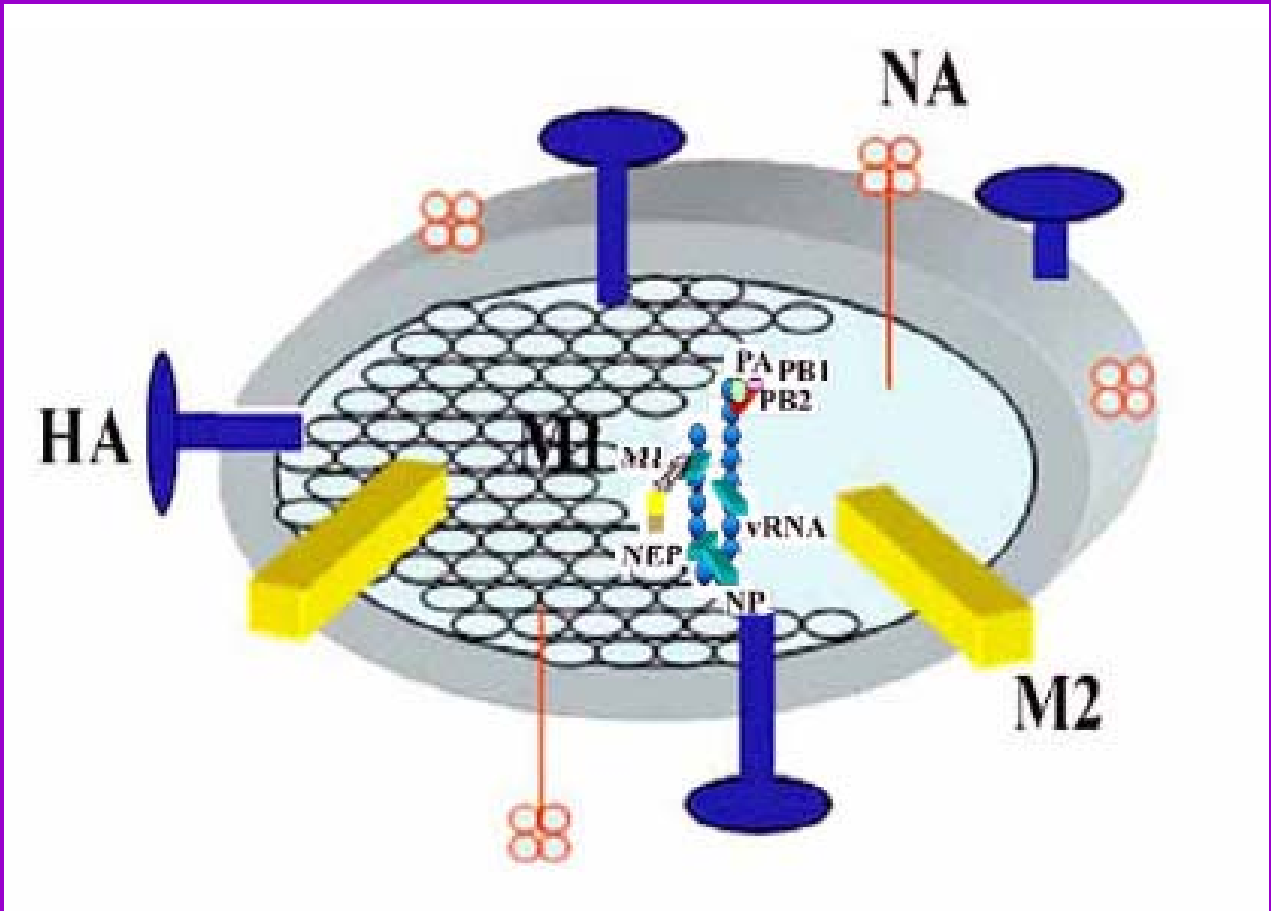
High-pressure assisted in-gel tryptic digestion in label-free quantification of influenza virus proteins

Melkamu Getie-Kebtie
Division of Cell and Gene Therapy
CBER/FDA

21 May 2010

Overview

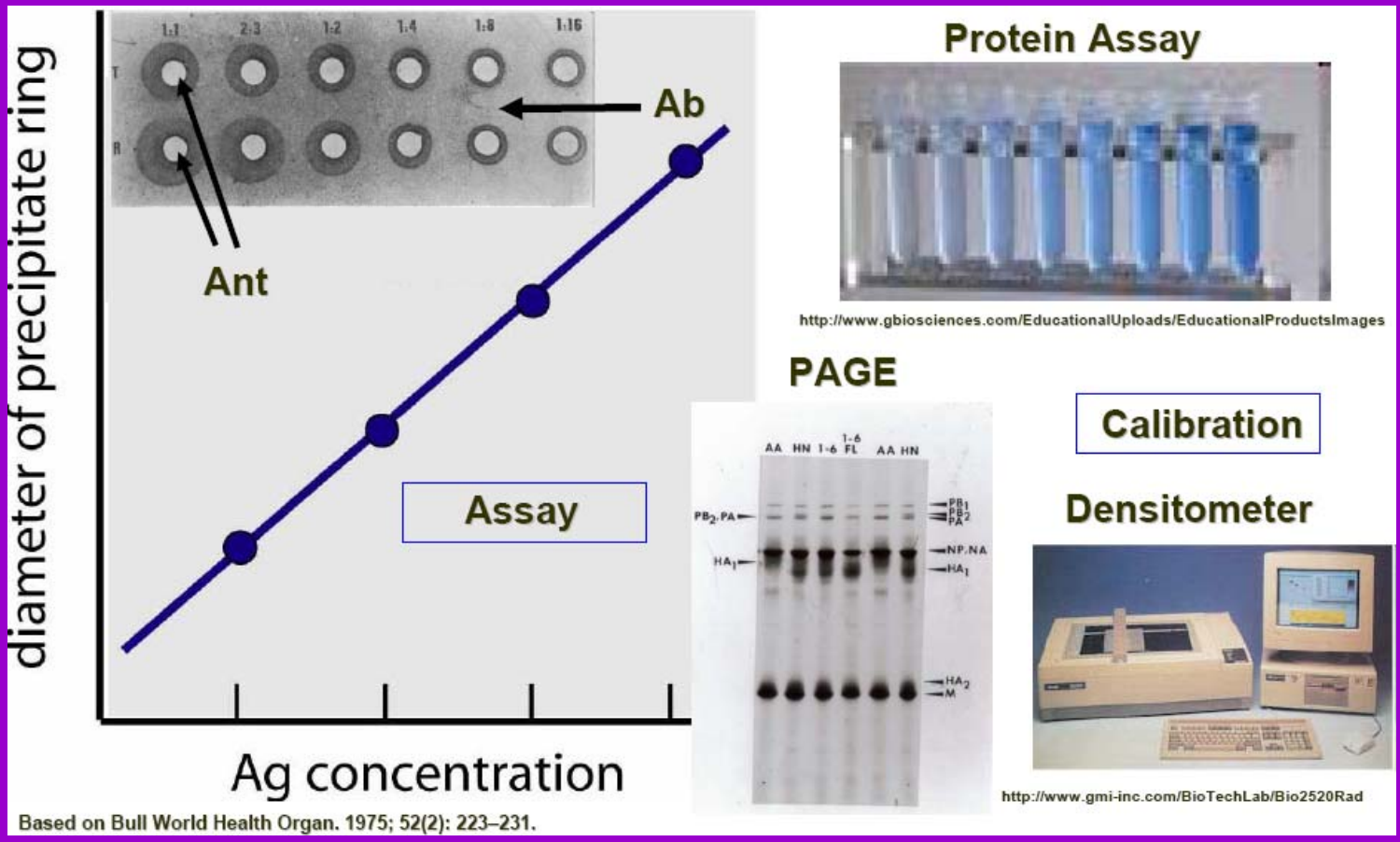
- Background on influenza
- Motivation for use of MS
- Comparison of standard Vs PCT tryptic digestion
- Quantitation of proteins in simple protein mixture
- Quantitation of HA in influenza virus preparation



Scheme of influenza A virus. Two glycoproteins, HA and NA, and the M2 protein are embedded in a lipid bilayer. The matrix protein, M1, forms a shell-like structure beneath the envelope. The ribonucleoprotein complex (RNP) consists of viral RNA associated with the nucleoprotein (NP) and three polymerase proteins (PB1, PB2, and PA). The nuclear export protein (NEP) is associated with RNP and the M1 protein is associated with both, the RNP and the viral envelope

Adapted from T. Betakova, ***Current Pharmaceutical Design,* 2007, *13,* 3231-3235**

Single Radial Immunodiffusion (SRID)



SRID

- Identity and quantitative assay for trivalent vaccine product
- Verifies the presence of all three HAs or their antigenically related variants

Pitfalls

- Requires the production and standardization of reference antigens and specific antibodies (time consuming and costly)
- Laborious and not high throughput
- Accuracy of the quantitative assay not satisfactory
- No information about other viral proteins or contaminants

Can MS help quantify HA in influenza virus preparations?

Objective:

Direct quantification of HA in SDS-PAGE bands

Comparison of standard in-gel tryptic digestion of proteins* vs high-pressure assisted tryptic digestion (PCT)

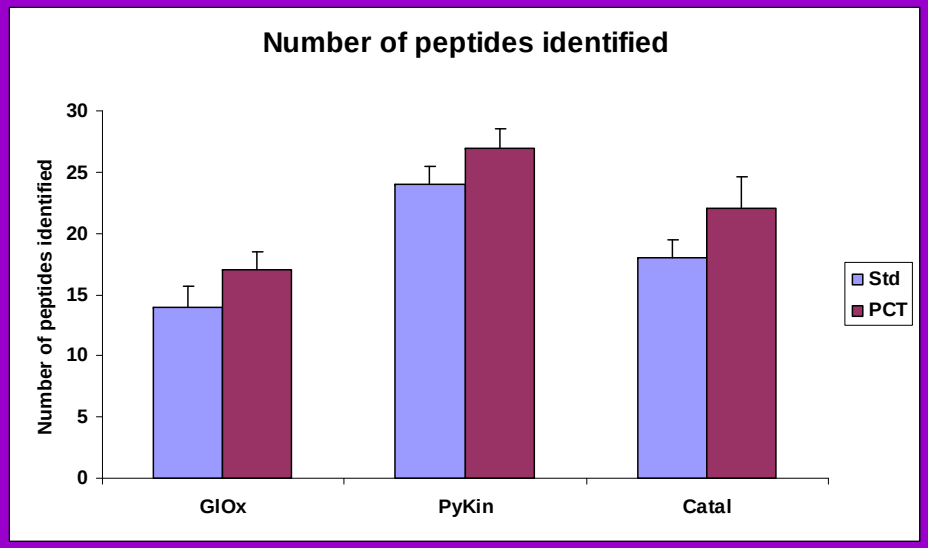
* Shevchenko *et al*. Nature Protocols (2006) 6: 2856-60

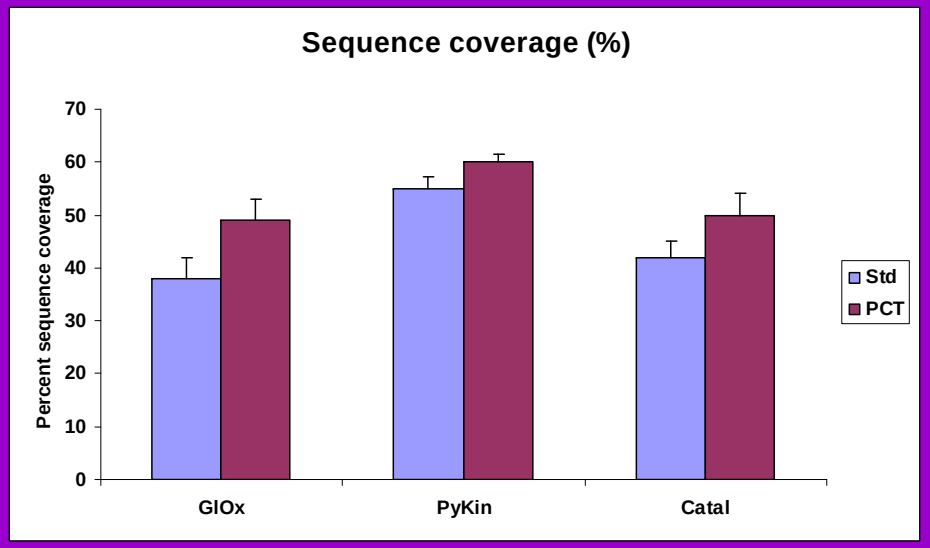
Destaining/reduction/alkylation as per Shevchenko *et al*

Standard Digestion
37 °C, 16 h

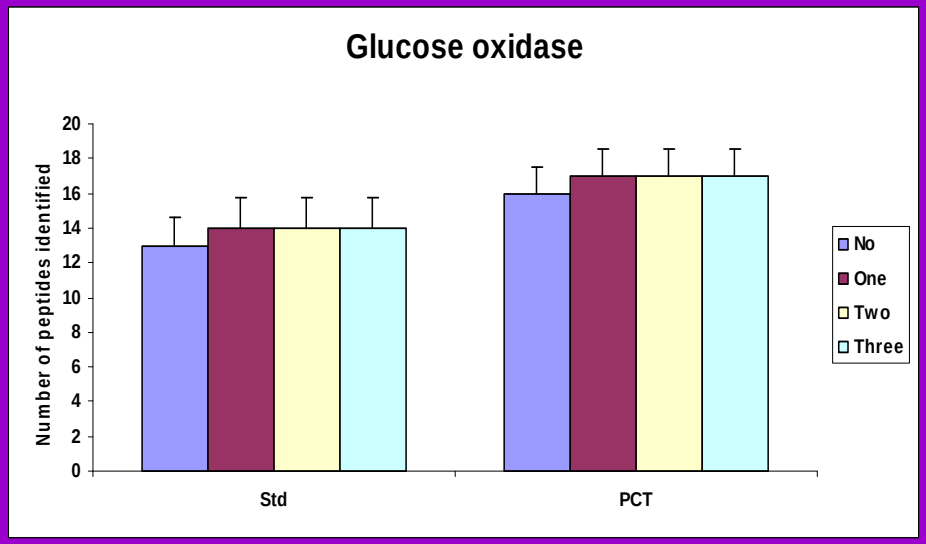
PCT Digestion
Barocycler, 37 °C, 45 cycles of high (35 kpsi for 55 s) and low (atm. pressure for 5 s)

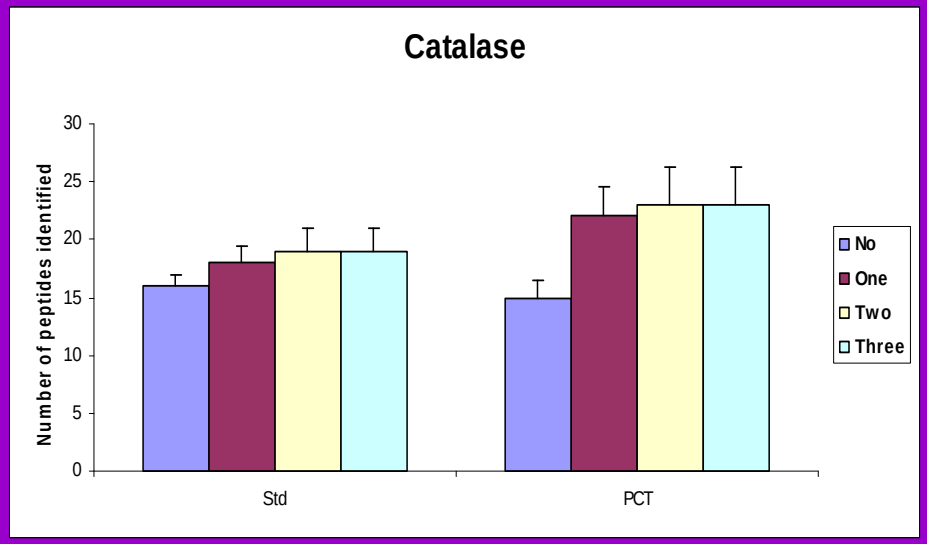
Comparison of Std vs PCT tryptic digestion (considering one missed cleavage, average of 3 experiments)

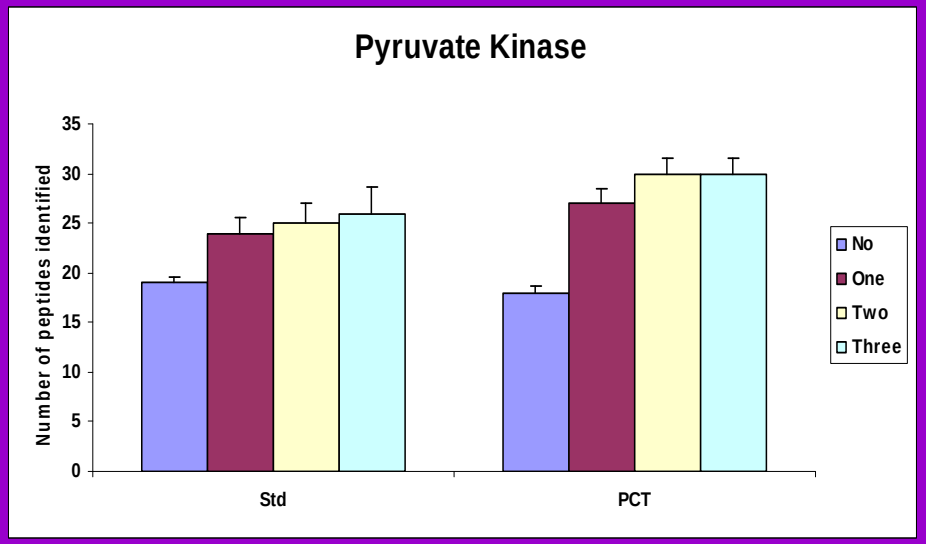




Comparison of Std vs PCT tryptic digestion with respect to number of missed cleavage peptides, n = 3







Label-free relative quantification of proteins separated by 1DE

Current Methods

Labeling approaches

Metabolic labeling

- Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC)

Chemical labeling

- Isotope Coded Affinity Tags (ICAT)
- Isobaric Tags for Relative and Absolute Quantification (ITRAQ)
- Stable isotope labeled peptides as internal standard (CDC paper)

Enzymatic labeling

- ^{18}O labeling

Label Free approaches

- LC-MS Profiling
- Spectral Count
- Use of analogous peptide as IS (modified AQUA)
- **Universal Signal Response Factor**

Supplemental Material can be found at:
http://www.mcponline.org/cgi/content/full/M500230-MCP200/DC1

Absolute Quantification of Proteins by LCMS^{E}

A VIRTUE OF PARALLEL MS ACQUISITION*[S]

Jeffrey C. Silva‡§, Marc V. Gorenstein‡, Guo-Zhong Li‡, Johannes P. C. Vissers¶, and Scott J. Geromanos‡

Molecular & Cellular Proteomics 5:144–156, 2006.

"This method is based on the discovery of an unexpected relationship between MS signal response and protein concentration: the average MS signal response for the three most intense tryptic peptides per mole of protein is constant within a coefficient of variation of less than 10%."

"Given an internal standard, this relationship is used to calculate a universal signal response factor. The universal signal response factor (counts/mol) was shown to be the same for all proteins tested in this study."

Average signal response across six proteins

TABLE III

Summary of the absolute quantification results obtained from the analysis of the six-protein mixture described in Table I

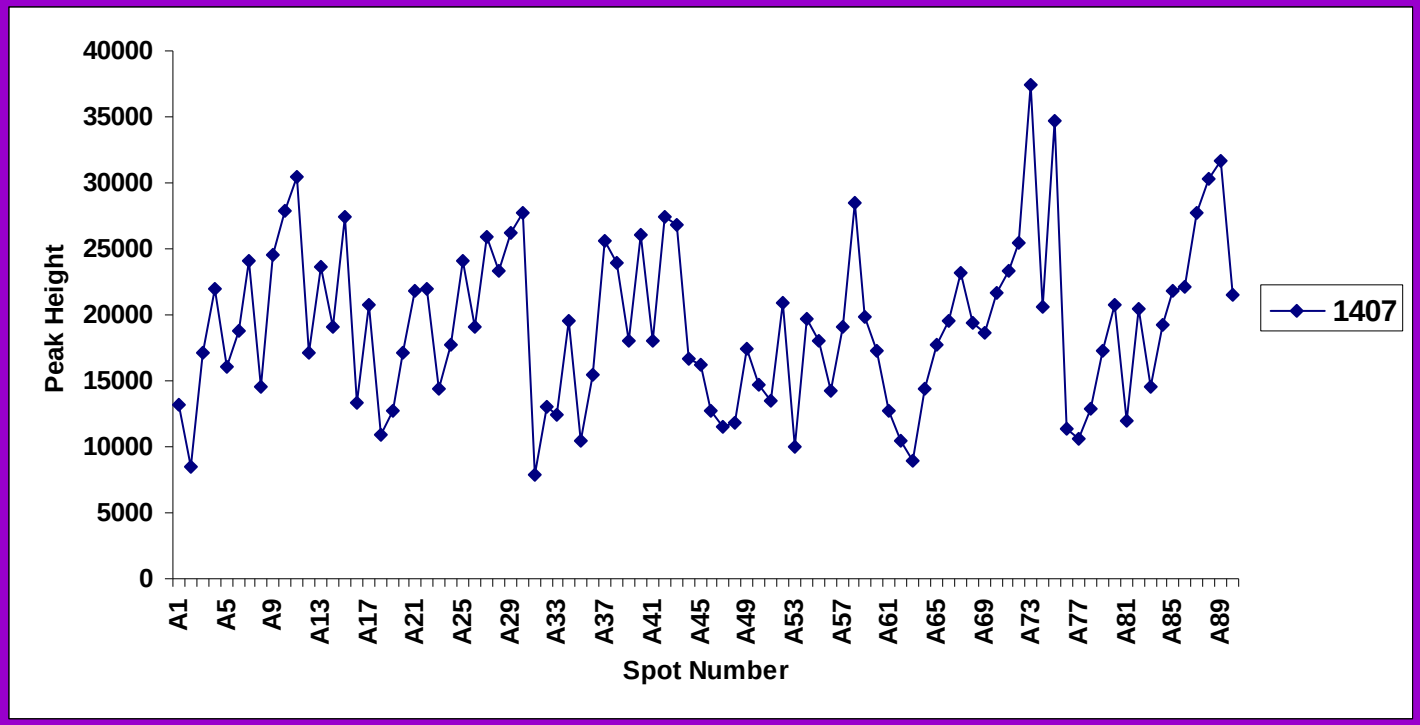
Alcohol dehydrogenase (bold) was used as the internal reference standard in both studies as discussed in the text.

Relative ratio	Average SR of top three peptides	Protein	Theoretical	Calculated	Error	SR/pmol
			pmol	*pmol*	%	
1.47	395,716	Enolase	15.0	14.7	−2.2	26,381
1.25	337,505	Serum albumin	12.5	12.5	0.1	27,000
1.00	**269,861**	**Alcohol dehydrogenase**	**10.0**	**10.0**	**0.0**	**26,986**
0.60	161,116	Phosphorylase B	6.0	6.0	−0.5	26,853
0.48	129,280	Hemoglobin (β)	5.0	4.8	−4.2	25,856
0.44	118,244	Hemoglobin (α)	5.0	4.4	−12.4	23,649
	269,861	Normalization		Average SR/pmol		26,121
				Cv		4.9

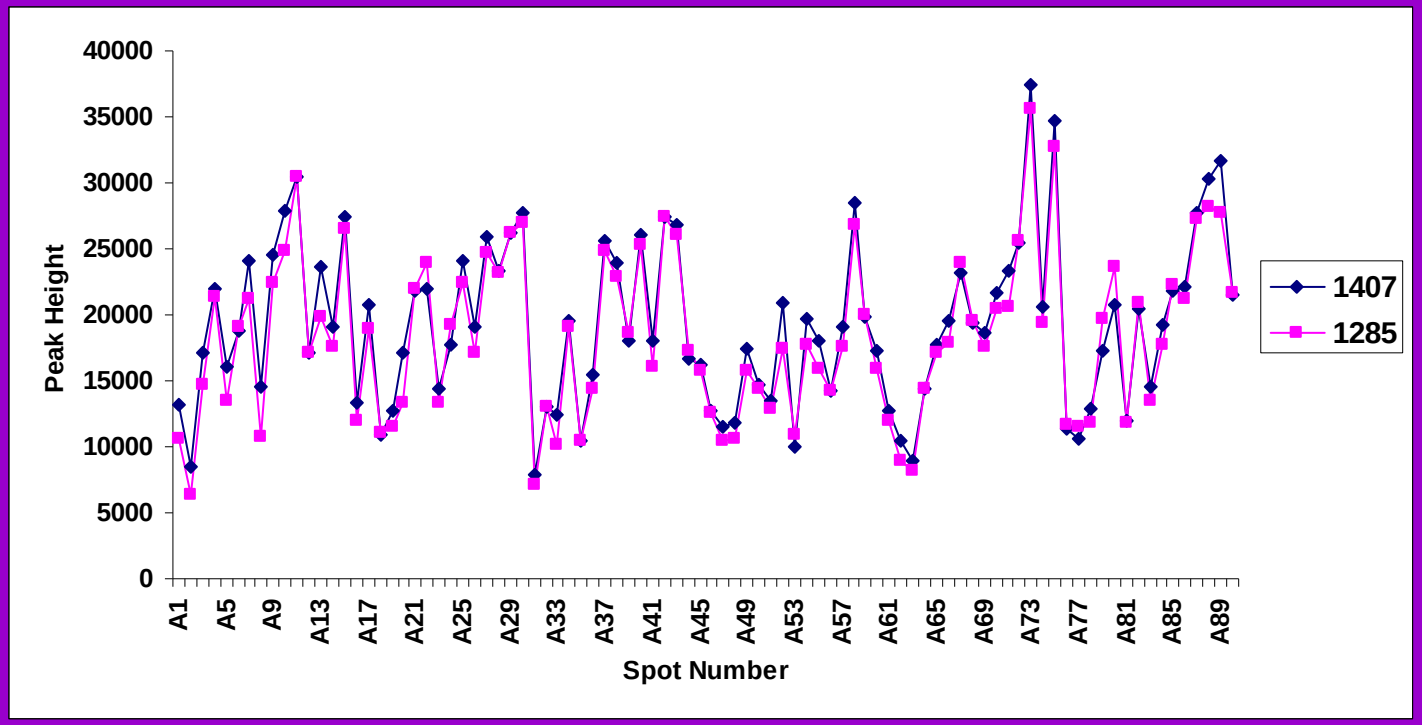
- An average signal response of 26,121 counts was consistently associated to 1 pmol of protein on column with a CV of 4.9%
- This relationship appears to be independent of the protein molecular mass

Molecular & Cellular Proteomics 5:144–156, 2006.

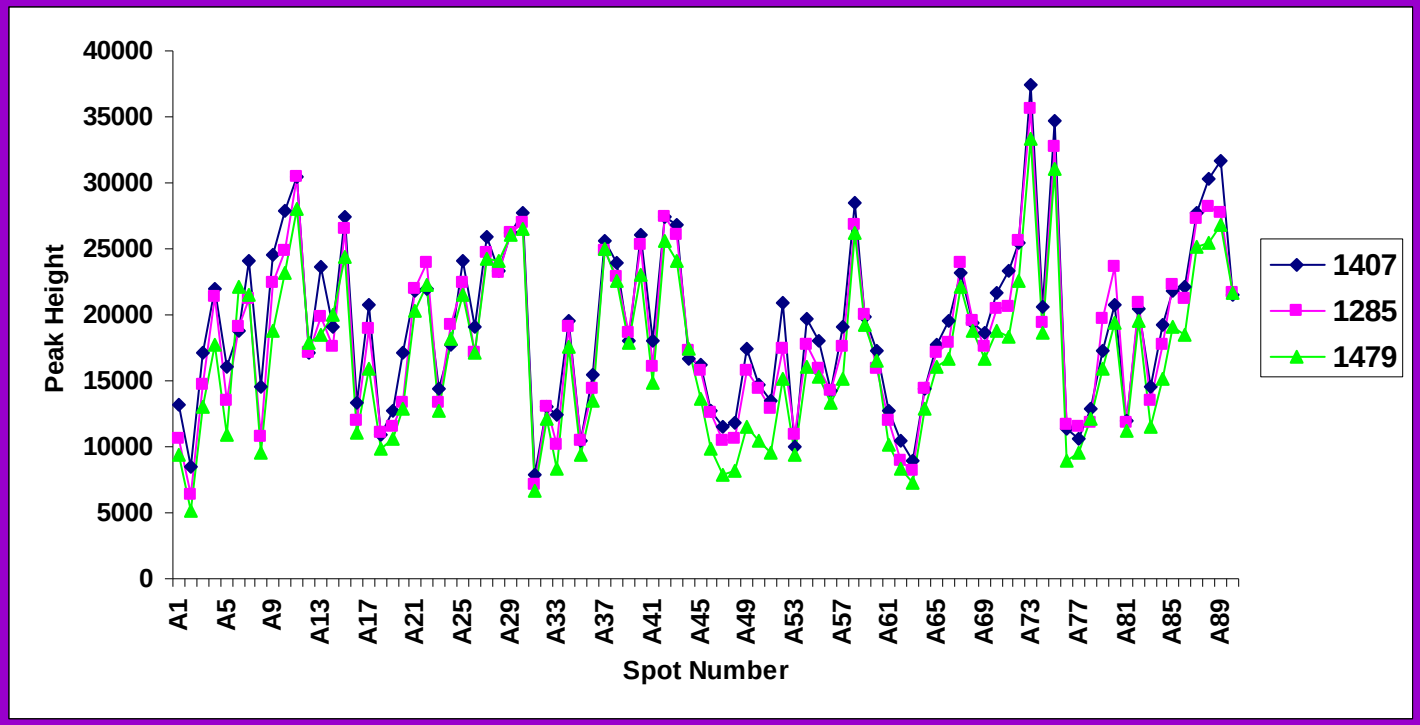
MALDI is not inherently quantitative



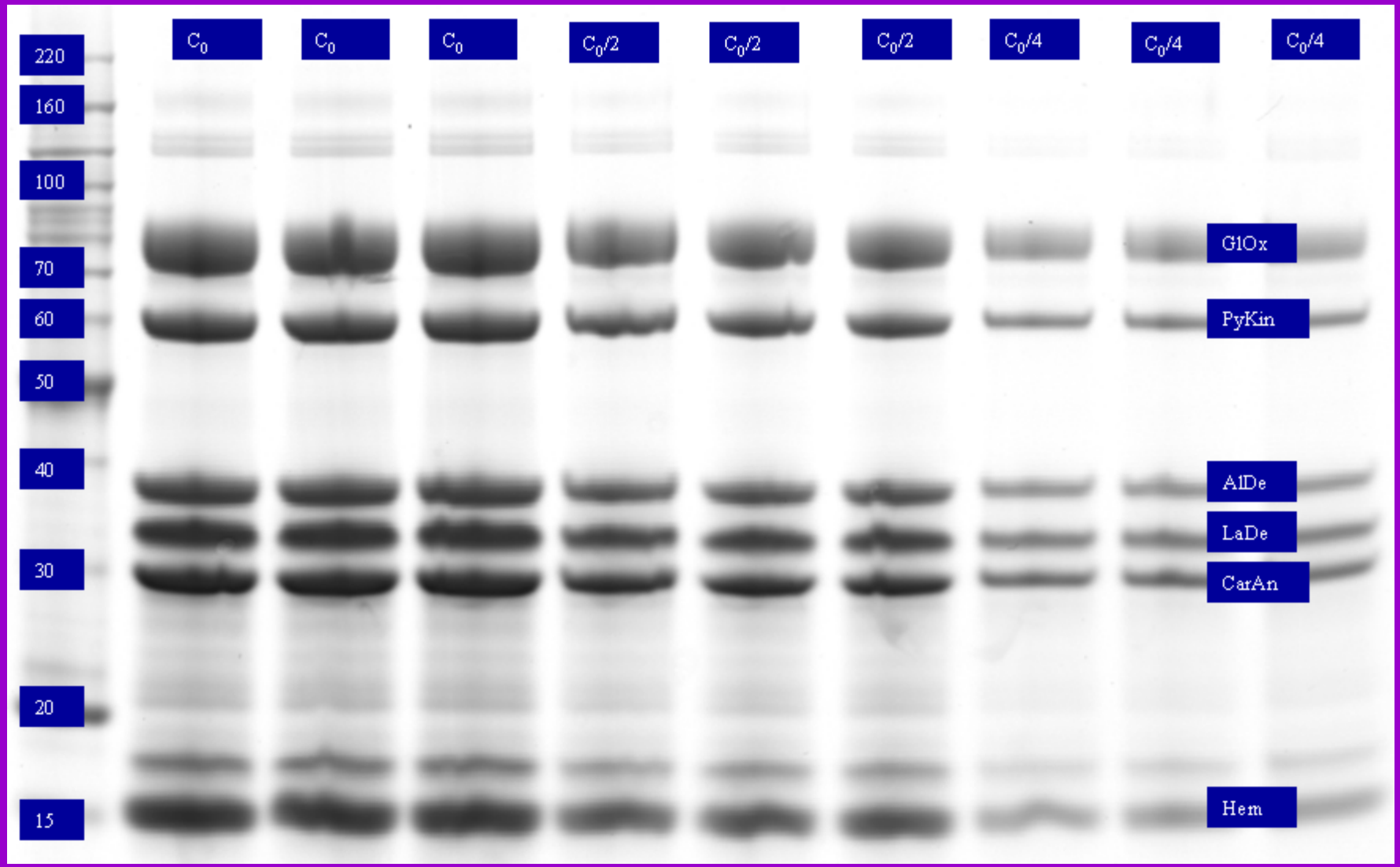
MALDI is not inherently quantitative



MALDI is not inherently quantitative



Relative quantification of proteins from simple protein mixture separated on SDS PAGE

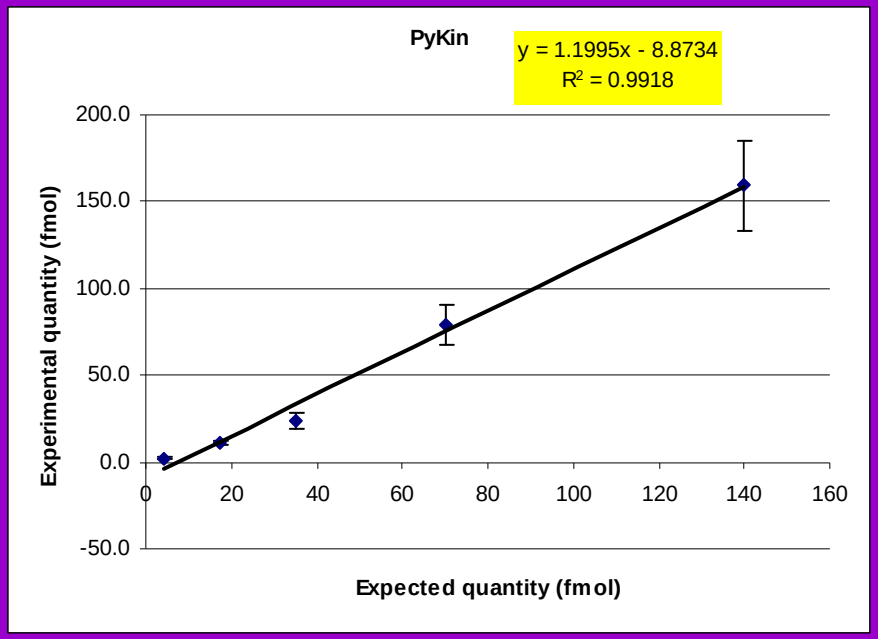


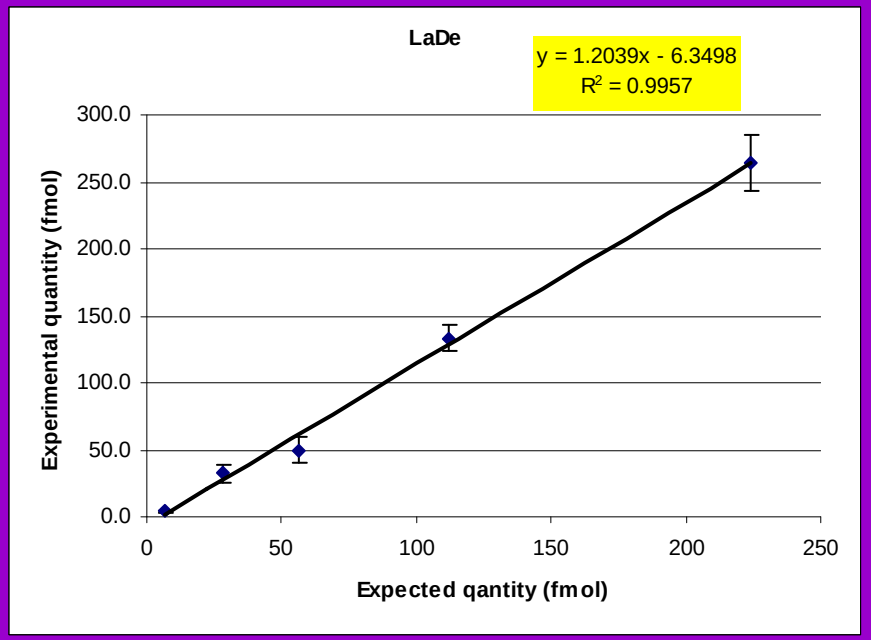
Summary of spot-to-spot and lane-to-lane repeatability for the highest concentration (C_0)

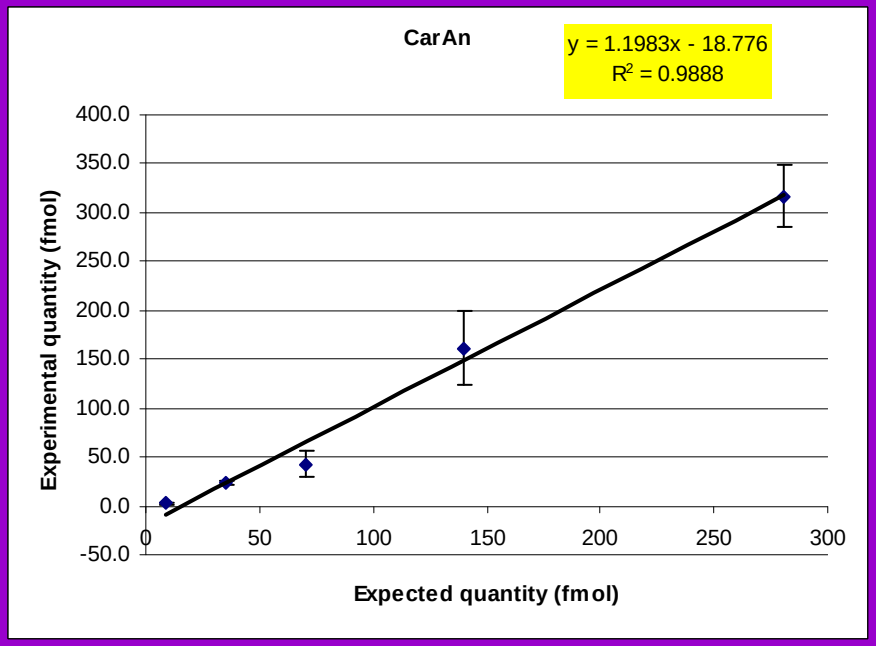
Protein	Lane-1		Lane-2		Lane-3		Lane-1 to Lane-3	
	Aver. Quant. (fmol)	% CV	Aver. Quant. (fmol)	% CV	Aver. Quant. (fmol)	% CV	Aver. Quant. (fmol)	% CV
AlDe	405.0	4.5	312.2	5.4	331.1	6.2	349.4	14.0
CarAn	280.2	1.0	339.4	8.4	330.3	2.2	316.6	10.1
GIOx	322.5	5.1	299.4	4.0	333.4	3.0	318.4	5.4
Hem	332.9	7.4	336.5	6.3	309.3	1.2	326.3	4.5
LaDe	241.4	4.0	266.5	9.0	283.2	5.0	263.7	8.0
PyKin	154.6	1.8	135.6	9.3	187.5	6.4	159.3	16.5

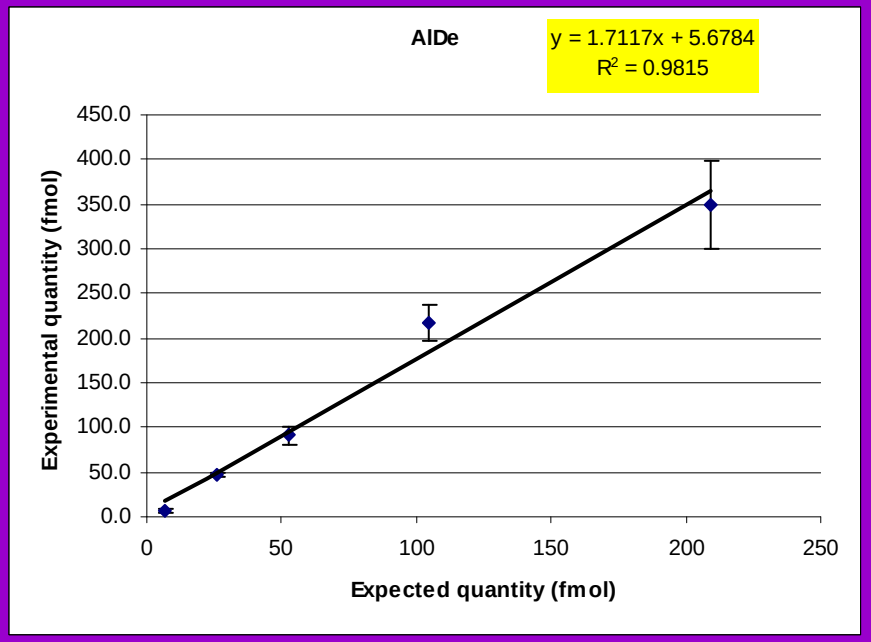
Summary of spot-to-spot and lane-to-lane repeatability for the lowest concentration ($C_0/32$)

Protein	Lane-1		Lane-2		Lane-3		Lane-1 to Lane-3	
	Aver. Quant. (fmol)	% CV	Aver. Quant. (fmol)	% CV	Aver. Quant. (fmol)	% CV	Aver. Quant. (fmol)	% CV
AlDe	8.3	5.7	6.2	4.6	5.2	0.9	6.6	24.0
CarAn	2.9	4.6	2.4	3.3	2.2	1.3	2.5	14.7
GlOx	5.0	7.2	4.2	4.2	2.9	5.3	4.0	25.4
Hem	1.4	1.4	1.5	1.7	1.4	2.7	1.4	0.4
LaDe	3.6	0.9	3.8	2.9	4.5	4.7	4.0	12.2
PyKin	2.7	2.5	1.7	6.7	2.3	6.2	2.2	23.3


PyKin
y = 1.1995x - 8.8734
R² = 0.9918
Experimental quantity (fmol)
Expected quantity (fmol)
200.0
150.0
100.0
50.0
0.0
-50.0
0
20
40
60
80
100
120
140
160


LaDe
y = 1.2039x - 6.3498
R² = 0.9957
Experimental quantity (fmol)
Expected qantity (fmol)
300.0
250.0
200.0
150.0
100.0
50.0
0.0
0
50
100
150
200
250

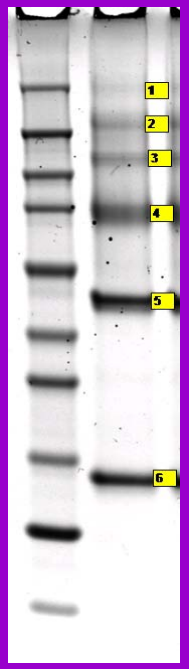

CarAn
y = 1.1983x - 18.776
R² = 0.9888
Experimental quantity (fmol)
Expected quantity (fmol)
400.0
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
-50.0
0
50
100
150
200
250
300


AlDe
y = 1.7117x + 5.6784
R² = 0.9815
Experimental quantity (fmol)
Expected quantity (fmol)
450.0
400.0
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
0
50
100
150
200
250

Label free quantitation of HA from primary liquid standard (pLS) preparation of A/Uruguay/716/2007 (H3N2)

Overview of protocol steps

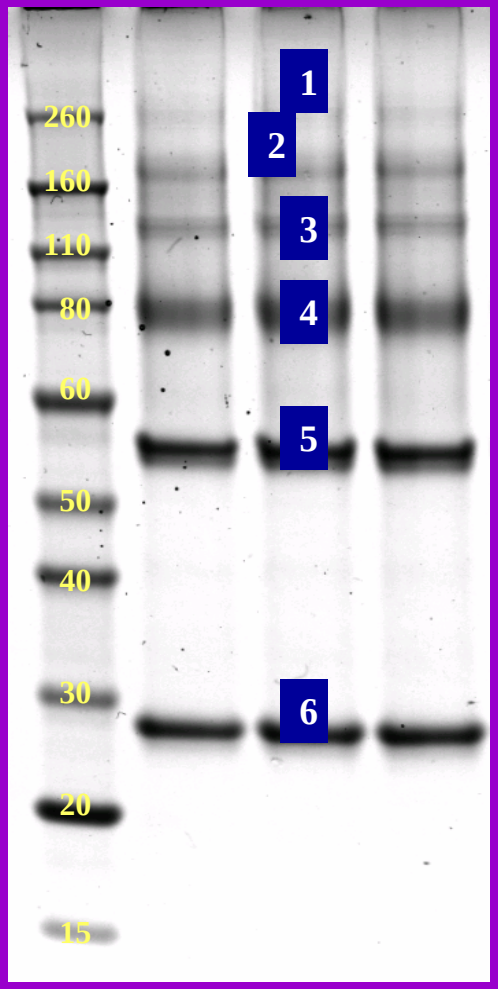
- ✓ Run pLS on SDS-PAGE

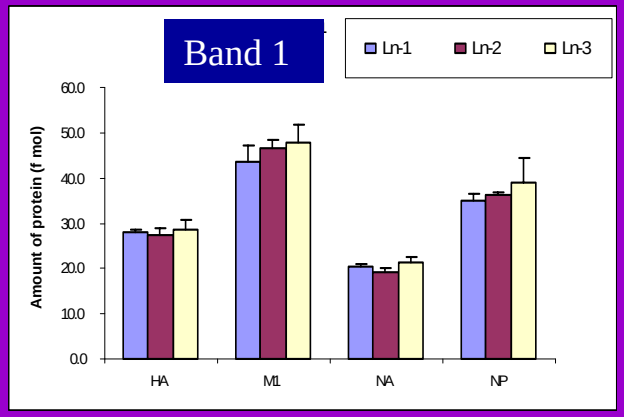


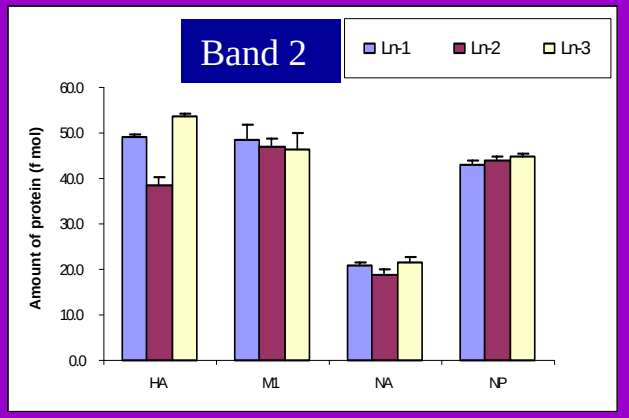
- ✓ Excise bands
- ✓ In-gel digest (PCT)
- ✓ Spike known molar amount of synthetic catalase peptides
- ✓ Identify peptides by MS/MS
- ✓ Determine signal response factor of catalase peptides per mole

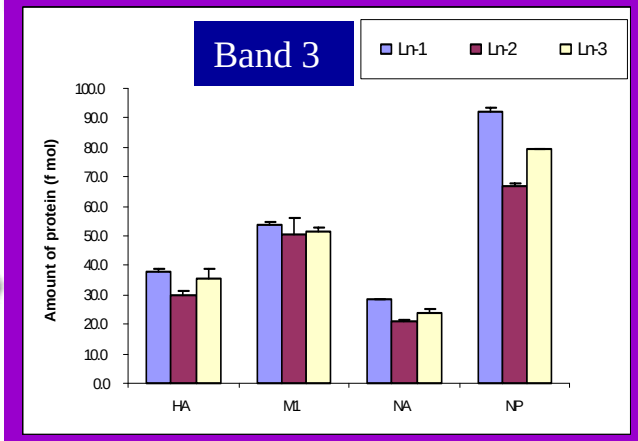
- ✓ Calculate absolute amount of each protein in each band
- ✓ Sum of HA in each band = total HA in volume loaded

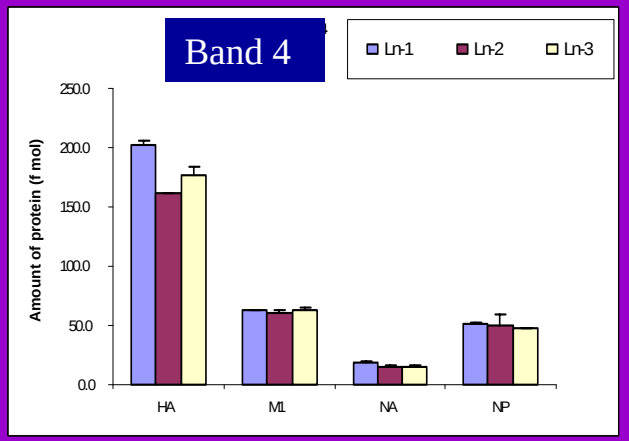
SDS PAGE of a primary liquid standard preparation of the influenza virus A/Uruguay/716/2007



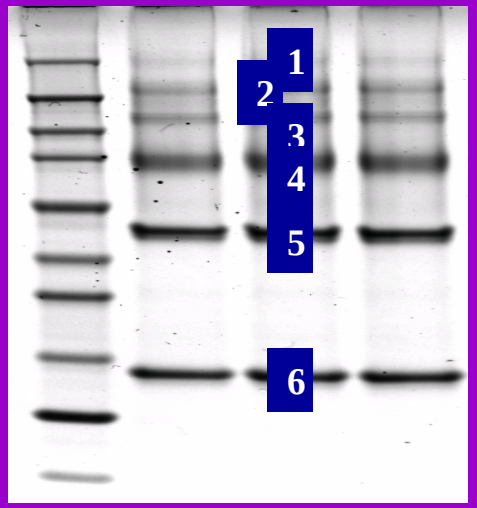


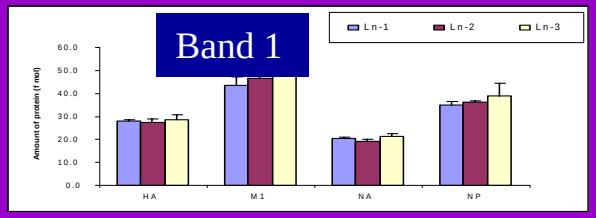


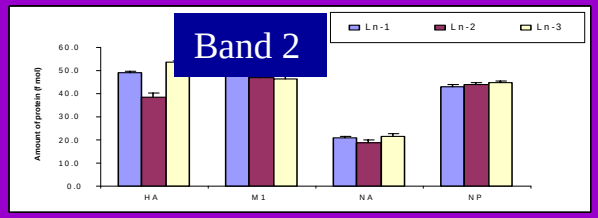


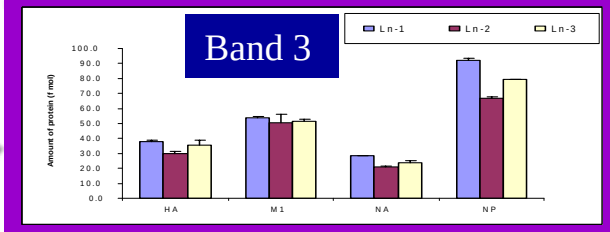


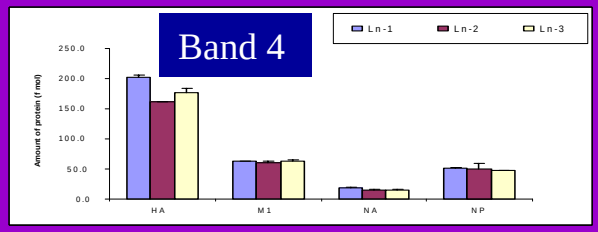
SDS PAGE of a primary liquid standard preparation of the influenza virus A/Uruguay/716/2007 (H3N2)











Quantification of co-migrating proteins (Band 3)

Protein	Lane-1		Lane-2		Lane-3		Lane-1 to Lane-3	
	Av. Quant. (f mol)	% CV	Av. Quant. (f mol)	% CV	Av. Quant. (f mol)	% CV	Av. Quant. (f mol)	% CV
HA	38	3	30	5	36	10	34	12
M1-	54	1	50	11	51	3	52	4
NA	28	1	21	1	24	6	24	15
NP	92	2	67	1	79	0	79	16

Quantification of HA from A/Uruguay/716/2007 (H3N2)

		Calculated after in-gel/MS quantification	
	Sample loaded	**Percent of total**	**Total µg**
	3 ul of stock solution		
Total protein	~7.5 µg	100	5.5 µg
HA	3 µg (40%)*	33	1.8 µg

*** Determined by densitometry**

Conclusions

- The pressure assisted tryptic digestion technique accelerates in-gel trypsin digestion, without loss of downstream protein identification and quantification information
- For relative quantification, label-free mass spectrometry with added internal standard shows adequate level of precision
- The method is particularly useful for targeted protein quantification after 1-D gel separation
- The MS-based quantification can be a viable option when specific antibodies are unavailable

Acknowledgement

Michail Alterman, DCGT
Maryna Eichelberger, DVP
Rajesh Gupta, DPQ
Manju Joshi, DPQ
Alexander Lazarev, PBI

Funding
BARDA

Thank You!