper matching of the duplicate samples.
(iii) The instrumental method(s) used to analyze the samples.
(iv) The strategy used for relative quantification.

The majority of participants ($N = 8$) used either selected reaction monitoring (SRM, also referred to as multiple reaction monitoring; MRM) or an analysis based on data-dependent acquisition followed by post hoc analysis selected reaction retrieval (Fig. 4A). A depletion column for sample preparation prior to digestion and tandem MS analysis was used by 47% of the participants. Other methods for sample preparation included 1-D SDS-PAGE and chromatography as detailed in Fig. 4B.

Most participants were able to correctly assign the duplicate samples, indicating that a variety of different

approaches are able to analyze samples of this type (Fig. 4C). The quantitative results that were closest to expected values (and that also showed the least variability amongst the respondent's measurements) were obtained for βhCG ($N = 14$ responding) followed by results for PSA ($N = 15$ responding) (Fig. 5). Although most of the respondents who reported quantitative data on the target proteins used depletion of abundant plasma proteins prior to subsequent analyses, the results showed that all instrument types interfaced with chromatographic separation could be used, with and without depletion, to obtain results that were in agreement with the expected values.

All participants reported using trypsin for proteolytic digestion. Successful MS scanning methods included SRM/MRM, peptide precursor/product ion chromatograms extracted from full scan tandem mass spectra, and MS^E. The PRG2009 study was rated as "difficult" by 11 and "moderate" by 6 of the participants, with the average confidence level of the participants' ability to perform the analysis remaining at "confident" to "very-confident" before and after the study. Most participants did not find the sample quantity to be limiting.

In preparation for the study, the PRG had PSA and βhCG levels in two sample sets determined by two clinical laboratories using standard ELISA techniques. With some exception, the results of the clinical laboratories were largely in agreement with the expected values calculated based on results from amino acid analysis (Fig. 4D and E). The exceptions were that PSA levels in samples B and F appeared to be below the detection limit for one of the clinical laboratories, and both clinical laboratories noted some difficulty in analyzing PSA levels in samples A and E.

3.4 Participants

All PRG studies are announced by postings on proteomics and mass spectrometry websites. As a result, participation in PRG studies has been global, with the majority coming from North America (mostly United States of America, with some from Canada) and Europe, but with a high representation from Spain due to the *ProteoRed* project [2]. Groups from Asia and Australia/New Zealand, and Middle East and South America, are also represented.

4 Discussion

Today, there is a wide range of powerful approaches available for qualitative and quantitative analysis of proteins. The

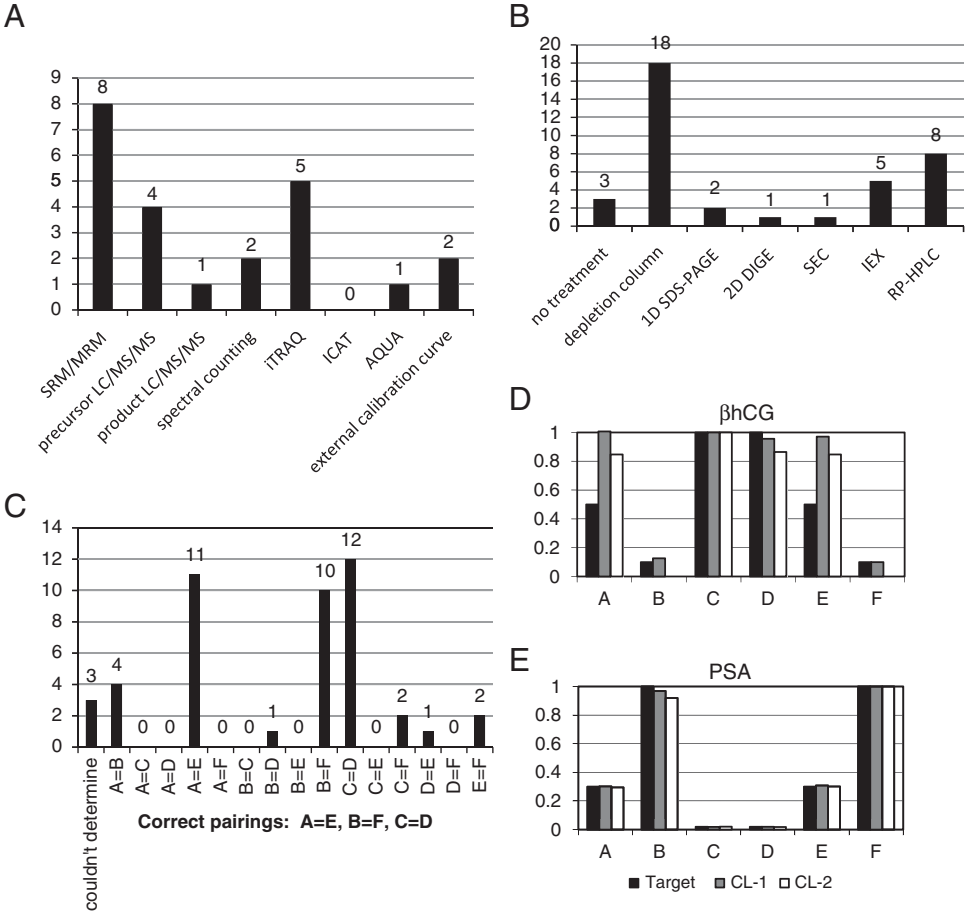


Figure 4. PRG2009 results. Mass spectrometry quantification (A) and sample preparation (B) techniques used by the study participants. (C) Tally of duplicate results as reported by the study participants via online survey, in response to the qualitative question, "These samples were provided as duplicates of three different dilutions of the four proteins. Please indicate below which pairs you think are the duplicates?" All 15 pair-wise choices for samples A–F were provided, in addition to "could not determine for any of them." (D and E) Relative amounts of prostate-specific antigen and β chorionic gonadotropin reported by clinical laboratories 1 and 2 (CL-1 and CL-2).

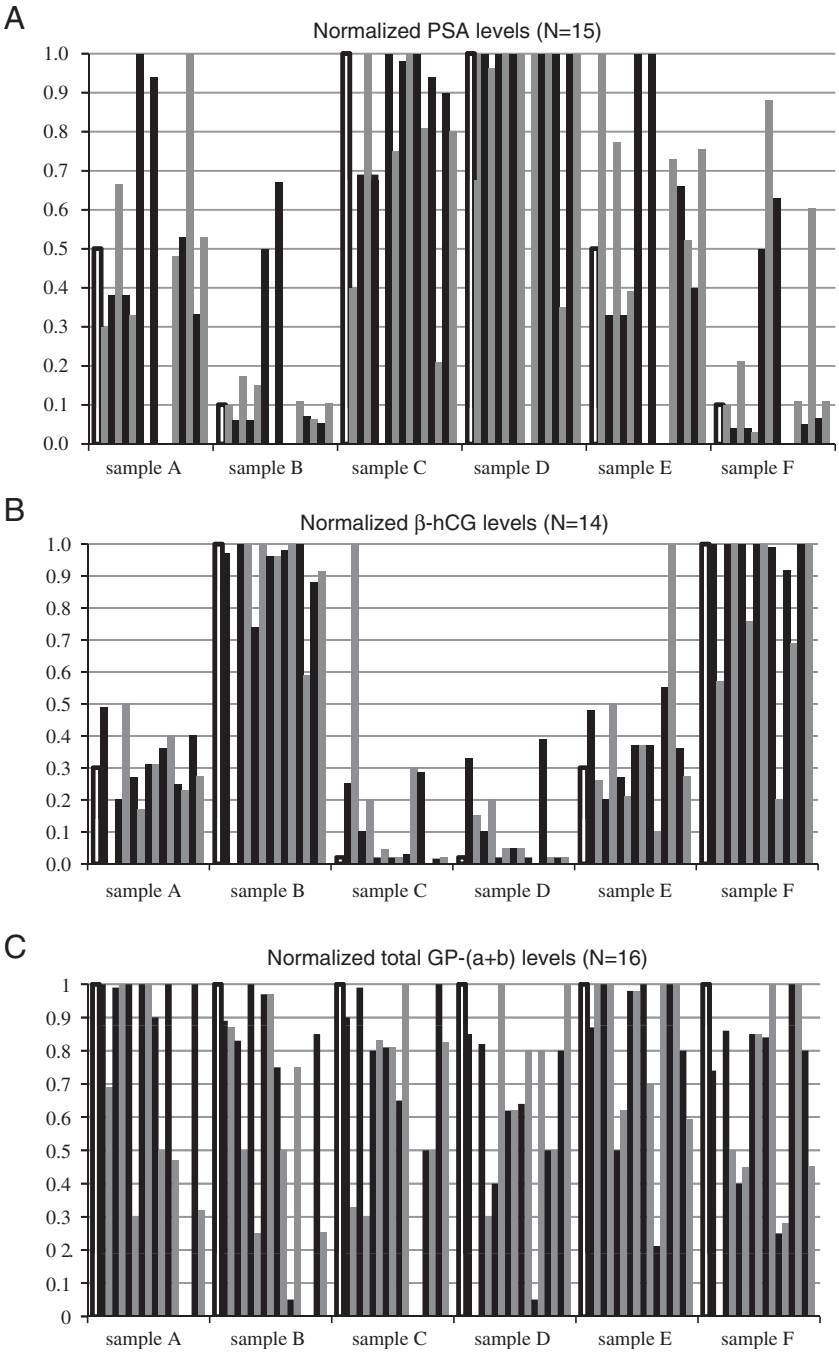


Figure 5. Detail of PRG2009 study quantitative results. Open bars (first in each series) reflects the target value for the indicated protein in each sample, followed by the results reported by the participants in alternating black and grey. Not every participant reported data for each sample, or for each protein. The total number (N) reporting data for PSA was 15 (A), 14 for βhCG (B) and 16 for glycogen phosphorylase, isoforms a and b combined (C).

results from the three PRG studies presented here indicate that protein/peptide separations and tandem mass spectrometry are still the methods of choice for performing standard proteomics analyses for discovery-based protein identification and for targeted approaches to gain specific information on a specific protein.

A vital function of a successful proteomics core facility is not only to generate sufficient information from an analysis to deliver the desired results, but also to identify potential sources of unanticipated variation that may impinge on the validity of an experiment. In the cases where unanticipated variation is found (e.g. truncations, contaminants and missing components), such information can be instrumental in helping an investigator modify the experiment and/or reagents to attain the desired results.

To provide information on such unanticipated variation, a key finding evident from these studies is that utilizing complementary approaches (e.g. intact protein separation by 1-D SDS-PAGE and peptide analysis by ESI-LC/MS/MS) is beneficial. This conclusion may seem obvious, but far too

often a single technique is employed and an incomplete answer is obtained. This was most apparent in the PRG2008 study where without the prior separation or detection of intact protein forms, many of the participants were unable to detect that truncated forms were present. As expressed by one participant, ''This study is a great example for how dreadful little you learn about protein structure if you run mere shotgun approaches.'' In cases where participants determined that truncations were present, they were more often able to determine the unique peptides that demarcated the truncation endpoints. This is a significant observation because this information was not revealed to participants ahead of time, and it demonstrates the utility of complementary approaches to be able to report crucial information regarding the efficacy of an experiment that contains unforeseen variants.

Uncovering unanticipated variants was also a theme of the PRG2010 study, where *E. coli* YodA (a protein from the expression system) was purposefully introduced into a sample in place of the target expressed protein (human Siah1). In this case, detection of the substitution was complicated by the fact that the proteins were ^{15}N-labeled. The inclusion of the ^{15}N-labeled contaminant YodA mimicked actual experiments using these expressed proteins that eventually led to a published Siah1-dependent in vitro ubiquitination assay for β-catenin [4]. In this case, the ability to determine that Siah1 had been replaced by *E. coli* YodA was critical, enabling the original investigators to successfully express Siah1 simply by supplementing the medium with exogenous $ZnCl_2$ (data not shown), since Siah1 is a zinc-chelating protein and YodA is induced upon metal starvation [5].

Finding contaminants instead of expected proteins is a common occurrence, especially when proteins are expressed and purified from recombinant sources. In the case of the ^{15}N-labeled Siah1 and YodA proteins in the PRG2010 study, participants needed to be able to search MS data in an unbiased discovery-mode analysis to detect YodA. This requires using ^{15}N-labeled amino acid masses in the database search, but not all search algorithms can accommodate this easily. This presents a realistic scenario; as captured by our survey, 5 of the respondents currently analyze ^{15}N-labeled samples (most at less than 1% of total throughput), with another 11 reporting that they have plans to offer this service in the near future. Given that the nature of the ^{15}N-label was fully disclosed (as it would be in a real submission), a workable solution still needs to be in place to enable the detection of a true contaminant.

The quantitative nature of the PRG2009 study was more challenging than the qualitative/troubleshooting aspects of the other studies, most likely due to the complex nature of the plasma background, and this study was associated with a concomitantly higher reported difficulty level as assessed by the participants. For the PRG2009 study, it was necessary to use an SRM/MRM-based approach to obtain quantitative answers that were closest to expected. Yet even without specific experience with or instrumentation optimized for analysis by SRM, participants were usually able to ascertain which pairs of samples were duplicates, a valuable determination for very complex samples. Although the number of participants returning useful data was too low to enable any significant conclusions to be drawn with respect to approaches that were more successful than others, most participants were able to attain some quantitative measure of the actual levels for the three proteins using a variety of instrument types and methods, and in most cases depletion of the most abundant proteins was helpful. Participants who used SRM reported that prior knowledge of the transitions to monitor was very valuable, as exemplified in a comment by one of the participants: ''We tried MRM method without knowing the MS/MS spectra of the proteins were on ABRF web [sic]. Without the MS/MS information, our MRM was not successful.'' Despite the fact that representative tandem mass spectra were provided for participants to design SRM experiments, it is anticipated that the results of this study would have demonstrated even more effectiveness for the SRM technique had software tools such as *Skyline* [6] been more readily available at the time of the study.

Several of the participating laboratories detected substantial keratin contamination in the study samples, although keratin was not intentionally added to the samples (despite claims from some participants!). Other issues noted involved difficulty with resolubilizing the samples, and in some cases, sample degradation and the need for more material. Since all samples were prepared in bulk and aliquoted prior to shipping (including room-temperature stability tests), it is difficult to ascribe any of these sporadic reports with problems inherent to the samples per se. Although our studies were not specifically designed to account for these variables, it suggests that these anomalous results arise from individual laboratory technique, and underscores the level of care that must be taken in proteomics analyses to protect samples from common laboratory contamination during processing.

The PRG studies provide a valuable educational resource to research laboratories and core facilities. Many laboratories have submitted results that were consistent with the intended expectations alongside those that were unable to perform a complete analysis. But overall, the PRG studies demonstrate that most of the participating laboratories were able to effectively address the challenges presented in the various studies detailed here. This is important because we have made no attempt to constrain participation to a predefined group of investigators using the same equipment or with similar years of experience. Indeed, participating laboratories may have different goals with these study samples, ranging from high-performance evaluation, to testing out new capabilities, to providing a learning experience to new lab members. To this end, our studies intentionally have different levels of challenges and are therefore useful both from an educational standpoint as well as for establishing good laboratory practices.

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6 Addendum

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