DEVELOPMENT OF ESSENTIAL SAMPLE PREPARATION TECHNIQUES IN PROTEOMICS USING ULTRA-HIGH PRESSURE

Alexander R. Ivanov

HSPH Proteomics Resource
Department of Genetics and Complex Diseases

Harvard School of Public Health
www.hsph.harvard.edu/proteomics

Sample Preparation:

- Proteolytic Digestion
  - In-solution
  - In-gel
  - On-membrane

- Cell Lysis
  - Pressure
  - Organic solvents

Workflow for Optimization of Digestion Protocol

Protein Mix

Conventional (In Incubator)

Pressure-Assisted, PCT (In Barocycler)

Tested variables in optimization of digestion

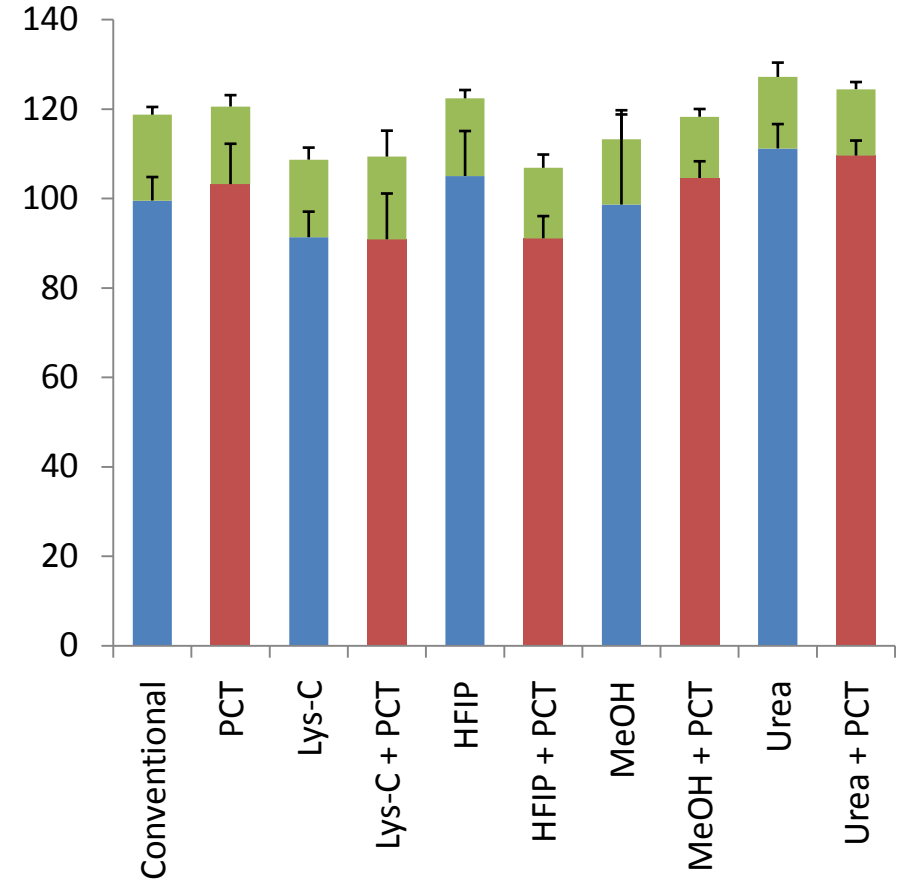
Dissolution additives: (HFIP, urea, methanol)	Time of digestion	Proteolytic enzymes: trypsin, Lys-C	Reduction reagent/ concentration: DTT vs. TCEP	Reduction environment: temperature and pressure

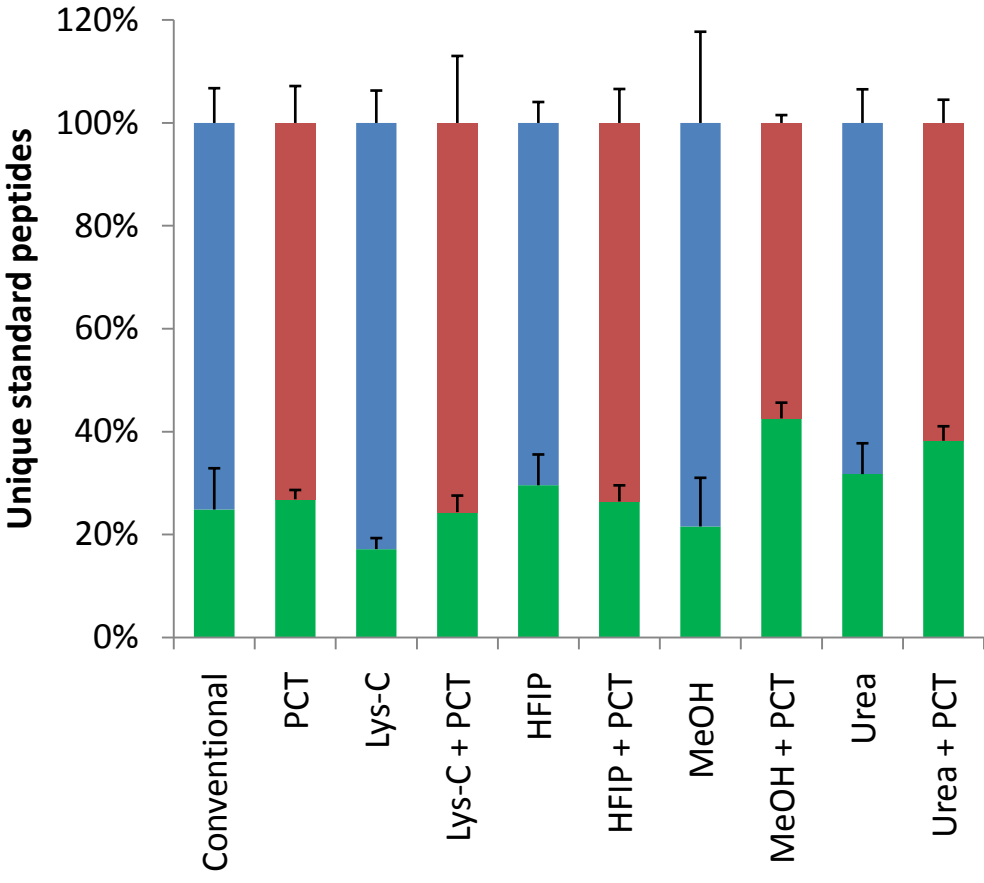
LC-MS/MS analysis and database searching (Scaffold, Protein Prophet, Peptide Prophet)

Protocol Variables

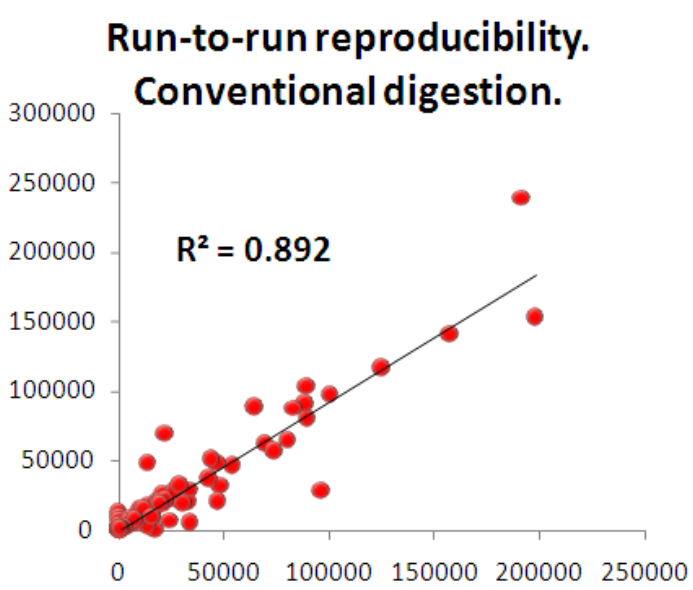
Protocol		Dissolution Additives:			Digestion conditions							
					In Incubator (37˚C)			In PCT (45˚C)				
Shorthand protocol name	Brief description	HFIP	MeOH	Urea	1-trypsin aliquot, 12-hrs,	2-trypsin additions, 40-hrs	1-Lys-C, 1-trypsin, 40-hrs	1-trypsin aliquot, 1-hr	2-trypsin, 2-hr	1-Lys-C, 1-trypsin, 2-hr	2-trypsin, 4-hr	2-trypsin, 8-hr
I-A_	Standard protocol					x						
I-C_	Standard in PCT								x			
I-D	Standard (small volume)					x						
II-A	Lys-C						x					
II-C	Lys-C in PCT									x		
III-A	HFIP (Hexafluoroisopropanol)	x				x						
III-C	HFIP +PCT	x							x			
IV-A	MeOH		x			x						
IV-C	MeOH +PCT		x						x			
V-A	Urea			x		x						
V-C	Urea +PCT			x					x			
VI-A	DTT for Reduction					x						
VI-C	DTT for Reduction in PCT								x			
VII-A	Urea/HFIP	x		x		x						
VII-C	Urea/HFIP/PCT	x		x					x			
I-A-1	Standard, only (1) 12-hr tryp				x							
I-A-2	Standard (I-A)					x						
I-C-1	Only 1 PCT digest							x				
I-C-2	Standard in PCT (I-C)								x			
I-C-4	4 PCT digests										x	
I-C-8	8 PCT digests											x
I-A*	Standard, only (1) 12-hr tryp				x							
I-AC*	I-A, digestion in PCT							x				
I-C*	I-C, 1 tryp digest							x				
VI-A50	VI-A with 50mM DTT					x						
VI-C50	VI-C with 50mM DTT								x			

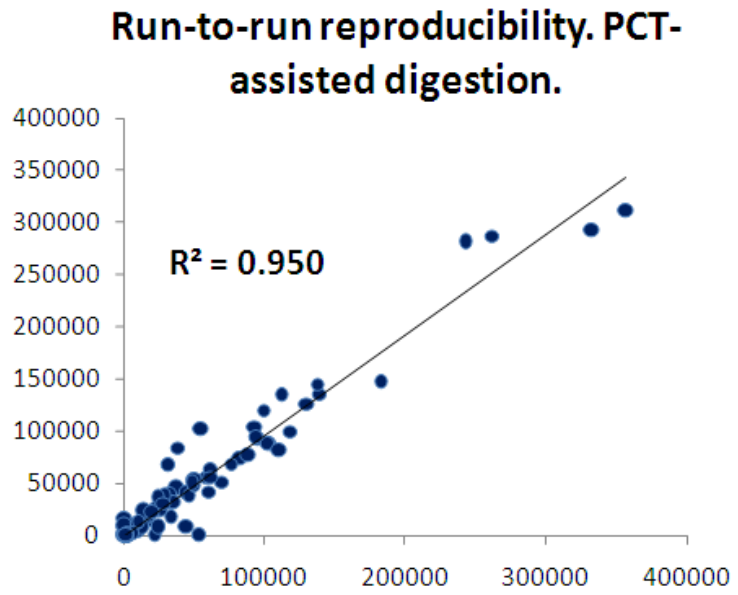
In-Solution Tryptic Digestion, 100 fmol/analysis

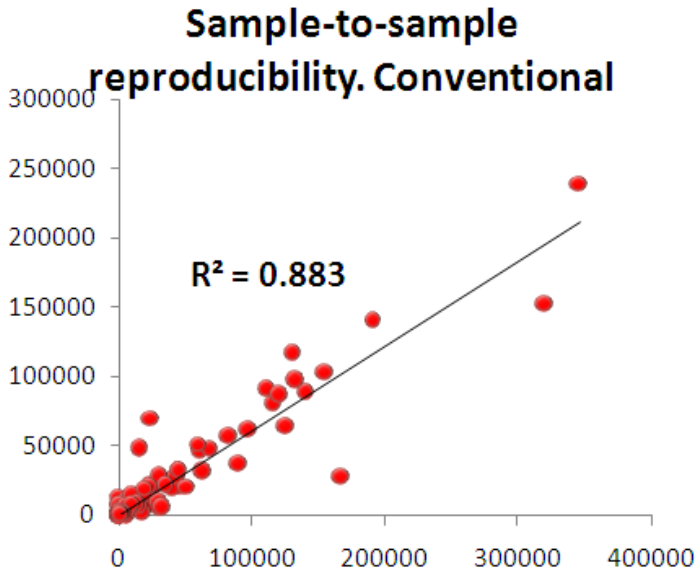


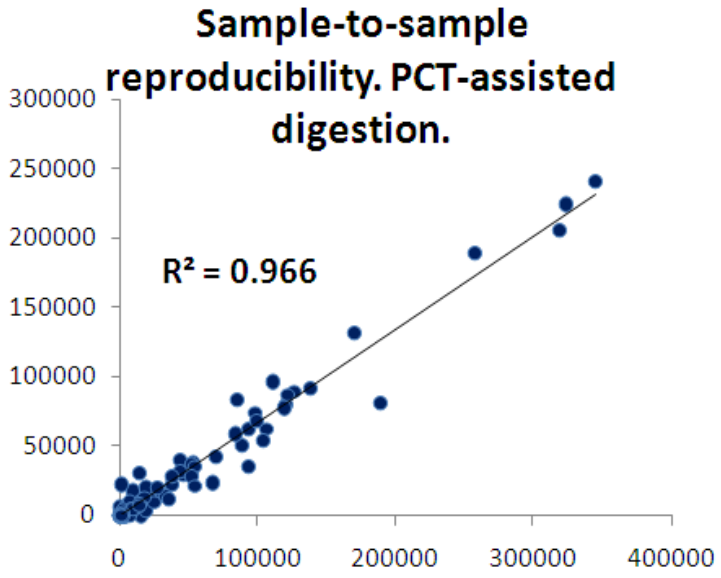


In-Solution Tryptic Digestion: Reproducibility of Peptide Abundances

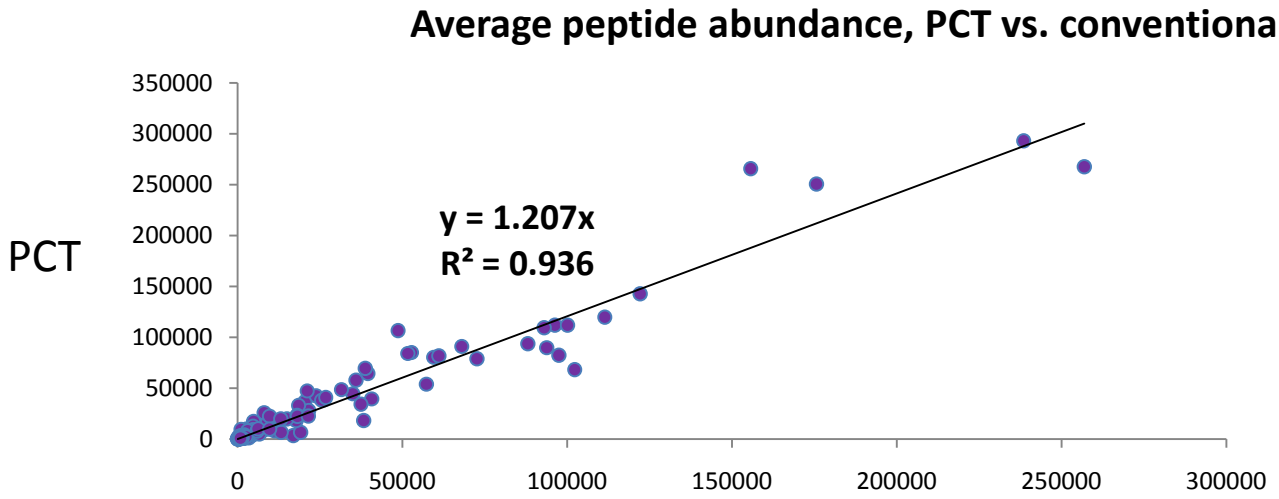






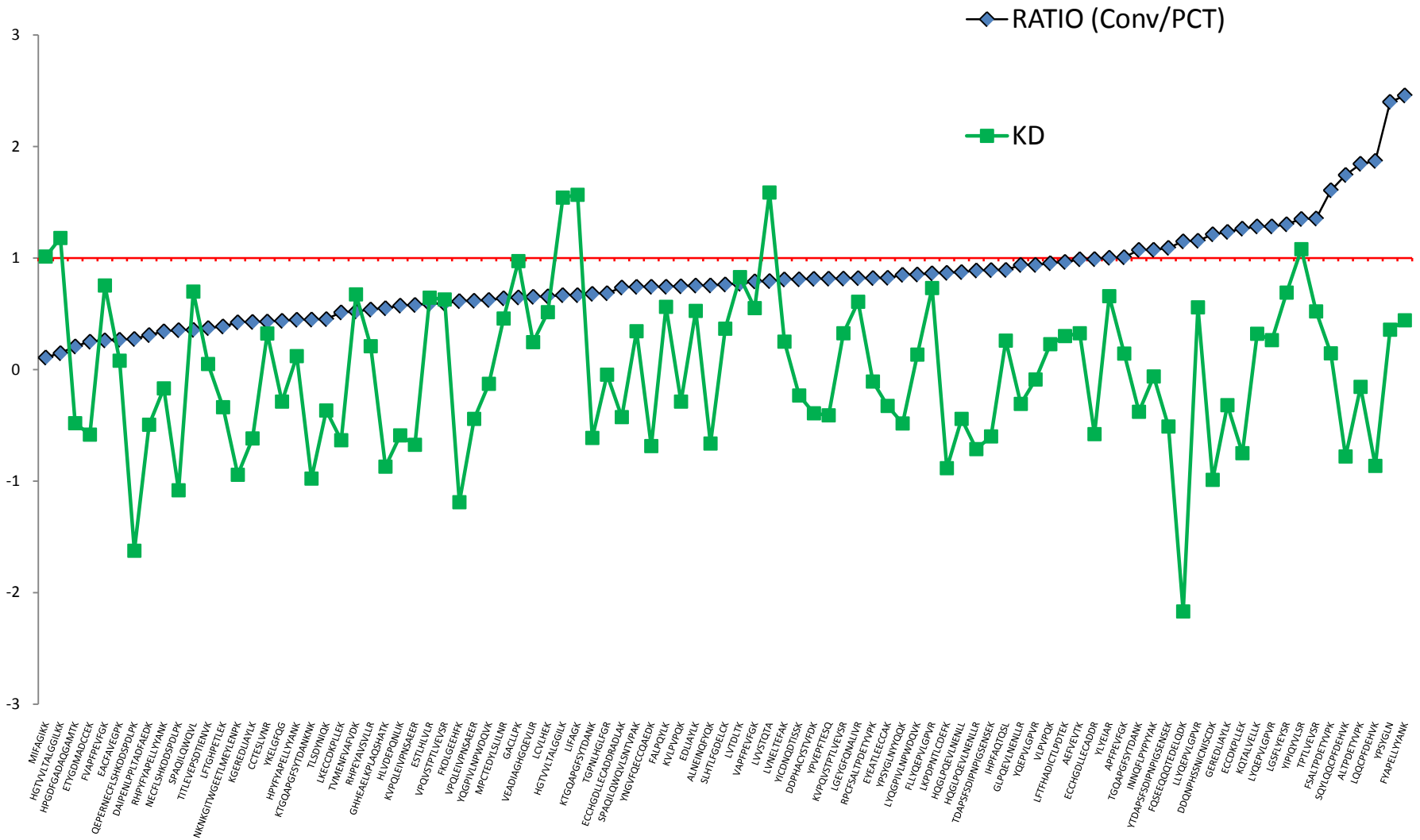


In-Solution Tryptic Digestion: Reproducibility of Peptide Abundances

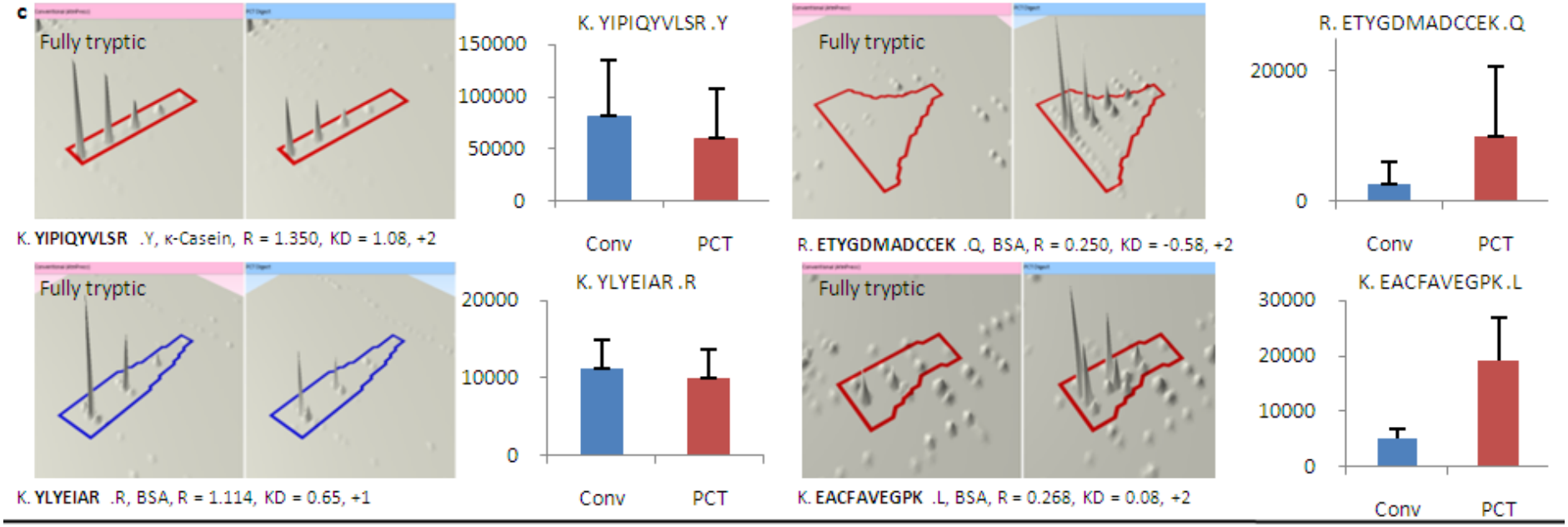


Run-to-run reproducibility ($R^{2)}$	LC-MS Runs 1 and 2, R^2	LC-MS Runs 2 and 3, R^2	LC-MS Runs 1 and 3, R^2	Mean R^2	CV
Conventional Digestion	0.945	0.815	0.892	0.884	7.4%
PCT Digestion	0.908	0.938	0.950	0.932	2.3%
Sample-to-sample reproducibility,(R^2)	Samples 1 and 2, R^2	Samples 2 and 3, R^2	Samples 1 and 3, R^2	Mean R^2	CV
Conventional Digestion	0.862	0.918	0.883	0.888	3.2%
PCT Digestion	0.966	0.944	0.939	0.950	1.5%

In-Solution Tryptic Digestion: Differential Peptide Recovery

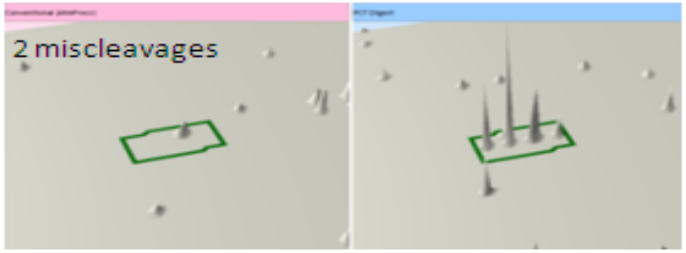


In-Solution Tryptic Digestion: Differential Peptide Recovery

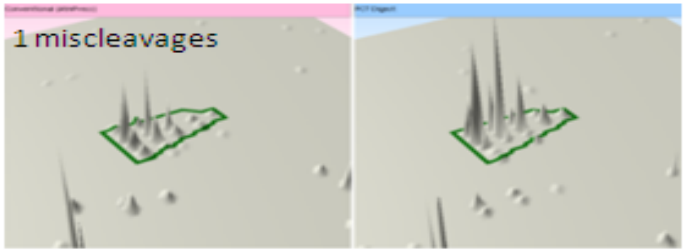


In-Solution Tryptic Digestion: Differential Peptide Recovery

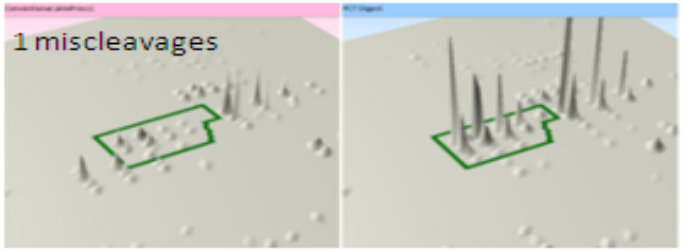
g

I. 

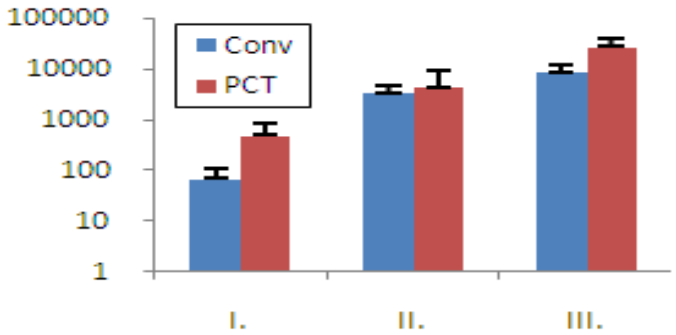
K. QEPERNECFLSHKDDSPDLPK .L, BSA, R = 0.139, KD = -1.62, +3

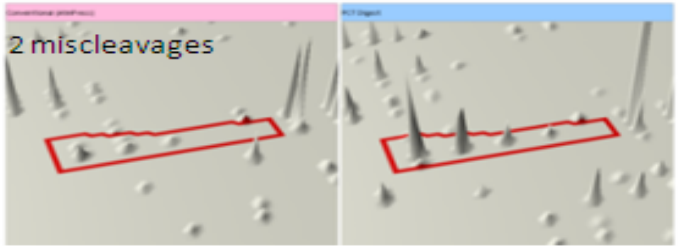
II. 

K. QEPERNECFLSHK .D, BSA, R = 0.747, KD = -1.72, +3

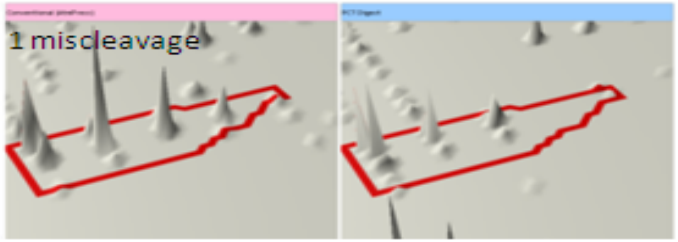
III. 

R. NECFLSHKDDSPDLPK .L, BSA, R = 0.307, KD = -1.08, +3



IV. 

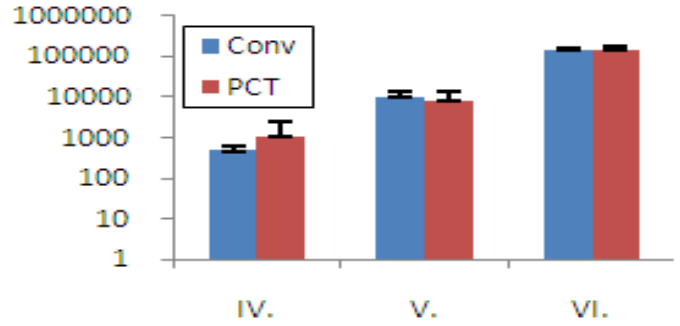
K. KGEREDLIAYLK .K, Cyt C, R = 0.427, KD = -0.61, +2

V. 

K. GEREDLIAYLK .K, Cyt C, R = 1.234, KD = -0.32, +2

VI.

R. EDLIAYLK .K, Cyt C, R = 1.04, KD = 0.52, +2

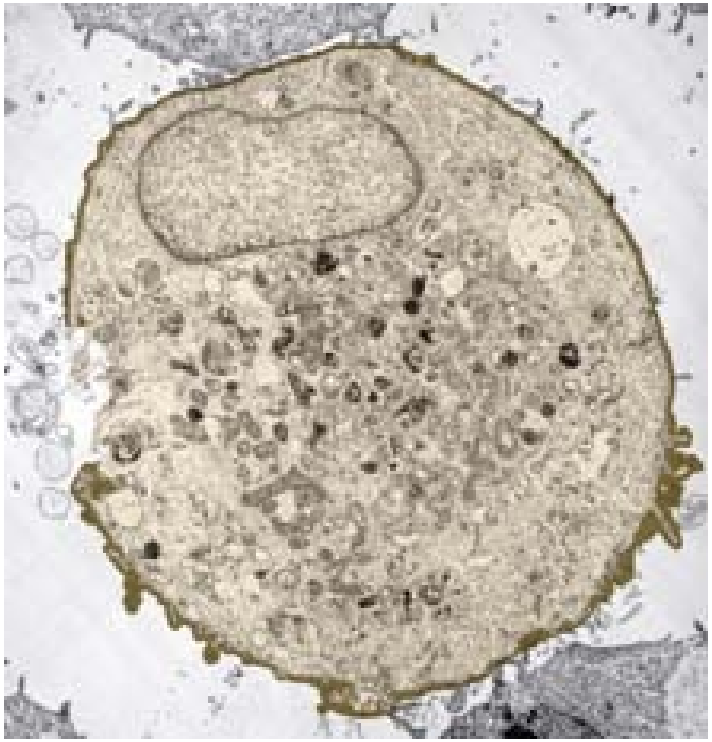


- Higher throughput (20x fold)
- Higher efficiency of proteolysis
- Lower preanalytical variability
- Better overall peptide recovery
- Absence of PCT-induced *in vitro* peptide oxidation
- Digestion specificity is not hampered by ultra-high pressure

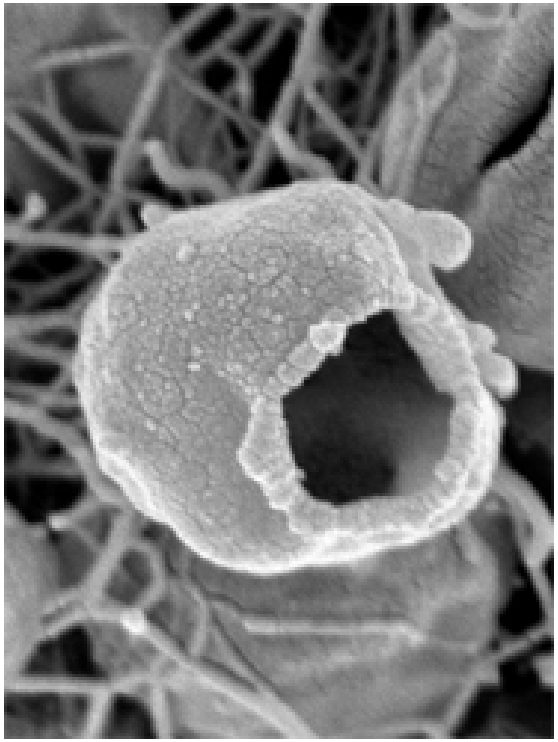
PCT-Assisted Cell Rupture

Conventional Cell/Tissue Rupture Approaches

Mechanical stress



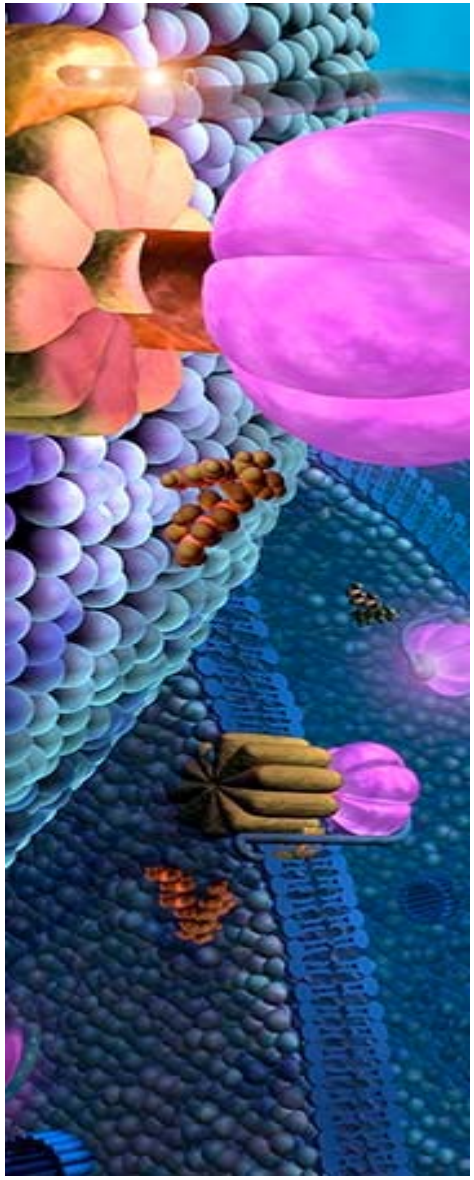
Ultrasound



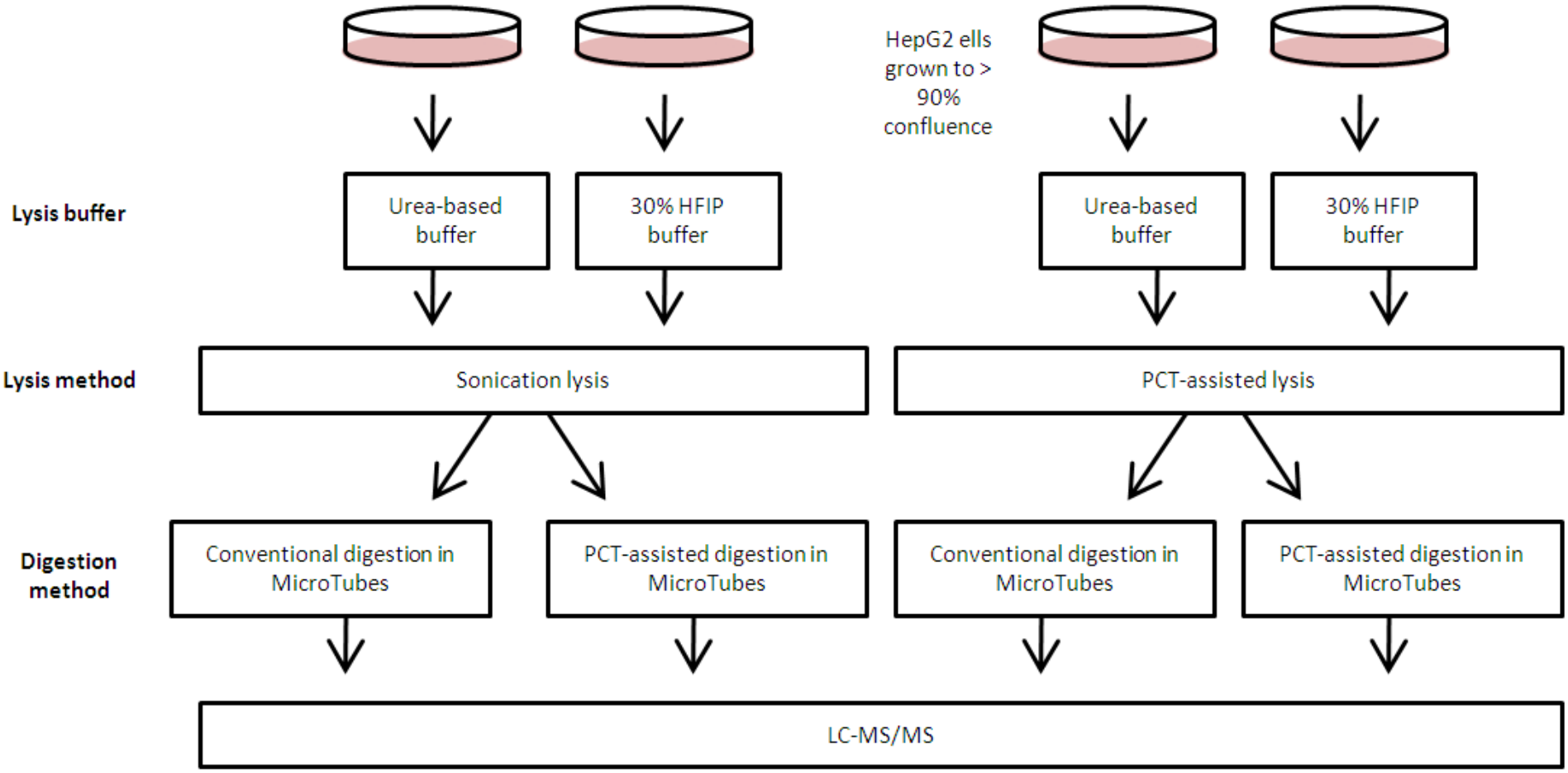
Osmosis

Credit: R. Schlicher, R. Apkarian, and M. Baran, www.cchem.berkeley.edu, www.sciencephotolibrary.com

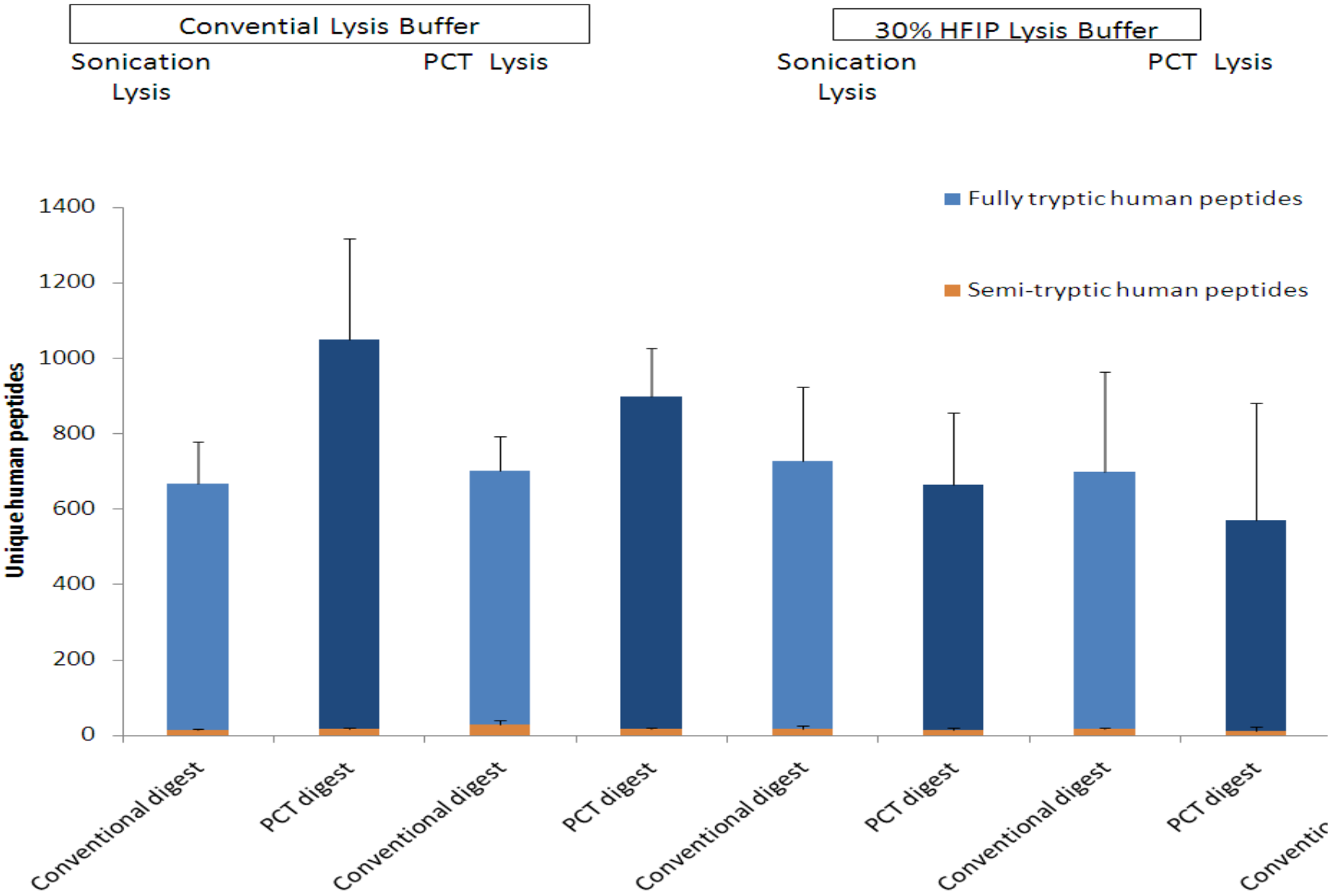
Pressure Cycling – Assisted Lysis and Protein Extraction



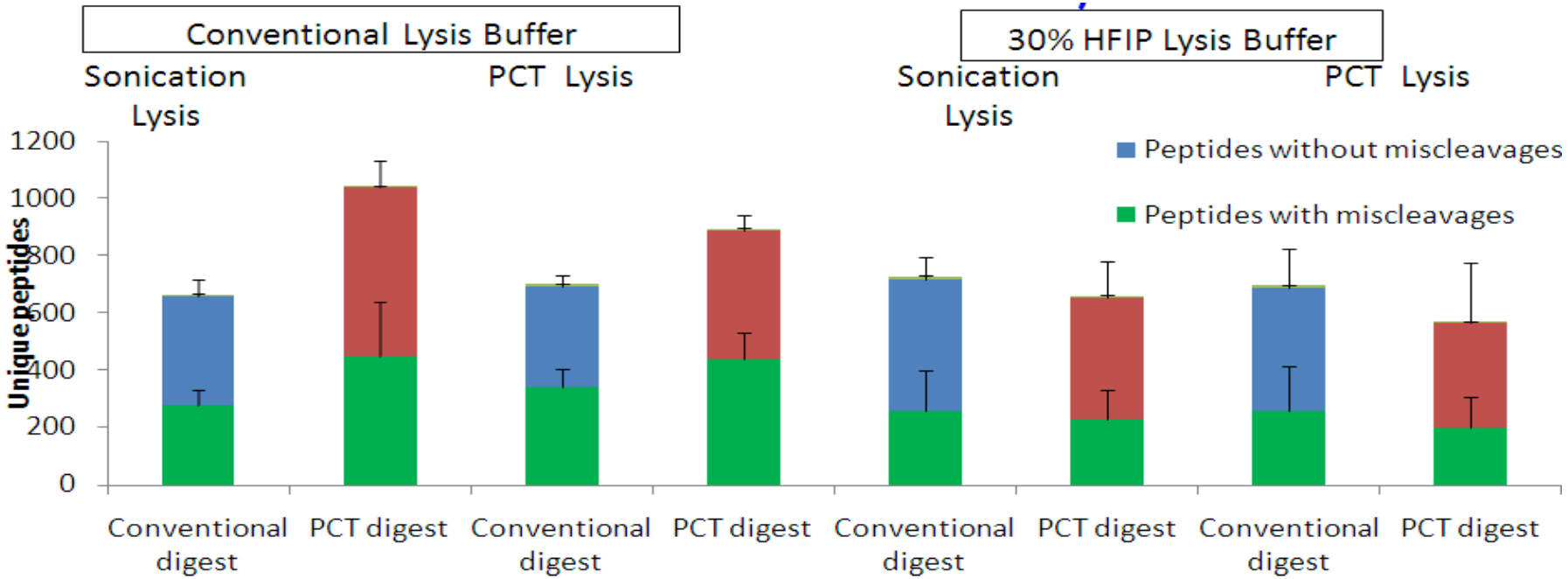
Pressure Cycling-Assisted and Organic Solvent – Assisted Cell Lysis

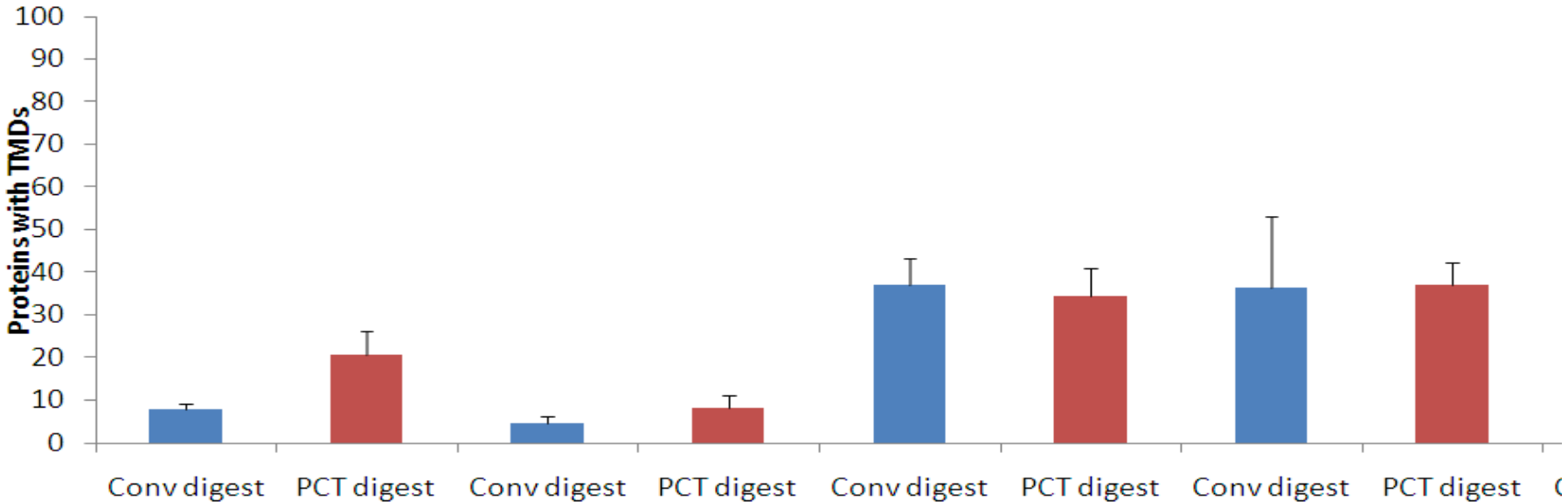


Pressure Cycling-Assisted and Organic Solvent – Assisted Cell Lysis



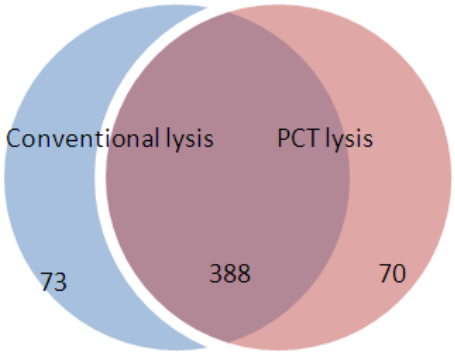
Pressure Cycling-Assisted and Organic Solvent – Assisted Cell Lysis

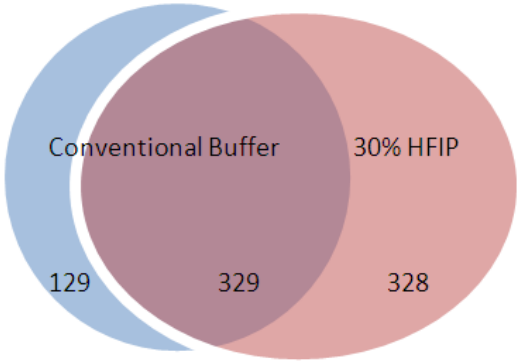


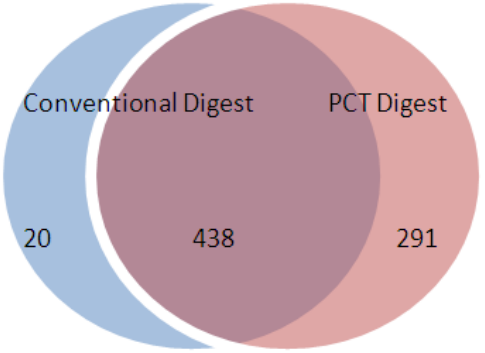


Pressure Cycling-Assisted and Organic Solvent – Assisted Cell Lysis

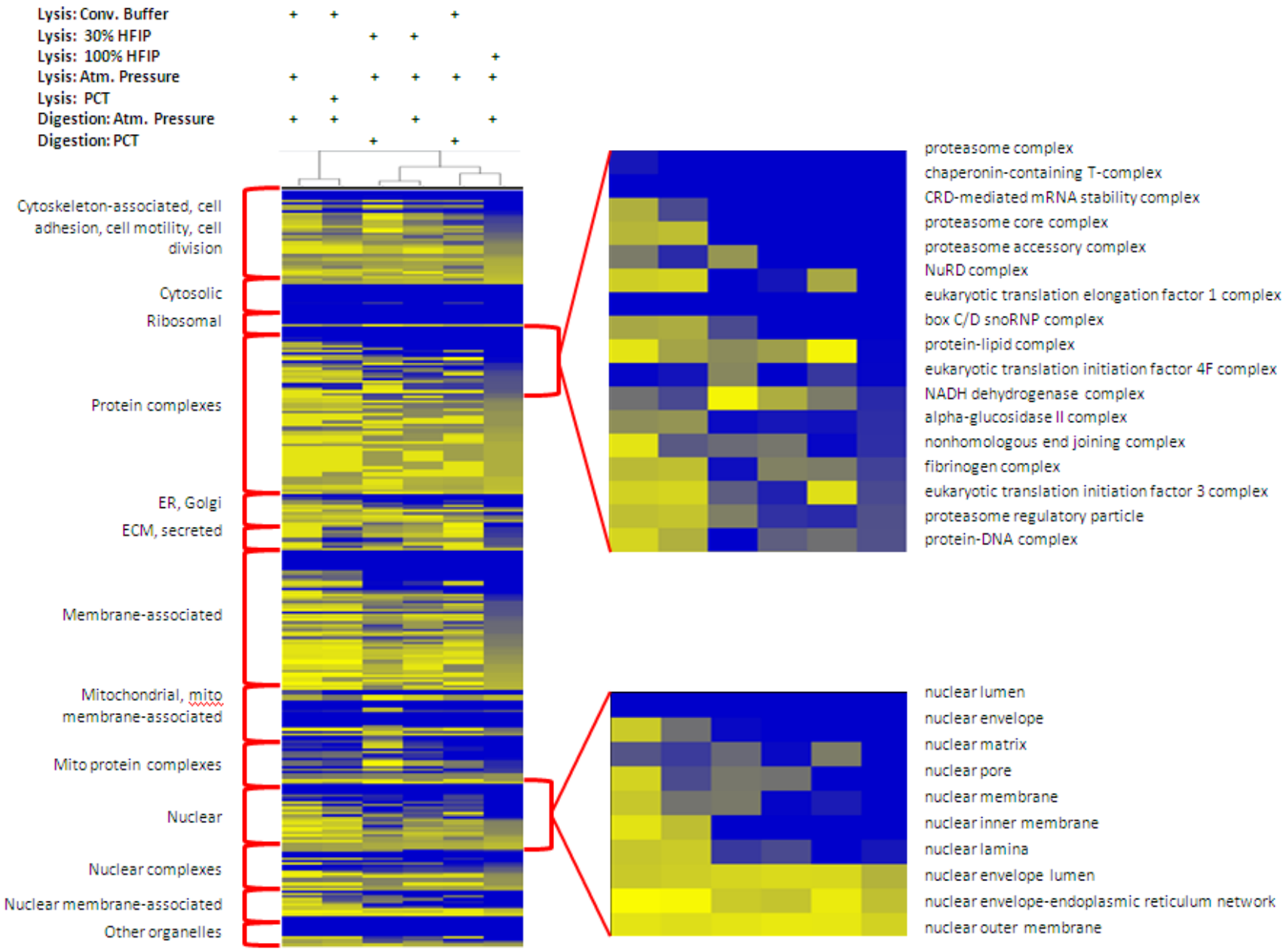
Lysis: Conv. Buffer	+	+	+		+	+
Lysis: 30% HFIP				+		
Lysis: Sonication	+		+	+	+	+
Lysis: PCT		+				
Digestion: AtmPr	+	+	+	+	+	
Digestion: PCT						+







GO Term Enrichment Analysis. Cellular Localization.



Conclusions

(1.) higher throughput;

(2.) higher efficiency;

(3.) superior reproducibility of enzymatic digestion;

(4.) more efficient cell lysis;

(5.) superior recovery of membrane, organelle, and complex forming proteins in comparison to the conventional protocols, as well as increased identification of proteins containing TMDs.

Acknowledgements

Emily Freeman
Alexander Lazarev
Vera Gross
PBI

Funding:
NIEHS, HSPH GCD Department,
CRDF, Harvard Catalyst