Optimized LC-MS/MS System Performance for High Throughput Analysis of Affinity Isolated Protein Complexes

Keiji G. Asano[1], Patricia K. Lankford[2], Tse-Yuan S. Lu[2], Catherine K. McKeown[2], Gregory B. Hurst[1], W. Hayes McDonald[1]

[1]Chemical Sciences Division, [2]Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN

OVERVIEW

- **Purpose**
  - Increase throughput of LC-MS/MS analysis of affinity isolated protein complexes without compromising sensitivity, dynamic range, and breadth of peptides to be analyzed
- **Method**
  - Systematic evaluation of variables, including gradient optimization, omission of desalting/sample cleanup, and direct loading onto analytical column
- **Results**
  - New protocol reduces analysis time by 55% while producing better results for number of proteins, peptides and spectra identified.

INTRODUCTION

- The Center for Molecular and Cellular Systems (CMCS) was established to elucidate protein-protein interactions in bacterial systems, such as *Rhodopseudomonas palustris (R. palustris)*, relevant to DOE for their potential in energy production and use in bioremediation and carbon sequestration.
- Development of "pipeline" analysis for soluble proteins in *R. palustris* that is also applicable to other organisms relevant to DOE energy and environmental missions.
- Improved throughput is needed for more rapid characterization of protein-protein interactions in either a particular organism or sets of pathways across organisms.
- Recent improvements/automation of the affinity isolation protocol have increased the rate of production.
- Improvements to current LC-MS/MS protocol needed to handle increased sample throughput.
- New protocol must maintain or improve data quality.

EXPERIMENTAL

- General "Pipeline" Work Flow (see poster 654 and mippi.ornl.gov for more information)
  - express a dual-affinity-tagged protein in a bacterial culture
  - Lyse cells
  - Isolate the affinity-tagged protein along with interacting proteins
  - Trypsin digestion and sample cleanup/desalting
  - LC-MS/MS identification of proteins
  - Data analysis
- Current LC-MS/MS protocol included digesting the protein, desalting (OMIX), and then autosampling directly onto a trapping cartridge followed by reversed phase separation on a column packed directly into a nanospray tip. Variables studied in optimizing this protocol to decrease total analysis time (summarized in Table 1) included omitting the initial desalting step, reducing sample volumes, and eliminating the trapping cartridge in favor of direct loading onto the analytical column.

Table 1

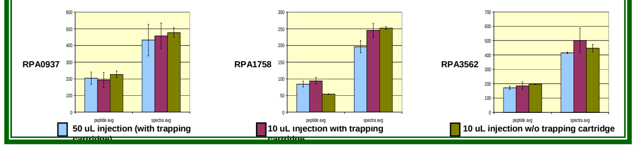
	Current Protocol	New Protocol
Instrumentation	LC Packings FAMOS Autosampler (50 uL loop)	LC Packings FAMOS Autosampler (10 uL loop)
	LC Packings Ultimate HPLC	Agilent 1200 HPLC
	LC Packings Switchos	
	ThermoFinnigan LCQ Deca XP Plus	ThermoFinnigan LQ Deca XP Plus
Solvent System		
Solvent A	95% H_2O, 5% acetonitrile, 0.1% formic acid	100% H_2O, 0.1% formic acid
Solvent B	30% H_2O, 70% acetonitrile, 0.1% formic acid	5% H_2O, 95% acetonitrile, 0.1% formic acid
Solvent C	5% H_2O, 95% acetonitrile, 0.1% formic acid	NA
Analytical Column	15 cm, 100µm i.d. Jupiter, C18, 5µm (Phenomenex)	20 cm, 100µm i.d. Jupiter, C18, 3µm (Phenomenex)
Flow Rates	300 nL/min at spray tip	300 nL/min at spray tip
Repeat Count for dynamic exclusion in data dependent acquisition	2	1
Method		
blanks	190 min solvent blank	70 min "blank" using PSM
sample	160 min analysis time	140 min analysis time
	190 min total run time	140 min total run time

- Standards and Samples
  - digested *R. palustris* (rpal) proteome standard
  - *digested protein standard mixture (PSM)
  - archived pulldown samples – *R. palustris*
  - pulldown samples – *R. palustris*

RESULTS AND DISCUSSIONS

Small Volume Injection Experiments

Figure 1. Plots showing search results of various protein baits using different injection volumes (50 uL vs. 10 uL) and with and without a trapping cartridge prior to the analytical column.



Our results showed:
- No dramatic difference in chromatography and search results between 50 uL and 10 uL injections.
- Plugging of the ion transfer tube became an issue when injecting 10 uL directly onto the analytical column (no trapping cartridge) since the samples for these experiments were not desalted prior to analysis.
- Subsequently, the actual sample comparison experiments were performed on samples that had been desalted.

Gradient Optimizations

Figure 2. Gradient optimizations were performed for our new column and HPLC setup (see table 1). Both the start (5% and 10%) and end (25%, 30%, and 35%) concentrations of buffer B (95% ACN, 0.1% FA) were varied. Plots show both base-peak chromatograms from 3 ug of injected *R. palustris* proteome and density plots of protein identifications achieved per unit time for each gradient

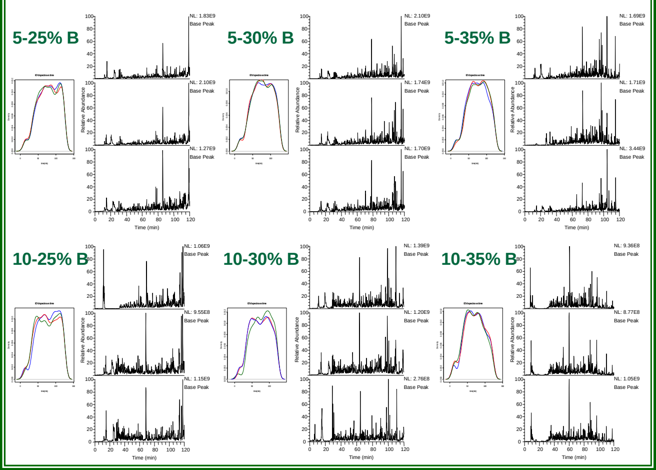
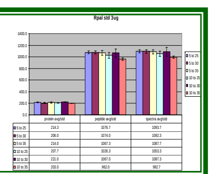


Table 2. Sequest search results for LC-MS/MS analysis of *rpal* standard using various gradients.



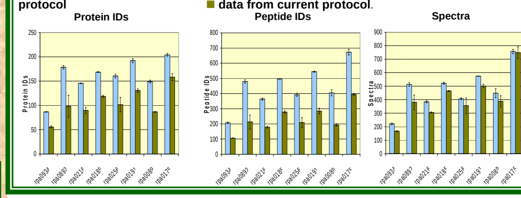
	protein avg/std	peptide avg/std	spectra avg/std
5 to 25	214.3	1076.7	1093.7
5 to 30	206.0	1074.0	1092.3
5 to 35	214.0	1067.3	1087.7
10 to 25	207.7	1026.3	1053.3
10 to 30	221.0	1067.0	1087.3
10 to 35	203.0	962.0	992.7

Our results showed:
- Little difference in the total number of protein, peptide, and spectral identifications across the various gradients,
- Differences in the rate of identifications per unit time suggest that these new gradients were more efficient than the current gradient at separating out the peptides that are most readily identified.
- A 10% to 30% B gradient was chosen

Real-world Samples: Pipeline "Pulldown" Comparison

- Equal amounts of material injected onto column
- Duplicate measurements for each sample with blanks between runs
- Total analysis time (8 samples (duplicate runs), blanks between each run and PSM QC samples at the beginning and end of a pipeline run – total of 37 runs)
  - current protocol: 4 days 21 hrs
  - new protocol: 2 days 16 hrs

Figure 3. Comparison of Sequest search results for various protein complex samples. Results are average of two replicates. ■ data from new protocol ■ data from current protocol.

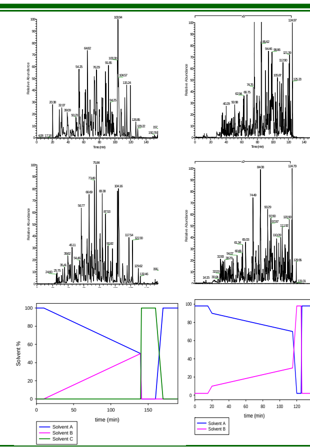


Our results showed:
- New protocol yields consistently greater numbers of protein, peptide, and spectral identifications for each of these real-world pipeline "pull-down" samples
- This was accomplished while reducing the total analysis time by ~ 55% and increasing the number of QC injections from 2 to 20

Real-world Samples Continued: RPA0175

Figure 4. Base-peak chromatograms (duplicate runs) from bait protein RPA0175 using current protocol (left) and new protocol (right).

Diagrams of final gradient conditions (below)



Our results showed:
- While the total number of proteins identified using the new protocol was greater, the sequence coverage of certain proteins (e.g. the target protein shown in Table 3) did not increase.
- However, other members of the protein complex did have both a greater number of peptides identified and a greater percentage of sequence coverage (Table 4).

Tables 3 and 4. Results comparing percent sequence coverage and specific peptides identified (SEQUEST scores for each peptide shown) for the target protein, RPA0175 (top), and two other subunits of the complex, RPA0843 and RPA0844 (bottom).

		Current protocol		New protocol	
Table 3	RPA0175 atpC (ATP synthase epsilon chain)	18.50%	18.50%	18.50%	18.50%
	K.IVVLGGIAEVSDK.G +1	3.47	3.16	3.36	3.71
	K.IVVLGGIAEVSDK.G +2	4.81	4.67	4.50	4.78
	R.PGILTVTAGANK.Q +1			3.04	3.10
	R.PGILTVTAGANK.Q +2	3.50	3.69	3.78	3.94
Table 4	RPA0843 atpF1 (ATP synthase B chain)	22.10%	14.10%	33.70%	28.20%
	K.AKLEDFVAR.R +2			2.63	
	K.GNLADELLSK.G +2	2.99	3.15	3.06	3.53
	K.IALAEAQALADVR.A +2	4.67	4.69	4.46	4.73
	R.AAAAEAAVAAASR.I +1			2.94	2.81
	R.AAAAEAAVAAASR.I +2	4.58			4.39
	R.EAQAIVDSAK.A +1			2.93	3.05
	R.EAQAIVDSAK.A +2			2.78	
	RPA0844 atpF2 (ATP synthase, subunit B')	23.80%	23.80%	38.40%	34.10%
	K.AVDSAVDASLK.G +2				3.39
	K.AVDSAVDASLKG.- +1			2.49	2.75
	K.AVDSAVDASLKG.- +2	3.85	4.03	4.06	3.97
	K.AYEAELADAR.A +1			2.03	2.73
	K.AYEAELADAR.A +2	3.97	4.03		
	K.LKGEADDALK.A +1			2.09	2.47
	K.LKGEADDALK.A +2			2.90	2.81
	K.LNAQAEAER.K +2			2.72	3.01
	K.TIDGDLAAAQK.L +1			2.37	2.28
	K.TIDGDLAAAQK.L +2			4.22	3.81
	R.AQAIGAETR.E +1				2.08
	R.AQAIGAETR.E +2			2.82	3.01
	R.EKLNAQAEAER.K +2			3.02	3.16
	R.NIASDAASAIVQQLAGVTPDSK.A +2	5.99	5.87		
	R.NIASDAASAIVQQLAGVTPDSK.A +3	4.31	5.17		
	R.VGGVIEER.Q +2			2.77	

CONCLUSION

- Optimized and implemented an updated LC-MS/MS protocol for the analysis of isolated protein complexes
- Can eliminate trapping cartridge and use smaller injection volumes (less loading time) for direct column loading.
- Produced consistently better results in terms of number of proteins, peptides, and spectra identified
- Analysis time for 8 samples (in duplicate) with blanks and PSM QC samples (37 total)
  - current: 4 days 21 hrs
  - new: 2 days 16 hrs
- Performing QA/QC injections as "blanks" in between all of our analytical injections

ACKNOWLEDGMENTS

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