

Pressure BioSciences, Inc.

**The Challenges of Automating
Sample Preparation in the
Proteomics Era**

**ALA 2011
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Pressure BioSciences**

**Gary Smejkal
Harvard University**

Discovery Starts With Sample Preparation

PBI

Advantages of Automation

- Process Control
- Accuracy
- Precision
- Safety
- Throughput
- Data Output
- System Integration
- Validation

Diversity, Complexity, Targets and Goals

Vast Number and Array

Virus

Prokaryotes

Eukaryotes

Complex Matrices

Soil

Water

Parasites

Targets

DNA and RNA

Proteins

Lipids

Small Molecules

Organelles

Complexes

Goals

Extraction

Biomolecules

Active Molecules

Complexes

Insertion

Biomolecules

Drugs

Line Balance

Throughput

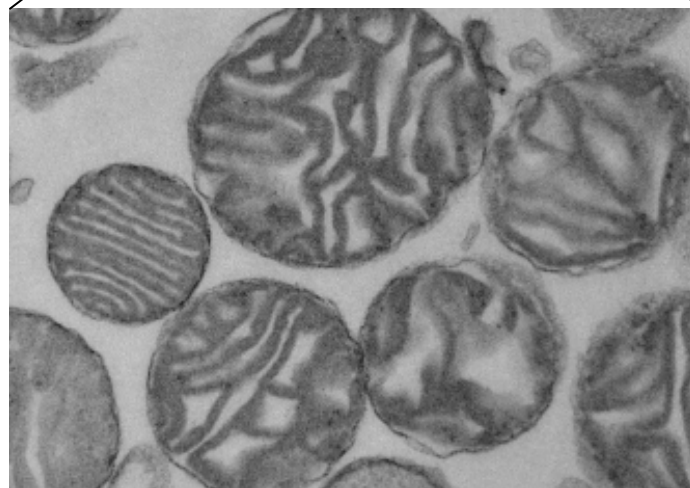
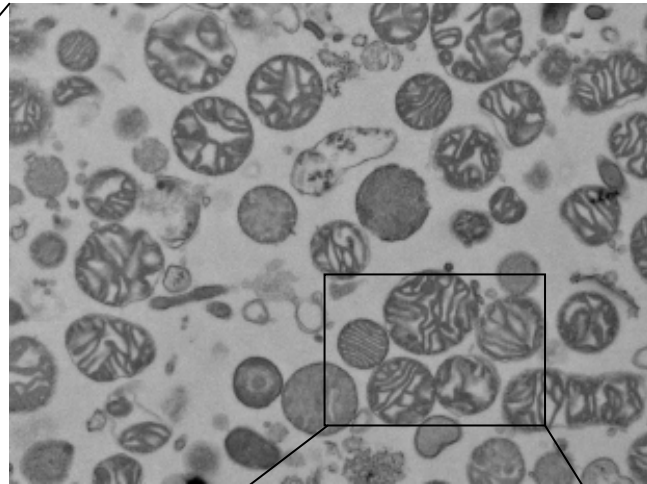
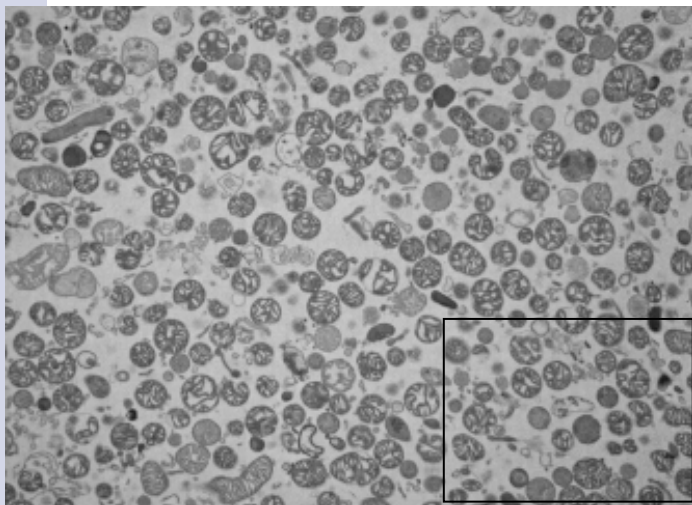
Data Analysis

Sample Preparation Tools

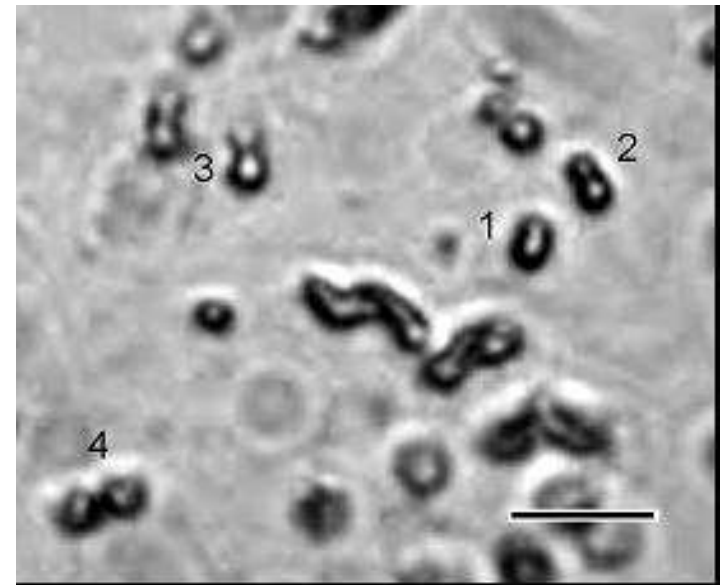
“To a man with only a hammer in the toolkit every problem looks like a nail”

Abraham Maslow

Active Kidney Mitochondria Isolated by PCT



Insects and Yeast in 30 Million Year Old Amber



*Raw Lobster Separated from Shell
by High Pressure*



Current Extraction Methods

- Mortar & Pestle
- Dounce homogenizer (glass on glass)
- Potter-Elvehjem homogenizer (Teflon on glass)
- Enzymatic Digestion
- Polytron shearing homogenizers
- Blenders
- Bead Beating
- Sonication
- Repeated Freeze/Thaw cycles
- French Press (≤ 2000 PSI)
- Robotics



State of the Art?

“A collaborator at a major university in a multi-million dollar Proteomics Facility, equipped with the most advanced instrumentation...

...but they use mortar and pestle.”



Native American Indian
Mortar and Pestle

circa 1000 AD

Pressure BioSciences

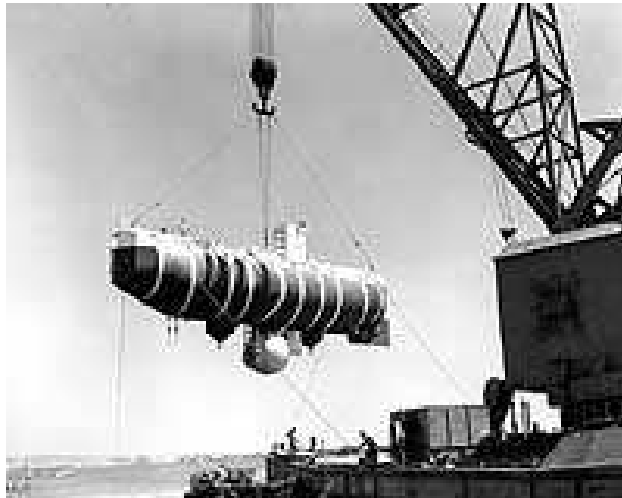
Pressure Cycling Technology (PCT)

PBI

Pressure Cycling Technology (PCT)

PCT is a Novel, Enabling Technology that Uses Cycles of Hydrostatic Pressure Between Atmospheric and Ultra-high Levels (up to 35,000 psi and greater) to Allow for the Precise Control of Biomolecular Interactions

Understanding Hydrostatic Pressure



U.S. Navy Bathyscaphe
Trieste (1958-1963)



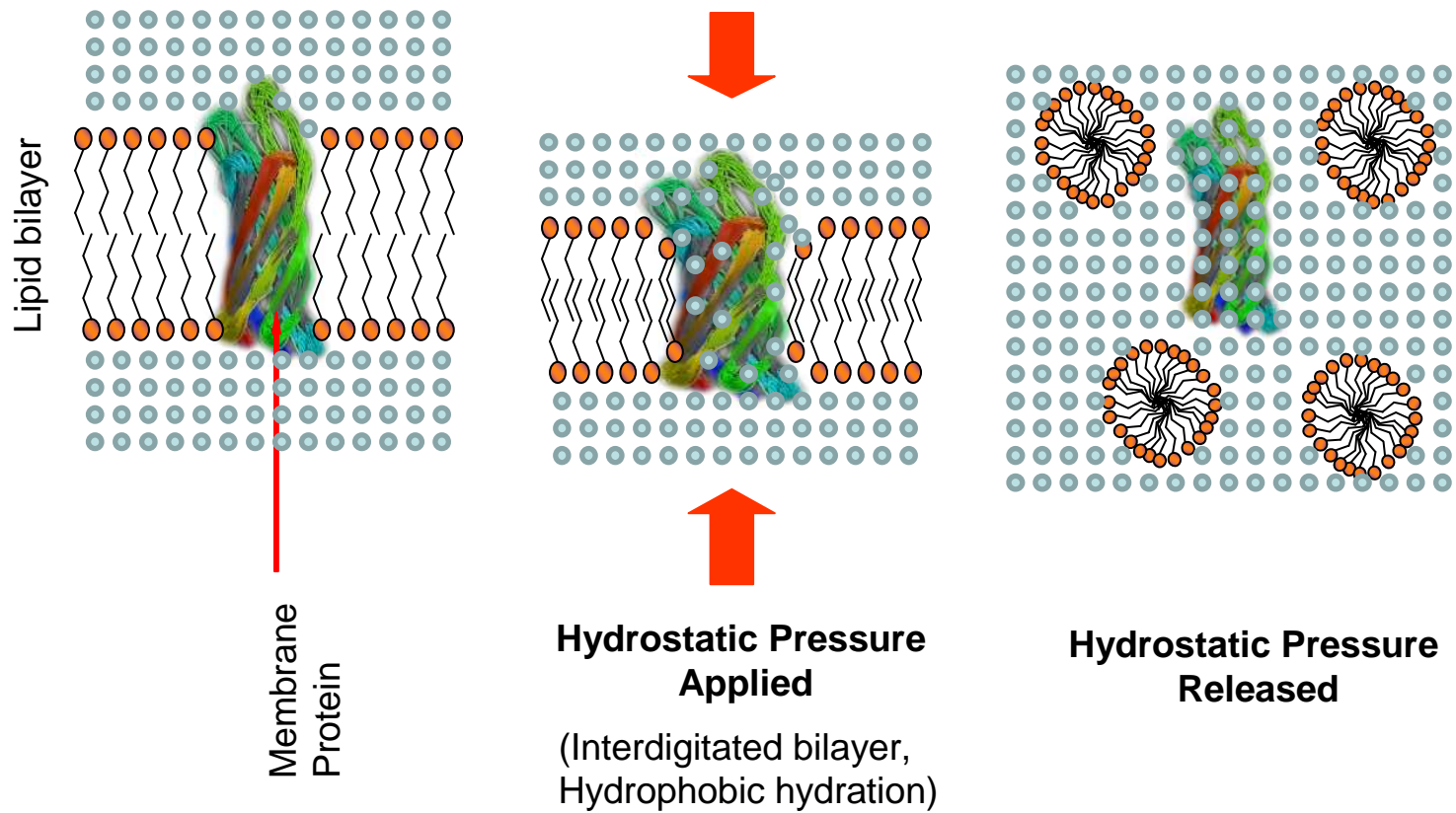
Marianas Trench:
38,713 ft (11,800m) deep
16,000 PSI (120MPa)

Significant portion of the Global Biosphere is
subjected to high hydrostatic pressure!

Why It Works

- Pressure is a Thermodynamic Process
- Compressibility of Water
- Synergy of Pressure, Temperature and Chemistry

Pressure Cycling Destabilizes Biological Membranes



PBI Products

Barocycler™ NEP3229



Barocycler™ NEP2320

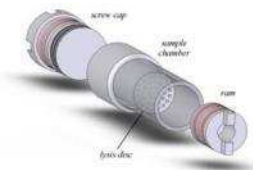


Barocycler™ HUB440

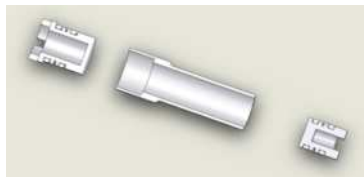


PCT Shredder and The Shredder SG3

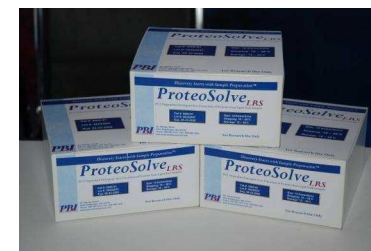
PULSE Tube FT500



PULSE Tube FT500-ND



PCT MicroTubes



Reagent Kits

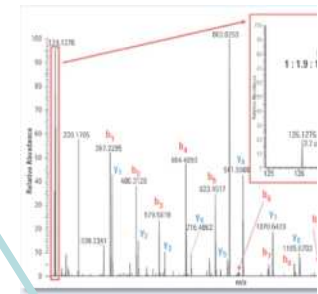
PCT Applications



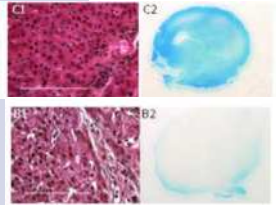
FORENSIC DNA EXTRACTION



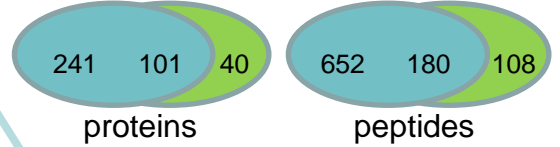
ACCELERATED ENZYMATIC DIGESTION



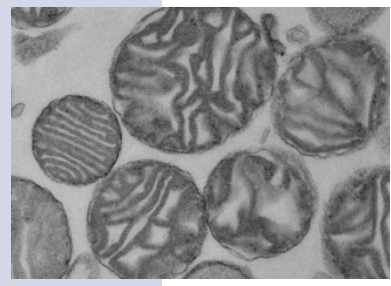
PATHOLOGY SAMPLE PREPARATION



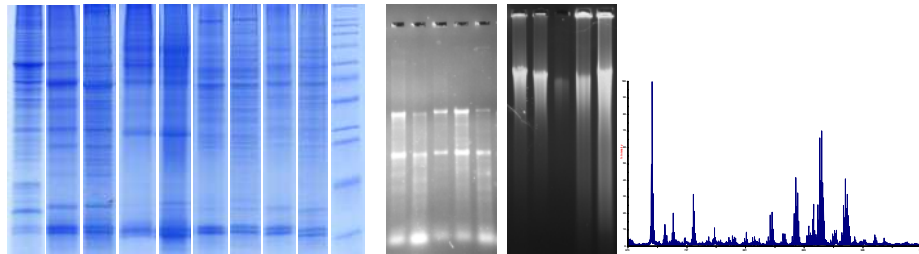
CELL AND TISSUE LYSIS



SUBCELLULAR FRACTIONATION



**EXTRACTION AND FRACTIONATION
DNA, RNA, LIPIDS AND PROTEINS**



PATHOGEN INACTIVATION



User-Adjustable Variables

- Pressure (up to 35 kpsi)
- Number of Cycles
- Cycle Profile
- Chemistry
- Temperature

Genomics

Release of DNA with the PCT Sample Preparation System (PCT SPS)

DNA Extraction

Agriculture

Improved Extraction of DNA of *Ca. Liberibacter* Species from Plants and Cultivated Cells Using Pressure Cycling Technology (PCT)

Dr. Norman Schaad (FDWSRU, USDA-ARS, Fort Detrick, MD USA)
APS Meeting 2090

Improved extraction of *Rhizoctonia* and *Pythium* DNA from wheat roots and soil samples using pressure cycling technology

Dr. Patricia Okubara (USDA, Pullman, WA)
Can. J. Plant Pathol. Vol. 29, 2007

Bioremediation

Analysis of Microorganisms in Oil Spills: Searching for Oil Eating Bacteria

Dr. Janet Jansson (Lawrence Berkeley Laboratories)
Work in Progress

Anti-bioterrorism

Use of Pressure Cycling Technology (PCT) in Sample Decontamination and Biomolecule Extraction for Analysis of the Anthrax Spore Proteome

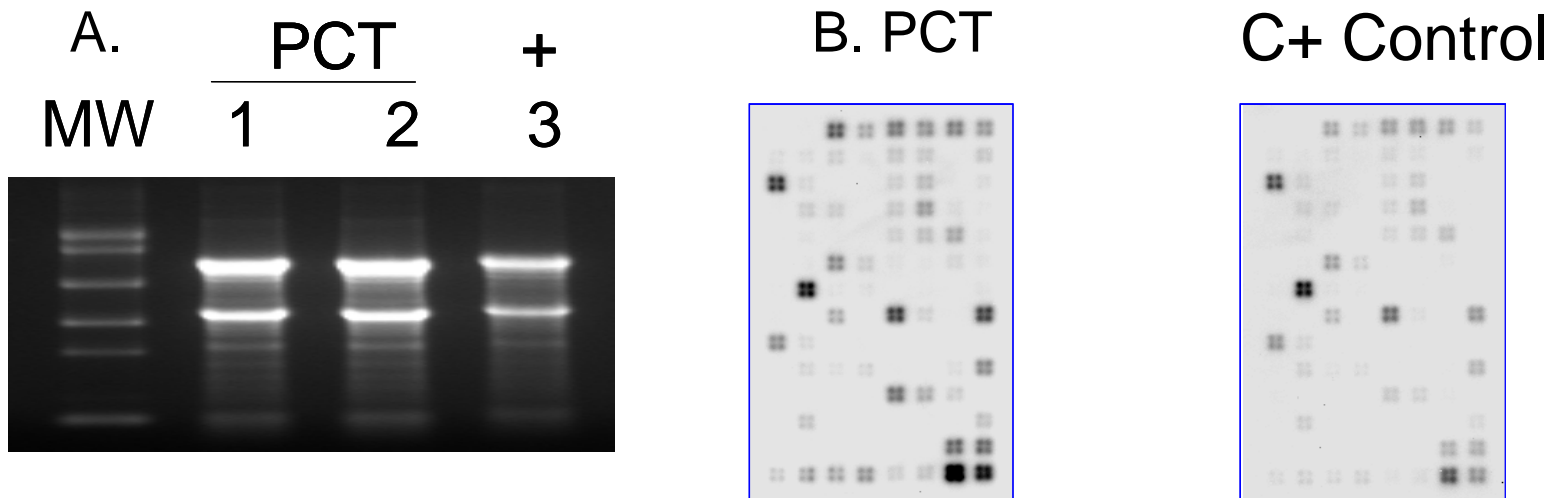
Bradford Powell, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD 21702, USA
Manuscript Submitted

Transcriptomics

Release of RNA with the PCT Sample Preparation System (PCT SPS)

Gene Expression Profiling

PCT Releases High Quality RNA for cDNA Microarray Analysis

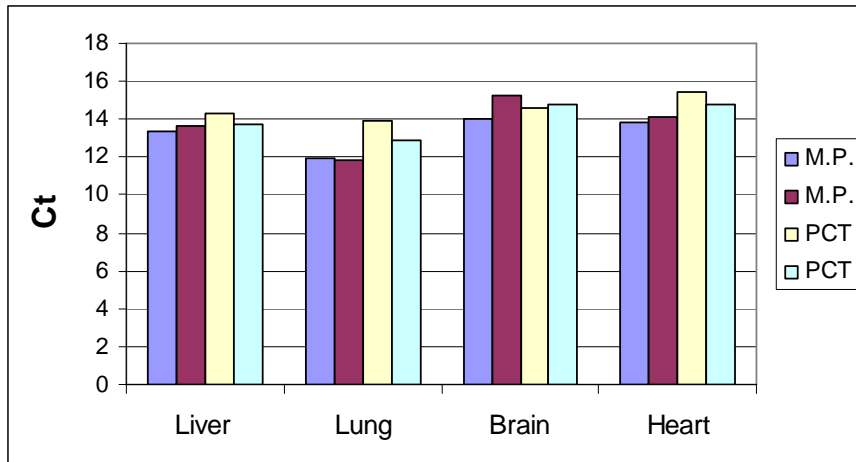


Sample: Rat Brain

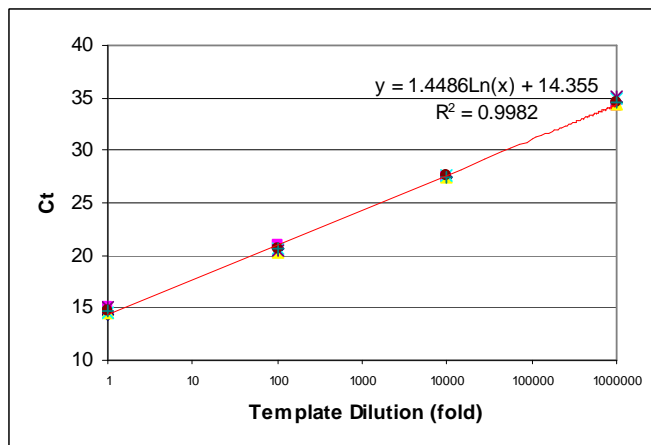
PCT Condition: 4°C, 5 x 1 min cycles, 35 kpsi

RNA Extraction Buffer: 1.1 ml 4M GTC/1% NP40

microRNA Detection from Rat Tissue Samples



- Both extraction methods yielded similar quality and quantitative RT/real-time PCR results
- PCT process is much easier to operate than M/P/H
- Excellent linearity on diluted real-time PCR templates were observed



Experimental Conditions:

PCT: 5 cycle, 35 kpsi, 4°C

miRNA Purified Using Ambion *mirVana* miRNA Kit

microRNA Assays Were Done with an hsa-miR-16 Probe Set on an ABI 9700 and 7500 Instruments

Proteomics

Release of Protein with the PCT Sample Preparation System (PCT SPS)

Complexity of Proteins and the Proteome

Primary, Secondary, Tertiary and Quaternary Structure

Post-translational Modifications

Conformation Changes

Globular, Fibrous, Membrane

Active Sites

Up and Down Regulation

Solubility

Low and High Abundance

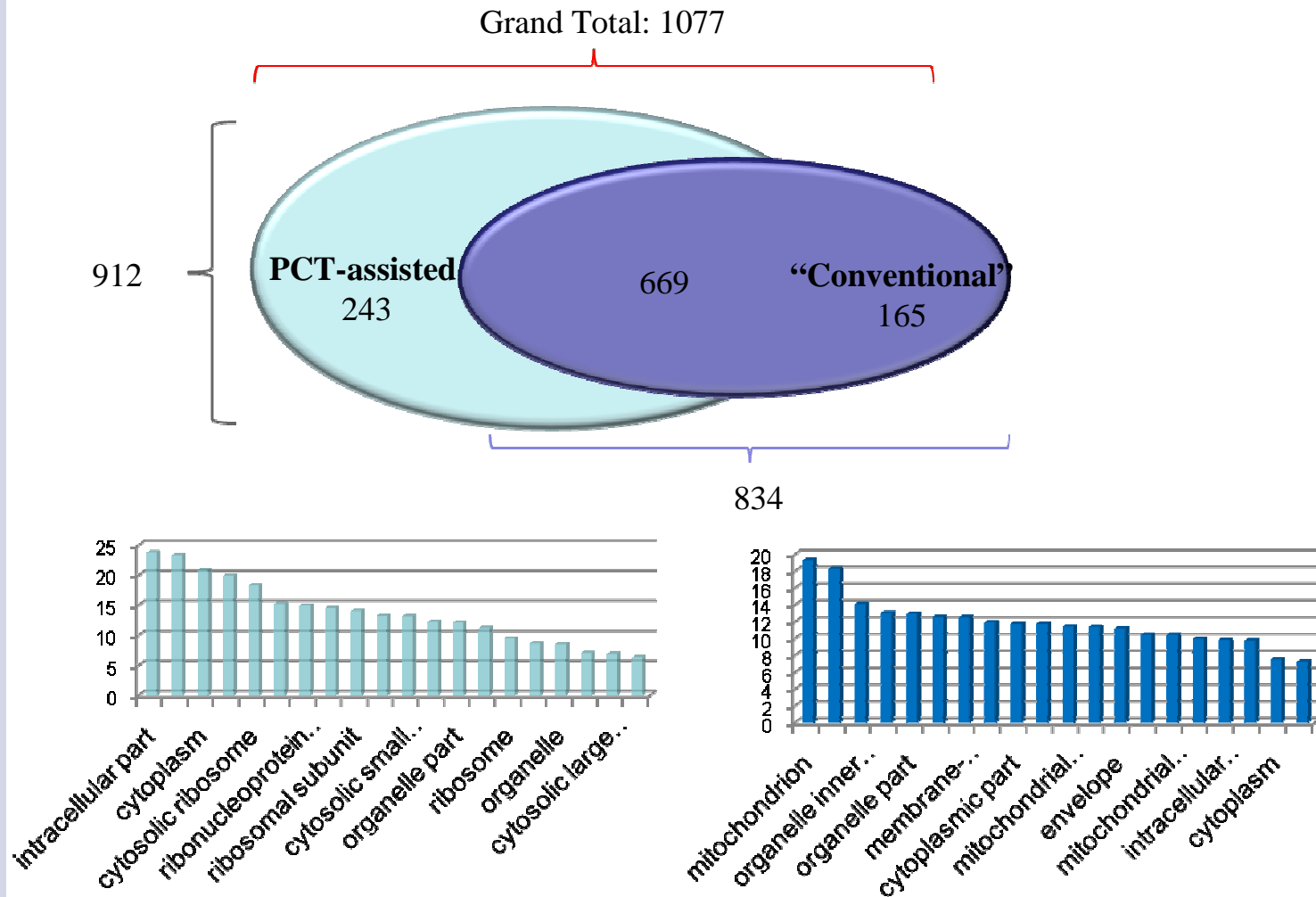
Complex Structures and Organelles

Not Amplified *In Vitro*

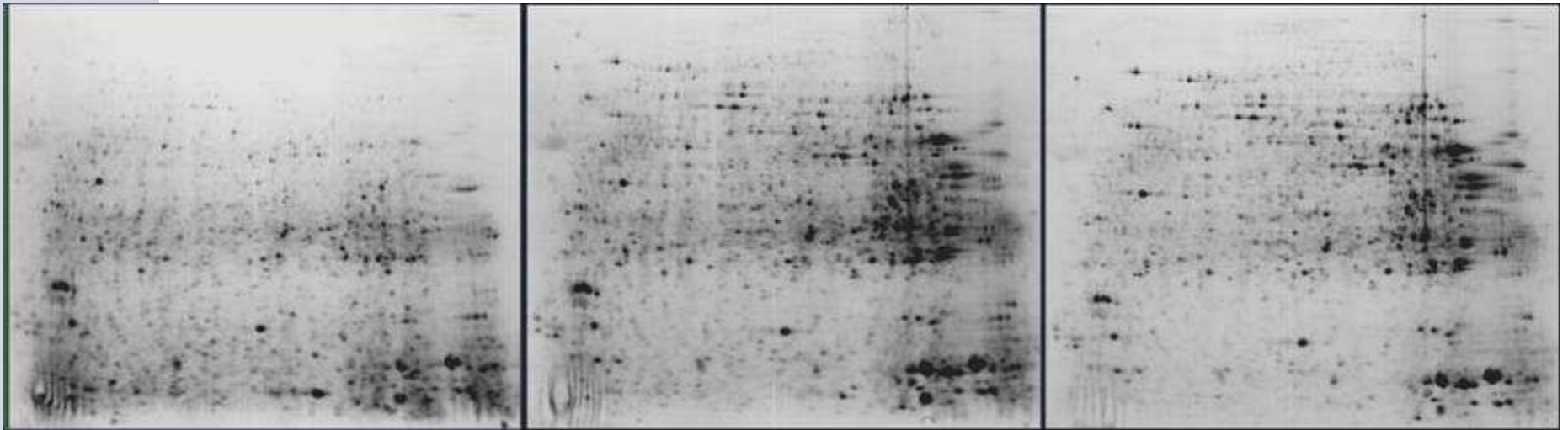
Proteins Under Pressure

- Pressure promotes **dissociation** of oligomeric proteins
- Pressure promotes protein **unfolding and re-folding**
- Unfolding leads to **hydration**, i.e. volume reduction
- Pressure activates most **hydrolytic enzymatic reactions**
- Pressure leads to **protein denaturation**
- Pressure **protects proteins from thermal denaturation**
- Pressure may act in **synergy with chemical denaturants**

PCT-assisted Cell Lysis in Detergent-free Buffer



Comparison of PCT, Sonication, and Grinding of Murine Liver

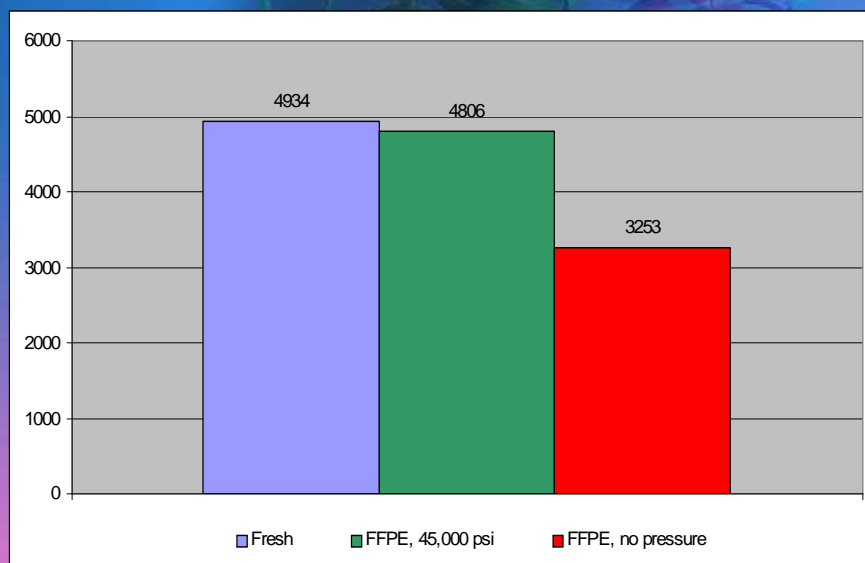


sonicator
1,739 spots

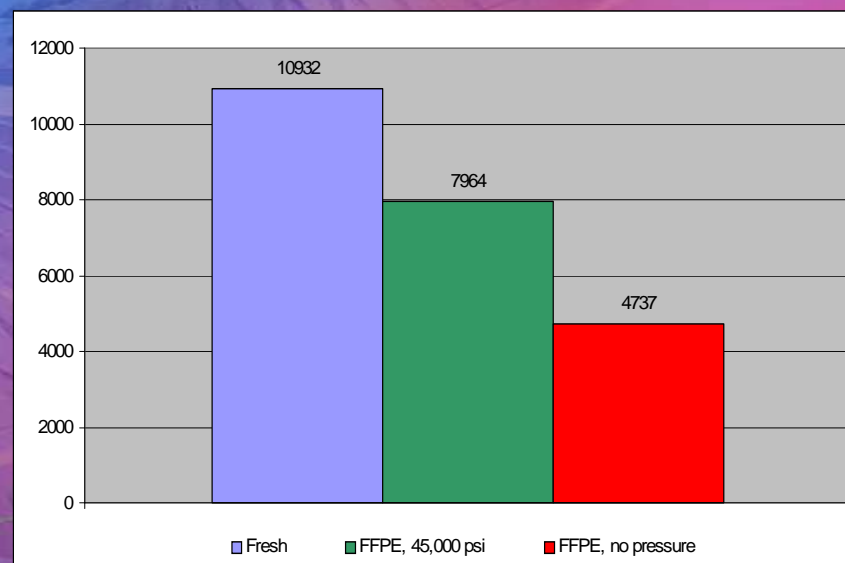
PCT
2,126 spots

ground glass
1,853 spots

Pressure Cycling Technology (PCT) Significantly Improves Recovery of Proteins/Peptides from Formalin Fixed Paraffin-Embedded (FFPE) Tissue



Unique Proteins Identified



Unique Peptides Identified

Data Courtesy of Dr. C. Fowler (AFIP)

"Our initial data show that for aorta samples, which are traditionally difficult to lyse, a greater amount of protein is recovered following pressure-enhanced FFPE extraction, compared to the standard non-pressure method."

Dr. J. Van Eyk, Director, The Hopkins NHLBI Proteomics Center, John Hopkins

Applications in Mass Spectrometry

Pressure-Enhanced Enzymatic Proteolysis

Some Pressure-Enhanced Enzymes

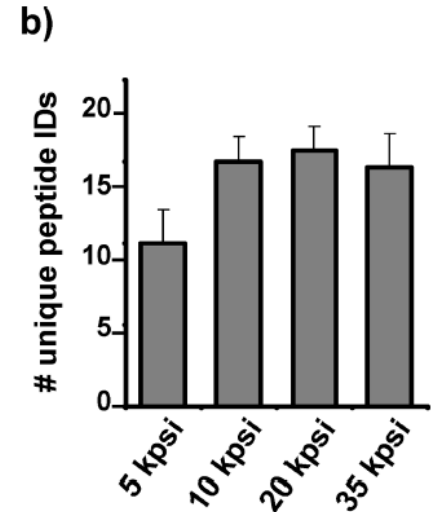
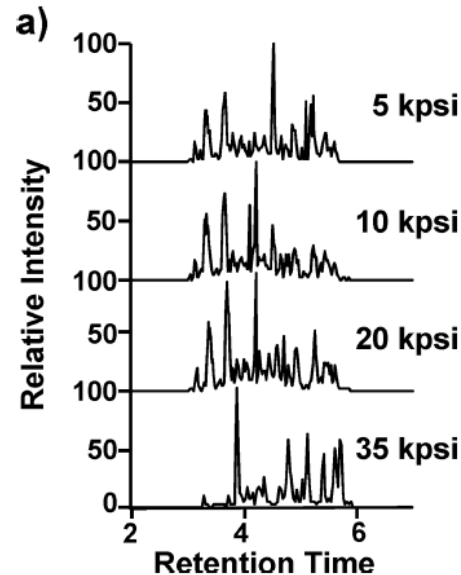
- **Proteinase K**
- **Lysozyme**
- **Trypsin**
- **Chymotrypsin**
- **Lys C**
- **PNGase F**
- **Pepsin**

PCT-Enhanced Tryptic Digestion of BSA

Pacific Northwest National Laboratories:
Application of Pressurized Solvents for Ultrafast Trypsin Hydrolysis in Proteomics: Proteomics on the Fly

Increase pressure can dramatically increase the rate of the enzymatic digestion.

PCT simplified sample preparation compared with other newer rapid digestion methods, such as MAPED and HIFU technologies.



Successful in-solution digestions of single proteins and complex protein mixtures were achieved in 60 seconds

PCT-assisted Lys-C Digestion of Monoclonal Antibodies

AMGEN:

A Comparison of Methods for Efficient Digestion of Protein Therapeutics

Conclusion:

This study demonstrated that pressure cycling provided the most effective method for digesting monoclonal antibodies. Complete digestion can be obtained in a short period of time without inducing modifications such as methionine oxidation. While the microwave technique has established applicability in a proteomics setting, the more stringent requirements of the biopharmaceutical arena suggest limitations of the technique with respect for characterization of protein primary structure

A Comparison of PCT and CTRL for Post-Nuclear Membrane Tryptic Digests

	Barocycler	Thermomixer
Unique Peptides	832	288
Unique Proteins	342	141
Integral Membrane Proteins	62	15

PCT enables a 2.5-fold increase in peptide identification compared to the conventional digestion procedure for post-nuclear membrane samples.

PCT is also more effective in the digestion of integral membrane proteins.

The Effect of Pressure Cycling on Proteolytic Cleavage Efficiency, Reaction Time and Protein Sequence Coverage

Eric Bonneil¹; Roger Biringer²; Julian Saba²; Andreas Huhmer²; Pierre Thibault¹

¹Institute for Research in Immunology and Cancer, Université de Montréal, Montréal, Canada

²Thermo Fisher Scientific, San Jose, CA

Deglycosylation of RNase B by PNGase F

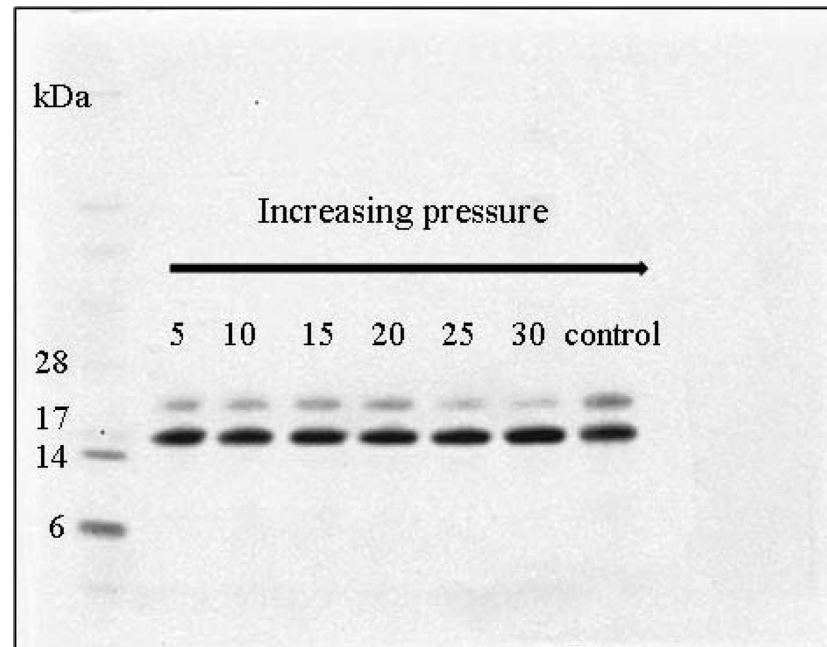


Figure 1. The effect of the maximum pressure level of pressure cycling on PNGase F-mediated deglycosylation of the N-linked sugars from RNase B (Coomassie Brilliant Blue stained SDS-PAGE image). The bands at 15 and 18 kDa represent the deglycosylated and intact forms of RNase B, respectively. Deglycosylation of denatured RNase B was carried out at 37 °C for 5 min with 1:2500 enzyme : substrate molar ratio in the presence of Triton X-100. Control: 5 min deglycosylation at atmospheric pressure and 37 °C. Pressure cycles: 50 s pressure/10 s atmospheric. (Left lane) SDS-PAGE protein sizing standards: aprotinin (6 kDa), lysozyme (14 kDa), myoglobin (17 kDa), and carbonic anhydrase (28 kDa).

Rapid Release of N-Linked Glycans from Glycoproteins by Pressure-Cycling Technology

Zoltan Szabo, Andras Guttman, and Barry L. Karger*

Barnett Institute, Northeastern University,

Boston, Massachusetts 02115

Anal. Chem. XXXX, xxx, 000–000

A Possible Mechanism

Untying the Gordian Knot



Summary

Pressure Cycling Technology (PCT)

- Employs an **orthogonal** sample preparation technique
Pressure, Temperature, Mechanical and Chemical Variables
- Has a wide variety of applications
Genomics, Transcriptomics, Proteomics, Enzymology, etc.
- Tool for **Discovery**