SILAC and iTRAQ Quantitation on an Orbitrap Using Protein Prospector

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Introduction

Mass spectrometry-based quantitative proteomics has proven to be a powerful approach to distinguish specific from non-specific protein interactions and determine biological process relevant changes in protein expression and posttranslational modifications. To quantify the MS data based on stable isotope labeling, the processing software needs to have the capability of generating quantitative information at both peptide and protein levels automatically. It also needs to have the flexibility of analyzing data from different instruments and different labeling methods. Towards this goal, we have developed several new features in the Search Compare program within Protein Prospector. In order to minimize the need for manual validation of ratios, we also improved the accuracy of the measurements with the option of averaging ratios across a given elution profile.

The new developments in PP for quantitative proteomics are illustrated in a study seeking to understand the changes of posttranslational modifications in proteasome complexes after oxidative stress using SILAC-based approach. The ability to acquire and quantify iTRAQ data using the LTQ-Orbitrap is also demonstrated.

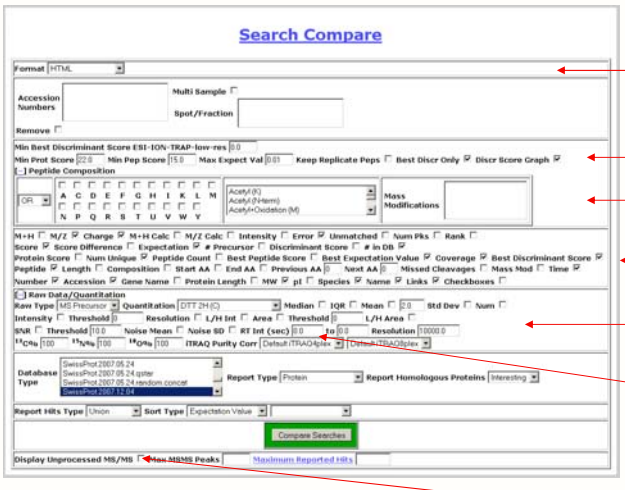
Experimental

1. **SILAC experiment:** The 26S proteasome complex was purified from cells expressing Rpn11-TAP. The cells grown in heavy media containing $^{13}C,^{15}N$ arg/lys were used as a control and the cells grown in light media containing $^{12}C,^{14}N$-arg/lys were treated with hydrogen peroxide. The cells were lysed and mixed in a 1:1 ratio prior to affinity purification. The purified proteasome complexes were then digested by trypsin and analyzed by 2-dimensional liquid chromatography and LTQ-Orbitrap XL MS. A cycle of one full FT scan mass spectrum (350-2000 m/z, resolution of 60,000 at m/z 400) followed by ten data-dependent MS/MS acquired in the linear ion trap with normalized collision energy (for details see Poster ThPVV 557).

2. **iTRAQ experiment:** The sample was part of study investigating differential protein composition in post-synaptic density preparations from different parts of the mouse brain (for more details attend presentation TOA 3:50pm).

3. **Data processing:** More than 100,000 spectra were acquired from LTQ-Orbitrap XL MS and searched using Batch-Tag within Protein Prospector. Search compare was used for results sorting, validation and quantification.

Search Compare Options in PPv5



Output format can be HTML or tab delimited text

Score filters

Composition filters

Report Columns

Raw data/quantitation options

Option that allows averaging of the precursor data

If this option is selected all subsequent MSMS spectra will be displayed using all the peaks in the centroid file

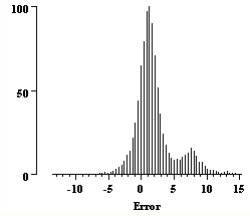
Representation of Quantitative Protein Report Using Search Compare Program

XW_1D_2D/acc

Rank	Uniq Pep	Acc #	Num Unique	Peptide Count	% Cov	Med L/H I	Q1	Q3	Mn L/H I	-1.0σ	+1.0σ	Num	Protein MW	Protein Name
1		YHR027C	204	1679	80.9	0.992	0.940	1.05	1.00	0.853	1.18	1427	109493.0	RPN1 SGDID:S000001069, Chr VIII from 164704-161723, reverse complement, Verified ORF, "Non-ATPase base subunit of the 19S regulatory particle of the 26S proteasome; may participate in the recognition of several ligands of the proteasome; contains a leucine-rich repeat (LRR) domain, a site for protein?protein interactions"
2		YIL075C	199	1519	66.0	0.984	0.935	1.03	0.994	0.819	1.22	1350	104222.0	RPN2 SGDID:S000001337, Chr IX from 220697-217860, reverse complement, Verified ORF, "Subunit of the 26S proteasome, substrate of the N-acetyltransferase Nat1p"
3		YOR117W	112	1045	74.7	0.973	0.918	1.02	1.07	0.573	2.01	911	48256.2	RPT5 SGDID:S000005642, Chr XV from 545020-546324, Verified ORF, "One of six ATPases of the 19S regulatory particle of the 26S proteasome involved in the degradation of ubiquitinated substrates; recruited to the GAL1-10 promoter region upon induction of transcription"
4		YER021W	112	976	75.9	1.00	0.954	1.06	1.02	0.873	1.20	846	60393.3	RPN3 SGDID:S000000823, Chr V from 196947-198518, Verified ORF, "Essential, non-ATPase regulatory subunit of the 26S proteasome lid, similar to the p58 subunit of the human 26S proteasome; temperature sensitive alleles cause metaphase arrest, suggesting a role for the proteasome in cell cycle control"
5		YDR427W	107	996	70.0	0.999	0.999	1.06	1.01	0.925	1.23	903	45702.9	RPN9 SGDID:S000002835, Chr IV from 1322197-1323378, Verified ORF, "Non-ATPase regulatory subunit of the 26S proteasome, has similarity to putative proteasomal subunits in other species; null mutant is temperature sensitive and exhibits cell cycle and proteasome assembly defects"
6		YDL097C	96	721	73.3	0.997	0.941	1.05	0.990	0.741	1.32	629	49774.4	RPN6 SGDID:S000002255, Chr IV from 286695-285391, reverse complement, Verified ORF, "Essential, non-ATPase regulatory subunit of the 26S proteasome lid required for the assembly and activity of the 26S proteasome; the human homolog (S9 protein) partially rescues Rpn6p depletion"
7		YDL147W	98	570	76.9	1.02	0.968	1.08	1.02	0.927	1.11	487	51768.9	RPN5 SGDID:S000002306, Chr IV from 190925-192262, Verified ORF, "Essential, non-ATPase regulatory subunit of the 26S proteasome lid, similar to mammalian p55 subunit and to another S. cerevisiae regulatory subunit, Rpn7p"
8		YOR259C	90	738	74.1	1.00	0.942	1.06	1.01	0.677	1.16	624	49408.6	RPT4 SGDID:S000005785, Chr XV from 813708-812395, reverse complement, Verified ORF, "One of six ATPases of the 19S regulatory particle of the 26S proteasome involved in the degradation of ubiquitinated substrates; required for spindle pole body duplication; localized mainly to the nucleus throughout the cell cycle"
9-1	84	YDR394W	86	846	88.1	1.07	1.02	1.14	1.15	0.651	2.03	750	47894.2	RPT3 SGDID:S000002802, Chr IV from 1261673-1262959, Verified ORF, "One of six ATPases of the 19S regulatory particle of the 26S proteasome involved in the degradation of ubiquitinated substrates; substrate of N-acetyltransferase B"
9-2	79	YGL048C	80	830	74.6	1.06	0.995	1.12	1.06	0.914	1.24	719	45272.1	RPT6 SGDID:S000003016, Chr VII from 411289-410072, reverse complement, Verified ORF, "One of six ATPases of the 19S regulatory particle of the 26S proteasome involved in the degradation of ubiquitinated substrates; bound by ubiquitin-protein ligases Ubr1p and Ufd4p; localized mainly to the nucleus throughout the cell cycle"
9		YDL007W	83	807	73.7	1.01	0.954	1.07	1.06	0.538	2.09	703	48828.6	RPT2 SGDID:S000002165, Chr IV from 438044-439357, Verified ORF, "One of six ATPases of the 19S regulatory particle of the 26S proteasome involved in the degradation of ubiquitinated substrates; required for normal peptide hydrolysis by the core 20S particle"
10		YKL145W	96	788	81.6	1.00	0.951	1.06	1.05	0.676	1.64	680	51983.4	RPT1 SGDID:S000001620, Chr XI from 174210-175621, Verified ORF, "One of six ATPases of the 19S regulatory particle of the 26S proteasome involved in the degradation of ubiquitinated substrates; required for optimal CDC20 transcription; interacts with Rpn12p and the E3 ubiquitin-protein ligase Ubr1p"

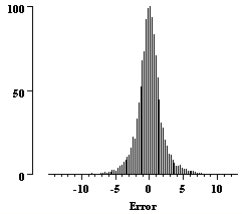
Note: Q1 and Q3 are the limits of the interquartile range.

Internal Calibration Using Peptide Hits



The distribution of mass errors from all the hit peptides shows a potential calibration problem in some of the fractions.

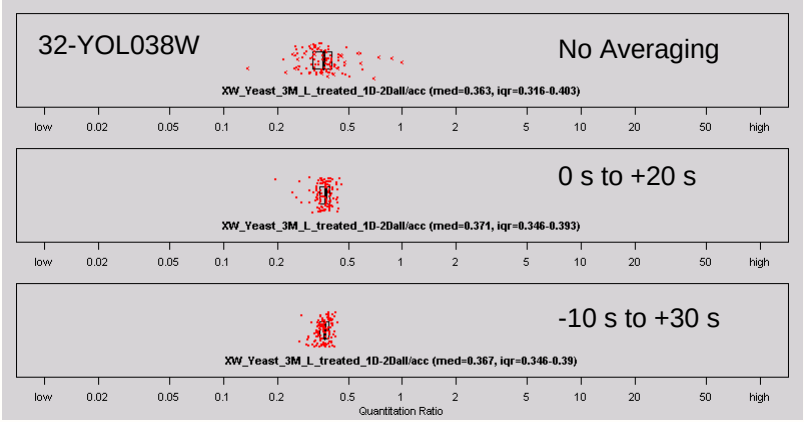
A calibration procedure shows the problem to be in mainly in fraction 1. It is possible to correct each fraction individually and research the data. The corrected error distribution is shown below.



Fraction	Num Points	Mean Error	StDev Error	3 StDev Error
1	2140	7.925	2.222	6.667
2	139	1.232	2.333	6.998
3	23	1.454	2.531	7.592
4	26	2.096	1.657	4.972
5	497	1.790	2.182	6.545
6	988	1.233	2.093	6.280
7	975	1.288	2.027	6.082
8	1417	1.006	1.968	5.903
9	1302	0.5369	1.909	5.727
10	1041	0.3847	2.002	6.007
11	1080	0.6062	2.017	6.050
12	1032	1.678	2.230	6.691
13	1188	1.213	2.079	6.237
14	1231	1.012	1.854	5.562
15	905	1.429	1.967	5.900
16	720	1.407	1.994	5.983
17	570	1.146	2.128	6.385
18	1453	1.093	1.783	5.349

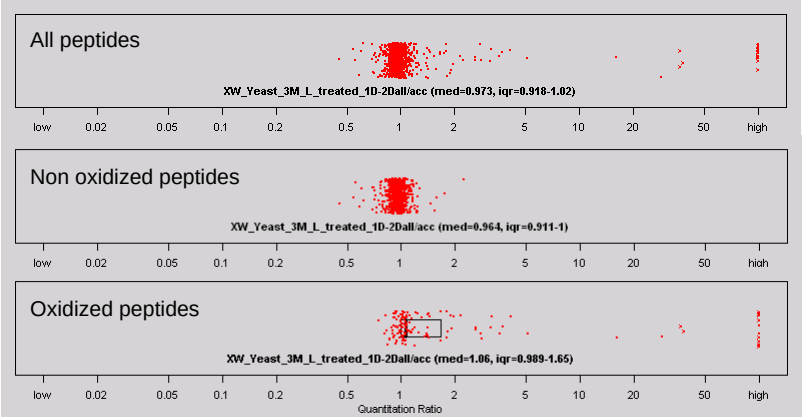
Scatter Plots of SILAC Ratios of Identified Peptides from the Selected Proteins

Improved Ratio Statistics Using MS Scan Averaging



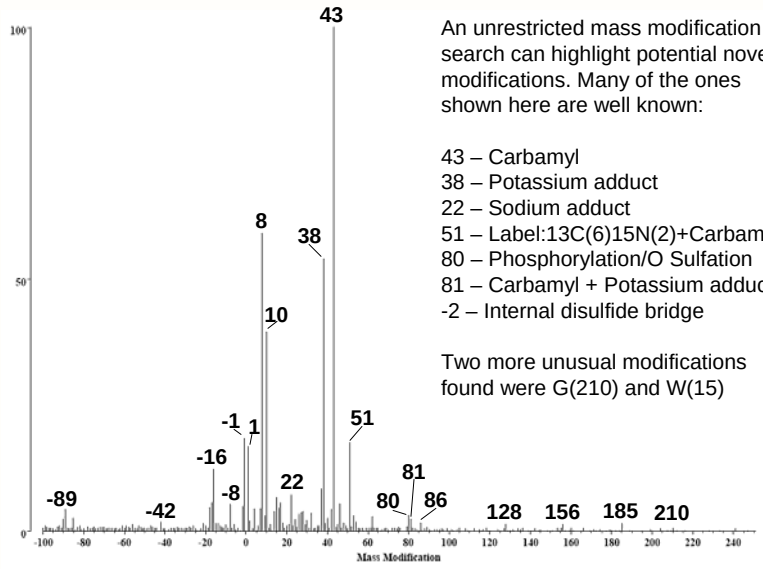
Search Compare now has a feature whereby the scans within a specified RT range are averaged before the ratio is calculated. This significantly reduces the spread of the results.

Comparison of Ratio Distributions of Normal Peptides and Oxidized Peptides from 3-YOR117W



•Variable levels of oxidation are responsible for the majority of peptide quantitation ratio outliers observed for measurements for this protein

Unrestricted Mass Modification Search



An unrestricted mass modification search can highlight potential novel modifications. Many of the ones shown here are well known:

43 – Carbamyl
38 – Potassium adduct
22 – Sodium adduct
51 – Label:13C(6)15N(2)+Carbamyl
80 – Phosphorylation/O Sulfation
81 – Carbamyl + Potassium adduct
-2 – Internal disulfide bridge

Two more unusual modifications found were G(210) and W(15)

Mass modification histogram from Search Compare output

Protein N-Terminus Myristoylation

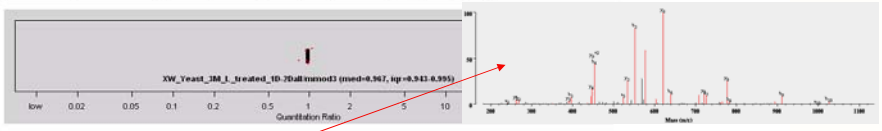
1 **Acc. #:** YDL007W **Species:** YEAST **Name:** RPT2 SGDID:S000002165, Chr IV from 438044-439357, Verified ORF, "One of six ATPases of the 19S regulatory particle of the 26S proteasome involved in the degradation of ubiquitinated substrates; required for normal peptide hydrolysis by the core 20S particle"

Protein MW: 48828.6 **Protein pI:** 5.8 **Protein Length:** 437

```
1   MGQGVSSGQD KKKKKGSNQK PKYEPPVQSK FGRKKRKGGP ATAEKLPNIY PSTRCKLKLL RMERIKDNLL LEEEFVSNSE
81  ILKPFEKKQE KEKKQLEEIR GNPLSIGTLE EIIDDDNAIV TSPTMPDYYV SILSFVDKEL LEPGCSVLLH HKTMSIVGVL
161 QDDADPMVSV KKNDKSPTES YSDIGGLESQ IQEIKESVEL PLTHPELYEE MGIKPPKGVI LYGAPGTGKT LLAKAVANQT
241 SATFLRIVGS ELIQKYLGDG PRLCPQIFKV AGENAPSIVF IDEIDAIGTK RYDSNSGGER EIQRTMLELL NQLDGFDDRG
321 DVKVIMATNK IETLDPALIR PGRIDRKILF ENPDLSTKKK ILGIHTSKMN LSEDVNLETL VTTKDDLSGA DIQAMCTEAG
401 LLALRERRMQ VTAEDFKQAK ERVMKKNVEE NLEGLYL
```

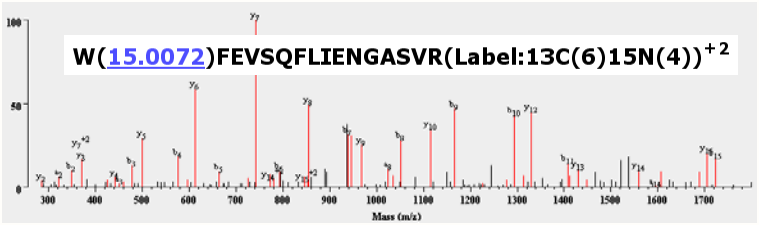
Num Unique	Peptide Count	% Cov	Med L/H I	Q1	Q3	Mn L/H I	-1.0σ	+1.0σ	Num
2	7	2.7	0.967	0.943	0.995	0.958	0.889	1.03	6

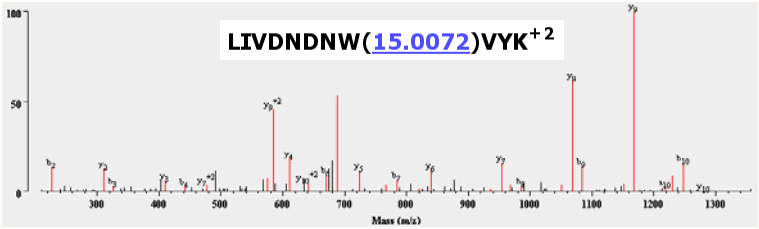
The G(210) modification turned out to be Protein N-Terminus Myristoylation. Search Compare allows these peptides to be quantitated separately



m/z	z	Da	Peptide	M Mod	Fraction	S	L/H Intensity	Score	Expect	# in DB
586.8303	2	0.049	M(Met-loss)G(210.1008)QGVSSGQDK	210	orbi031308_01	101.216	**0.959**	34.7	5.1e-5	1
650.8773	2	0.049	M(Met-loss)G(210.1008)QGVSSGQDKK	210	orbi031308_08	99.412	**0.943**	27.8	2.0e-4	1
650.8785	2	0.050	M(Met-loss)G(210.1008)QGVSSGQDKK	210	orbi031308_07	99.333	**0.975**	28.3	6.2e-4	1
650.8788	2	0.050	M(Met-loss)G(210.1008)QGVSSGQDKK	210	orbi031308_06	99.373	**0.995**	29.1	6.7e-4	1
650.8774	2	0.049	M(Met-loss)G(210.1008)QGVSSGQDKK	210	orbi031308_09	99.377	**1.05**	24.6	0.0019	1
650.8784	2	0.050	M(Met-loss)G(210.1008)QGVSSGQDKK	210	orbi031308_12	99.364	**0.839**	22.7	0.0033	1

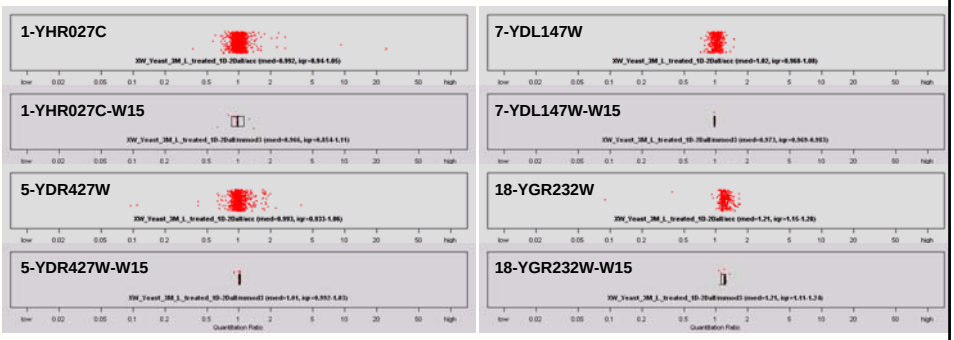
W(15) Modification





A modification of 15 Da to tryptophan was found in around 10 different peptides from 4 proteins in the sample. Some of these were labeled and some not. By searching one of the spectra again, adjusting the mass defect and considering the calibration offset for the relevant fraction the mass defect was found to by 14.978 (±0.006) Da. Two example spectra with the proposed tryptophan modification are shown above.

W(15) Modification – Quantitation Ratios



Distributions of the quantitation ratios for all the peptides and for just the W(15) modified peptides for the 4 proteins are shown above.

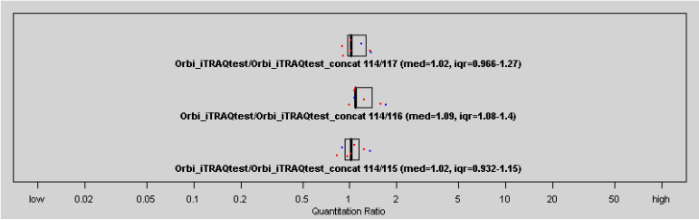
iTRAQ using HCD

•HCD fragmentation in the C-trap produces 'quadrupole-like' fragmentation with a full mass range, allowing observation of iTRAQ reporter ions.

33 **Acc. #:** Q9QYR6 **Gene:** MAP1A_MOUSE **Species:** MOUSE **Name:** Microtubule-associated protein 1A

Protein MW: 300142.3 **Protein pI:** 4.9 **Protein Length:** 2776

Num Unique	% Cov	Best Disc Score	Best Expect Val	Mn 114/115 A	-2.0σ	+2.0σ	Mn 114/116 A	-2.0σ	+2.0σ	Mn 114/117 A	-2.0σ	+2.0σ
7	1.9	1.88	1.3e-5	1.04	0.737	1.47	1.23	0.807	1.86	1.09	0.773	1.55



Red dots – Unique to this database entry
Blue dots – Occur in multiple entries in the database

iTRAQ Results

33 Acc. #: Q9QYR6 Gene: MAP1A_MOUSE Species: MOUSE Name: Microtubule-associated protein 1A

Protein MW: 300142.3 Protein pI: 4.9 Protein Length: 2776

Num Unique	% Cov	Best Disc Score	Best Expect Val
7	1.9	1.88	1.3e-5

m/z	z	ppm	Peptide	Fraction	S	Area 114	Area 115	Area 116	Area 117	Score	Expect	# in DB
540.3182	2	-0.63	iTRAQ4plex-AGSTALGSK(iTRAQ4plex)	T8011508	21.1365	258503	190452	151518	188534	25.8	1.3e-5	2
613.3274	2	1.6	iTRAQ4plex-SPWASDFK(iTRAQ4plex)	T8011506	46.2337	55585	57541	50881	60977	18.6	3.5e-5	1
561.3248	2	1.6	iTRAQ4plex-SETLQQK(iTRAQ4plex)	T8011508	17.6857	66846	65618	53816	65310	21.8	4.5e-5	1
580.8106	2	-4.4	iTRAQ4plex-AESFYQK(iTRAQ4plex)	T8011508	29.9789	132673	147471	122983	111476	23.4	6.2e-5	2
576.8218	2	1.1	iTRAQ4plex-TESSEALK(iTRAQ4plex)	T8011506	20.9712	146871	118537	93491	109133	27.8	9.0e-5	1
533.8096	2	-1.5	iTRAQ4plex-ELGFQGK(iTRAQ4plex)	T8011506	32.9731	409002	383005	379561	399883	23.0	2.0e-4	1
533.8030	2	0.41	iTRAQ4plex-NVTTSEK(iTRAQ4plex)	T8011508	15.0082	129386	155331	130702	143957	23.5	9.5e-4	1

- Uses raw data
  - Can report peak areas or intensities
- Protein Prospector can calculate mean and standard deviations or results can be exported as a tab-delimited file into spreadsheet for other statistical analysis.

- Single measurement
  - Generally gives larger standard deviations than SILAC.

Conclusions

♦The version of the Search Compare program in Protein Prospector version 5.0 is able to effectively analyze and quantify isotope labeled peptides such as SILAC-labeled and iTRAQ-labeled peptides measured from multiple instruments including Orbitrap and QSTAR. Due to high mass resolution, high signal to noise and high dynamic range, orbitrap data appears to work well for quantitation purposes.

♦Quantitation ratios can be obtained not only for peptides without modifications and with known modifications, but also for peptides with unknown modifications (i.e. mass modifications). Comparison of ratio distributions can be selectively plotted for chosen modifications.

♦Scan averaging leads to less spread in the ratio distributions and better statistics.

♦ A mass modification histogram can be generated which facilitates searching for potentially new modifications. These new modifications can later be quantitated.

Acknowledgements

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References

1. Wang, X, Huang, L.(2008) Mol Cell Proteomics, 7(1):46-57.
2. Baker P. R., Chalkley R. J., Trinidad J. C. and Burlingame A. L., New Protein Level Quantitation Features in Protein Prospector, 55th ASMS Conference of Mass Spectrometry and Allied Topics, Indianapolis, Indiana June 3rd - June 7 2007

A version of the Protein Prospector software used in this poster is freely available for use on the web at:

http://prospector2.ucsf.edu

This version does not by default allow the quantitation analysis, but if you would like to try it for quantitation it can be arranged by contacting ppadmin@cgl.ucsf.edu. Also, a version can be acquired for in-house use that has all the functionality demonstrated on this poster (again, contact ppadmin@cgl.ucsf.edu.)