PBI Pressure BioSciences Inc.

Life Under Pressure:
Application of Hydrostatic Pressure in Life Sciences

Alexander Lazarev, Ph.D.

NCI-Frederick

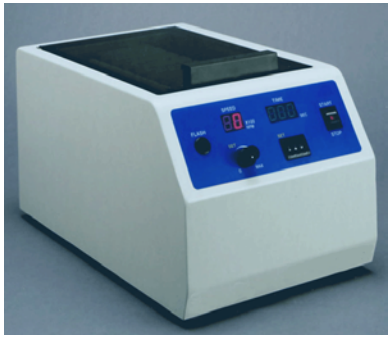
Presented at NCIFCRF on June 10th, 2008

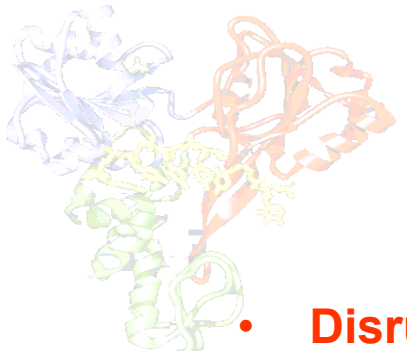
Sample preparation in the Era of "Analytical Arms Race"

Well-defined experimental goal and well-prepared sample are the foundation of success.

Conventional cell disruption methods

- Mortar & pestle or Dounce homogenizer (glass on glass)
- Potter-Elvenhjem homogenizer (Teflon on glass)
- Enzymatic Digestion
- Polytron shearing homogenizers
- Blenders
- Bead mills
- Sonication
- Repeated freeze/thaw cycles
- French press (≤ 2000 PSI)

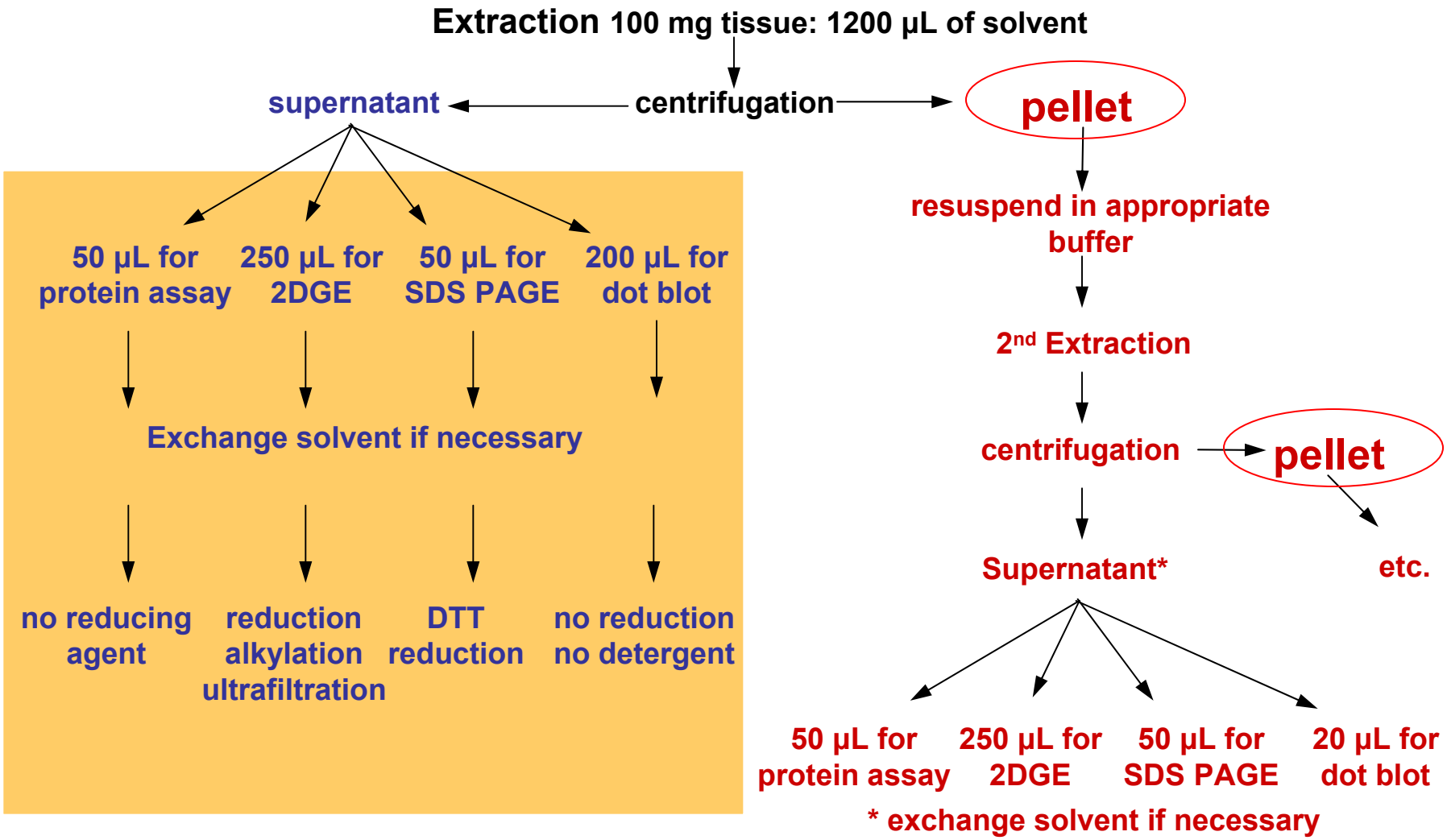




Ideal tissue and cell processor?

- **Disrupts lipid bilayer and molecular complexes**, but not covalent bonds (proteins, DNA, RNA, etc.)
- **Distributes energy** uniformly throughout the sample
- Facilitates **partitioning** of lipids, proteins and nucleic acid
- Does not depend on **aggressive extractions buffers**
- Yet, **compatible** with a wide variety of extraction buffers
- Prevents sample **cross-contamination**
- Keeps samples **enclosed** during the processing
- Provides precise **temperate control**
- Capable of processing **frozen samples** directly
- Processes samples with a **throughput** matching the downstream analysis.
- …

Multi-stage extraction approach employing orthogonal methods

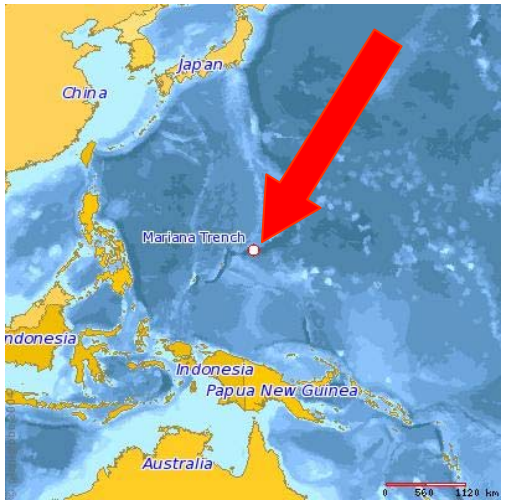


PRIMARY ANALYSIS

SECONDARY ANALYSIS

Understanding hydrostatic pressure

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



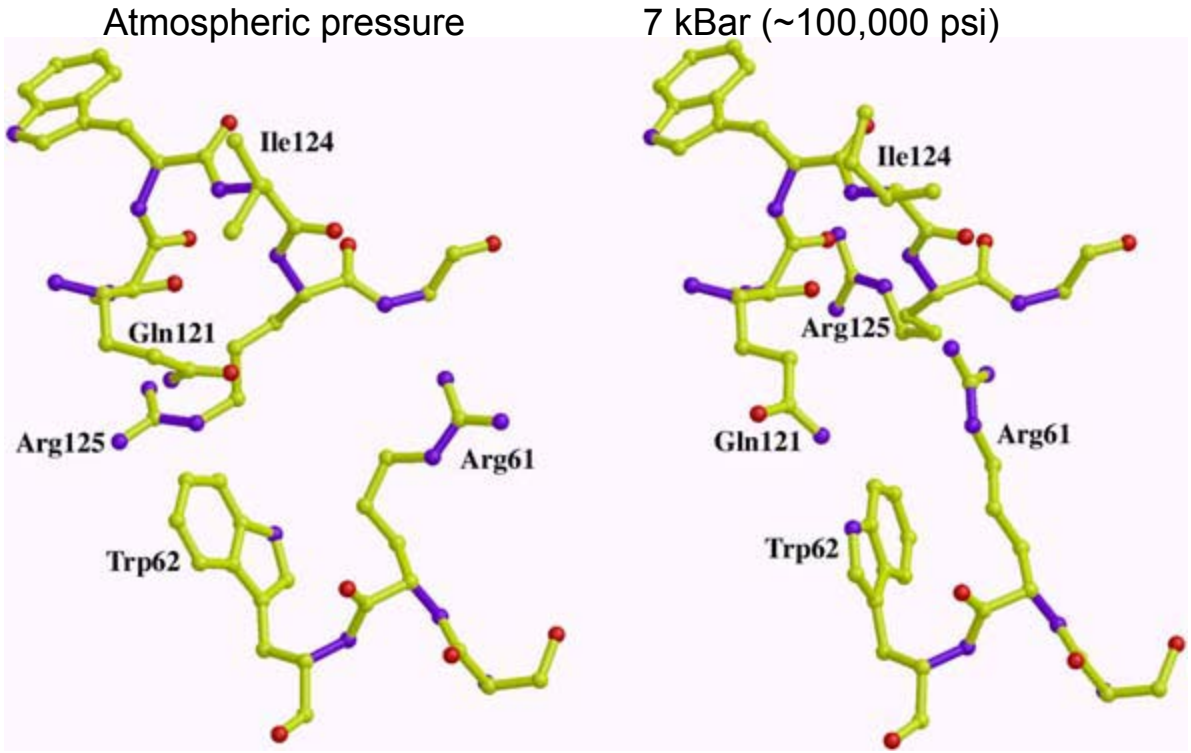
Marianas Trench:
38,713 ft (11,800m) deep
16,000 PSI (120MPa)

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

History of High pressure in Life Sciences

- 1623-1662: Blaise Pascal – described fundamental concepts of pressure and vacuum.
- 1895: H. Royer – pressure kills bacteria
- 1899: B.H. Hite *et al*. – pressure preserves milk
- 1914: P.w. Bridgman - pressure coagulates egg white
- 1989: High pressure processing of food products
- 2000: First International Conference on HPBB

Protein Crystallography at high pressure



Protein crystals of lysozyme under pressure exhibit more tightly packed structure.
The ordering effect of pressure may help to obtain quality crystals of "tough" proteins.

Data courtesy ESRF (www.esrf.eu)

HPP – High Pressure Food Processing

60,000 – 87,000 psi - selective denaturation of proteins

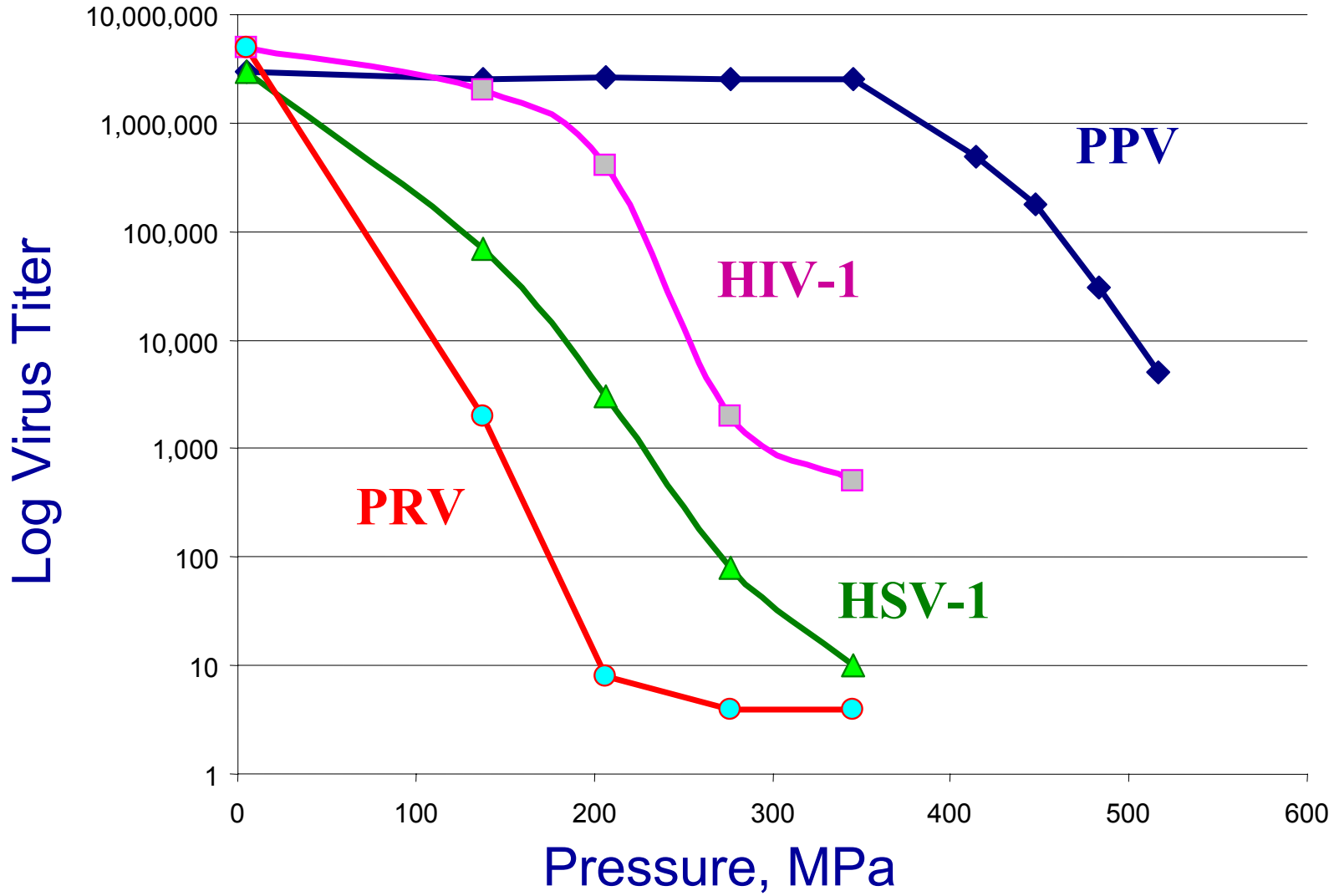
Commercial Scale Processing, 60,000 – 80,000 psi

- Inactivates food borne pathogens (listeria, e-coli, etc..) without heat or chemicals
- Extends shelf life
- Pre-packaged product is being processed
- No heat required
- Preserves chemical composition, texture, taste

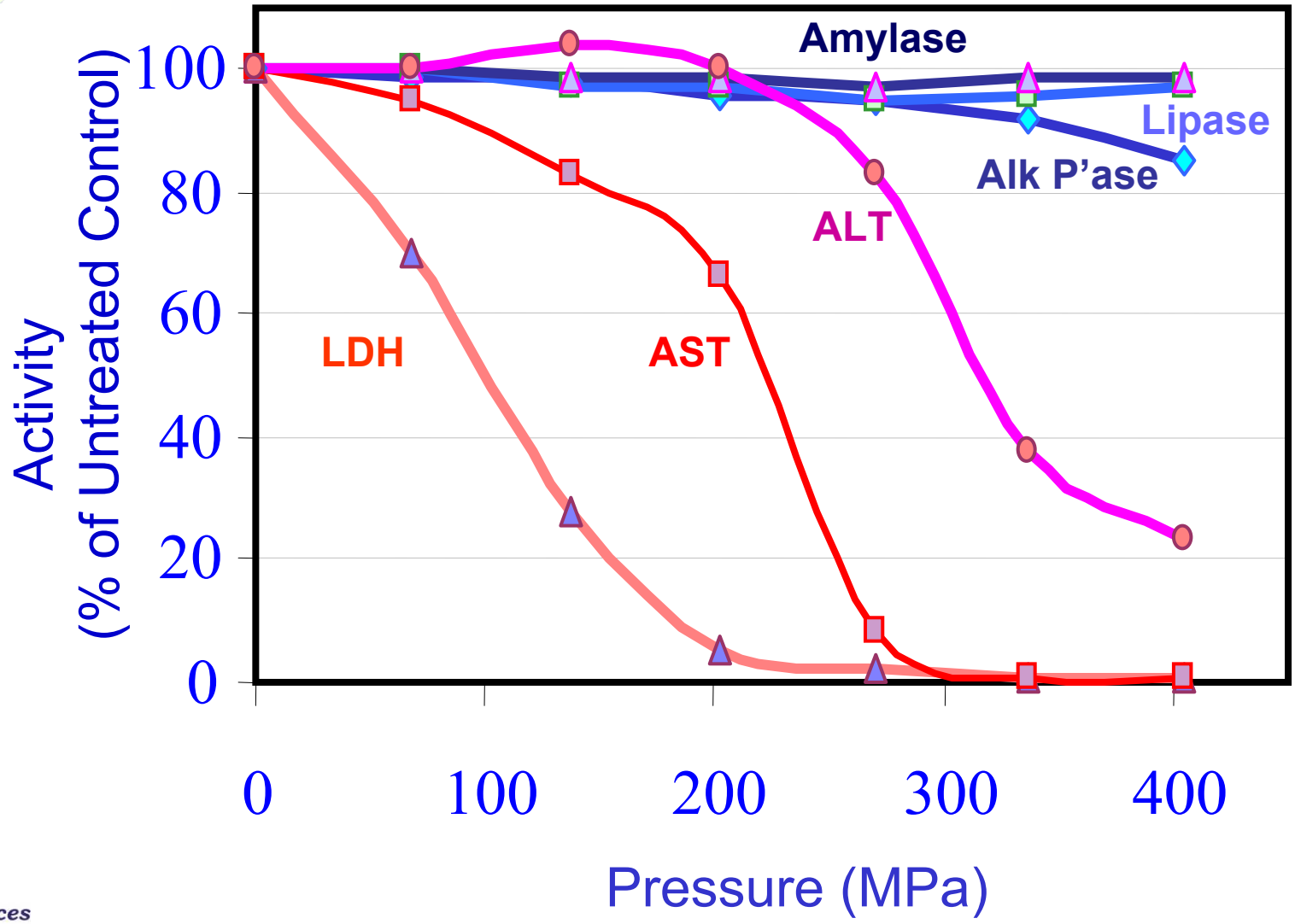
Hormel_sustainable

Viral inactivation by hydrostatic pressure

Note: 240 MPa = 35,000 psi



Effect of high pressure on enzymatic activity

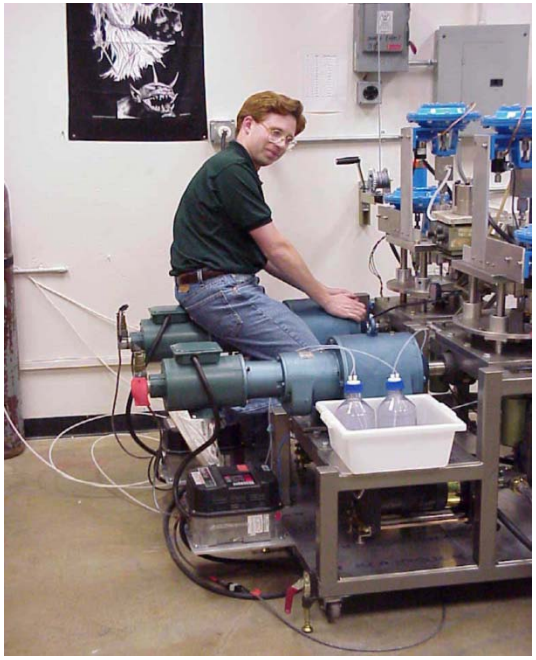


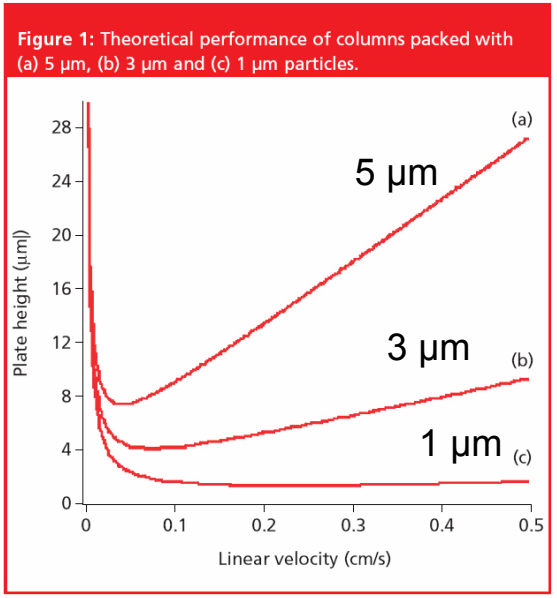
PBI Pressure BioSciences Inc.

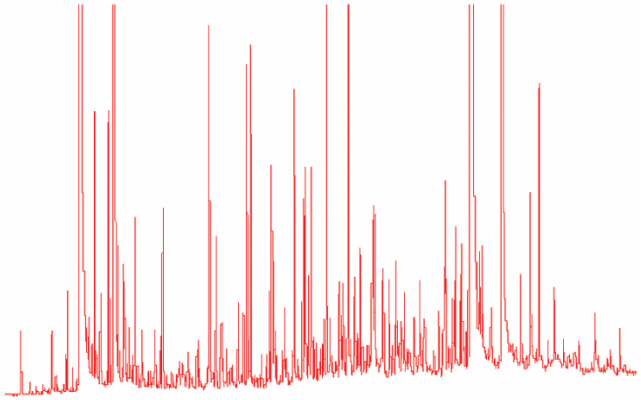
Ultra-High Pressure HPLC

James W. Jorgenson
W. R. Kenan, Jr. Professor of Chemistry, UNC, Chapel Hill

Ultra High Pressure HPLC
50,000 psi
Waters UPLC (15,000 psi)



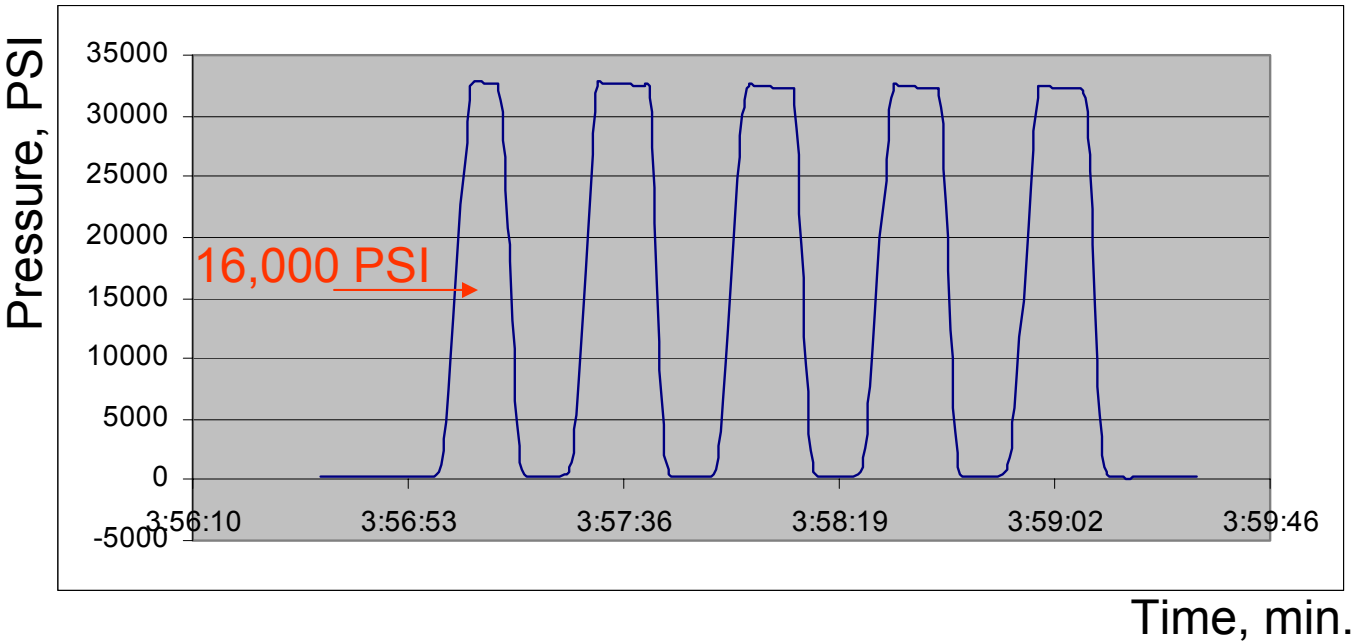




UHPLC gradient separation of a tryptic digest of bovine serum albumin. A peak capacity of 500 was obtained between 48 and 168 min.

Pressure Cycling Technology (PCT):

13 US patents
4 EU patents
1 AU patent



"Cycles of hydrostatic pressure between ambient and ultra high levels,
which allow for the precise control of molecular interactions"

PBI Pressure BioSciences Inc.

PCT Sample Preparation System

Hydraulic system
3 samples simultaneously
Optional temperature control

Barocycler™ NEP3229

PBI Pressure BioSciences Inc.

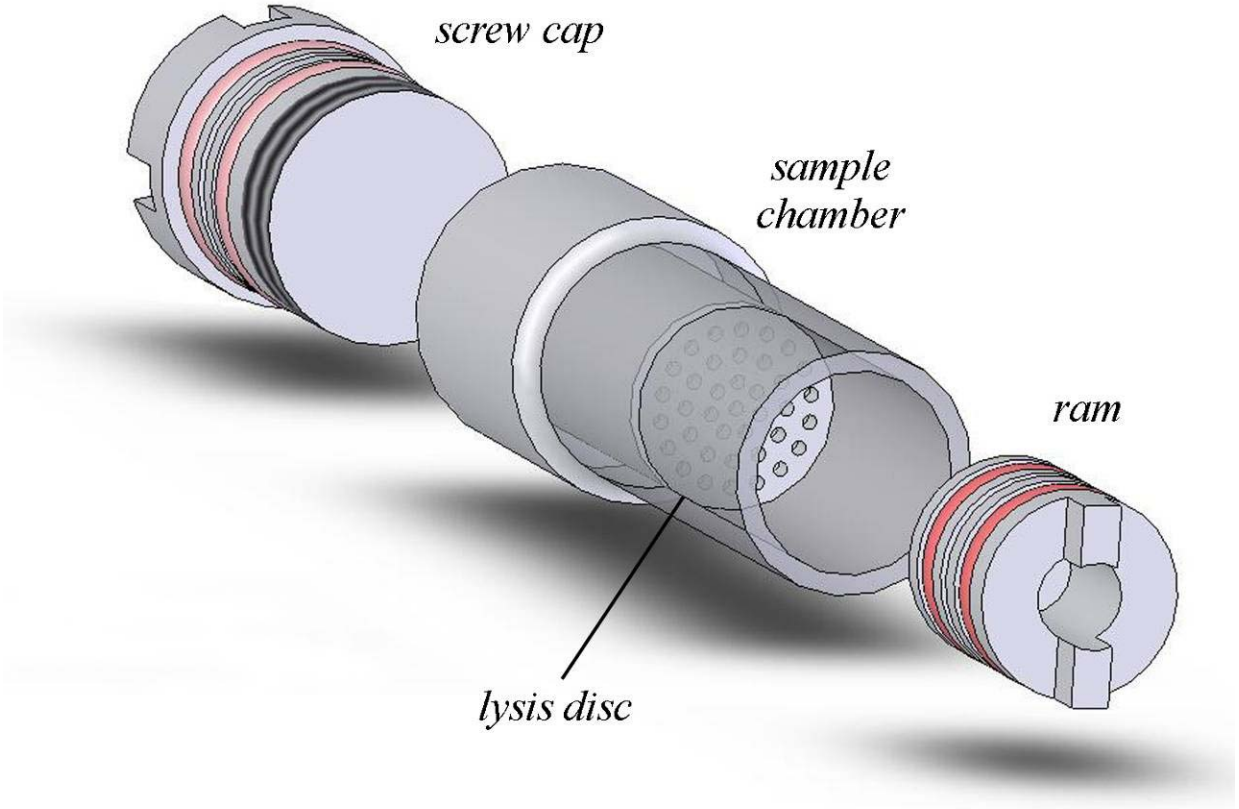
PCT Sample Preparation System

Pneumatic system
Single sample capacity
Optional temperature control

Barocycler™ NEP2320

PBI Pressure BioSciences Inc.

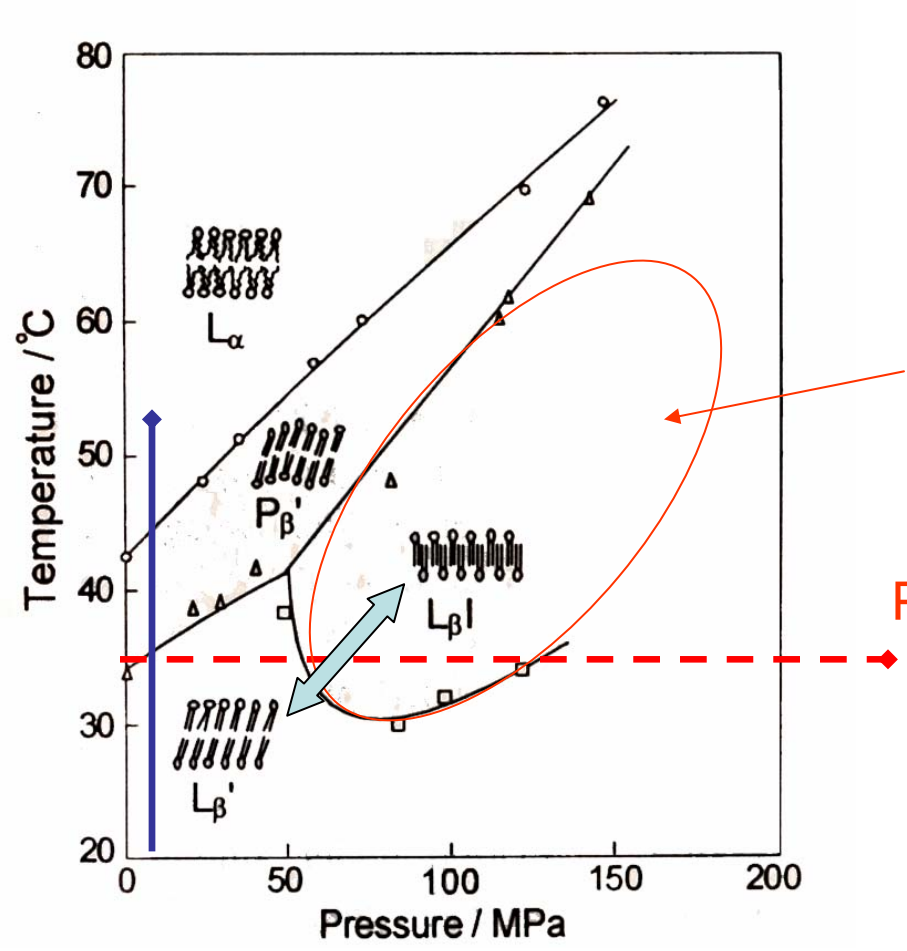
PULSE™ Tube: disposable sample container



Pressure Used to Lyse Samples for Extraction

PBI Pressure BioSciences Inc.

Thermodynamic impact on biological membrane structure



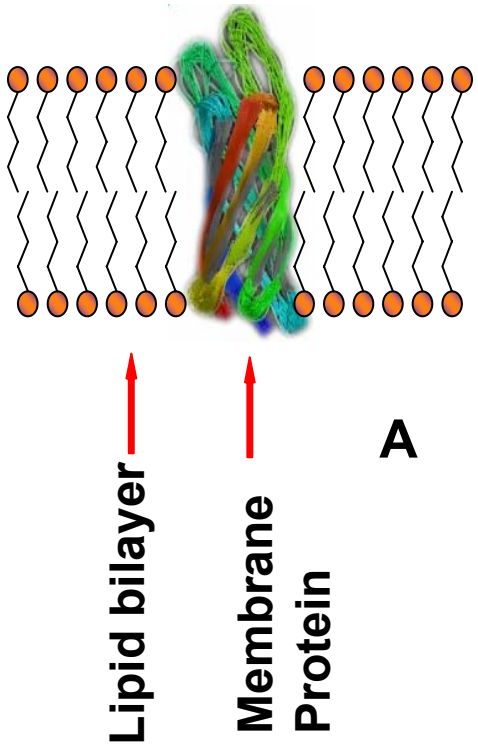
Pressure-induced interdigitation of lipid bilayers in an ester-ester linked HPPC bilayer: HP DSC data.

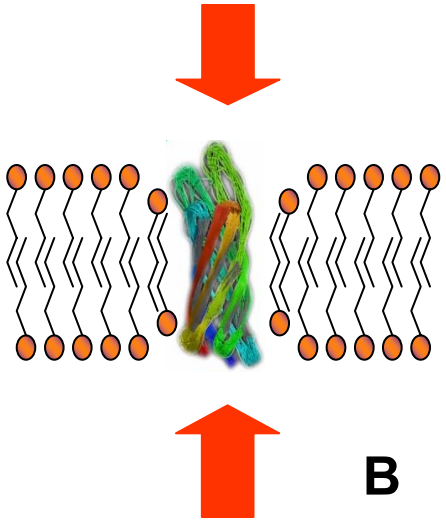
Interdigitated bilayer

Pressure cycling at 33 ºC

Ichimori H. *et al*., 1999; in: *Advances in High Pressure Bioscience and Biotechnology, Horst Ludwig (Ed.), Proceedings of the Intl. HPBB Conference, Heidelberg, 1998.*

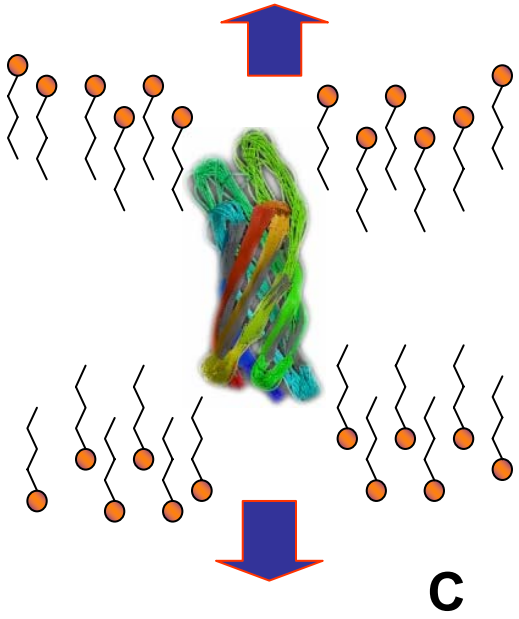
Pressure cycling acts directly on biological membranes





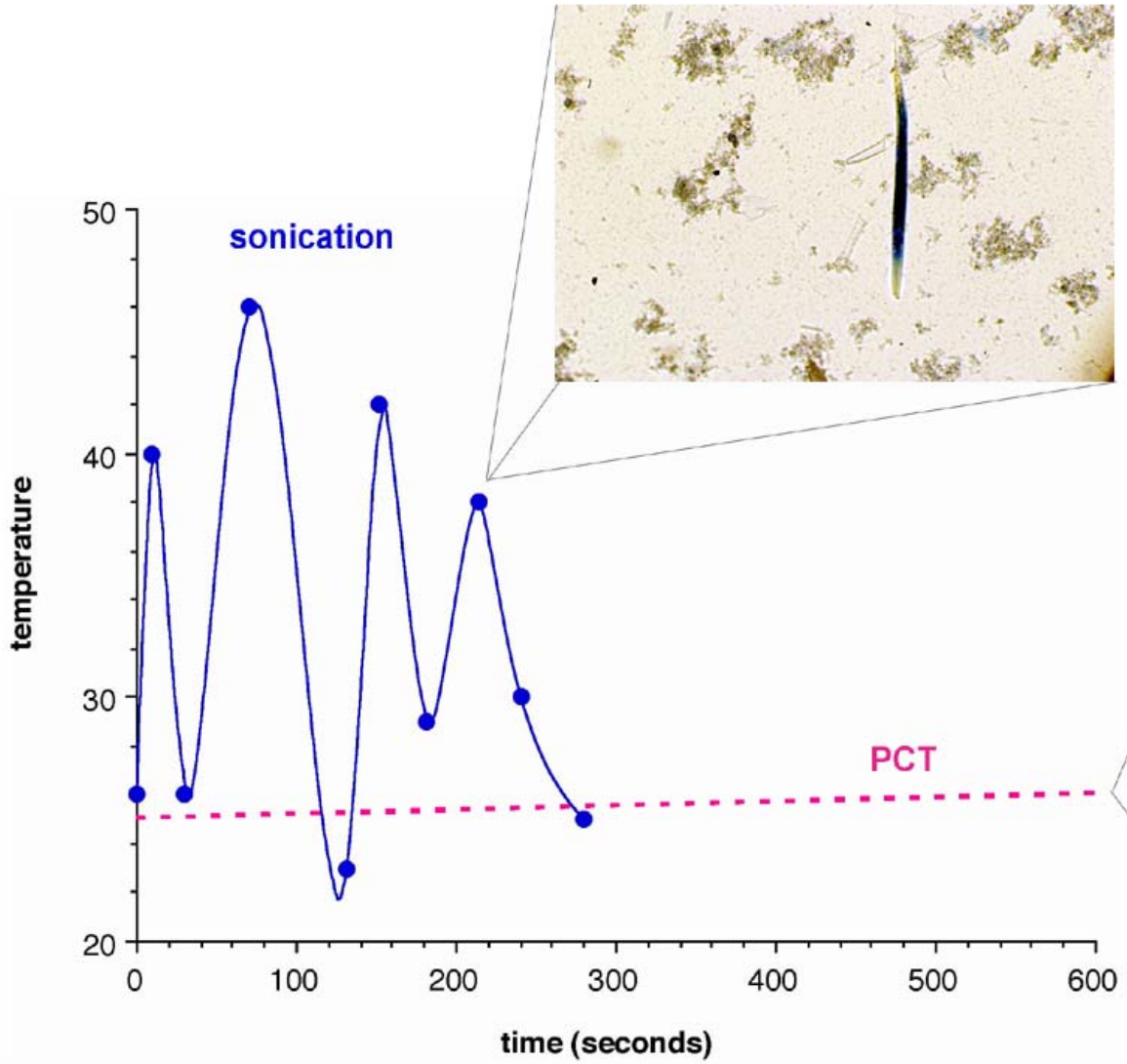
Hydrostatic Pressure Applied

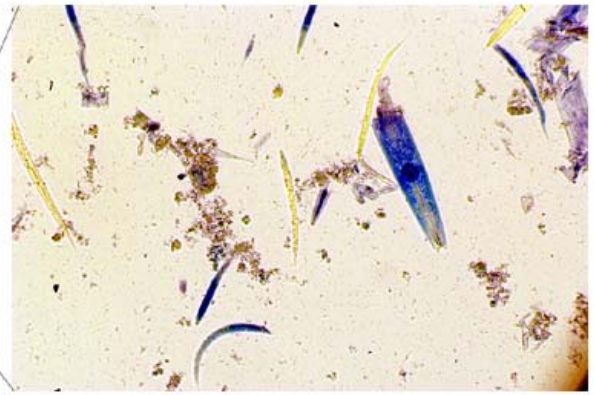
(Interdigitated bilayer)



Hydrostatic Pressure Rapidly Released

Caenorhabditis elegans extraction, temperature profile

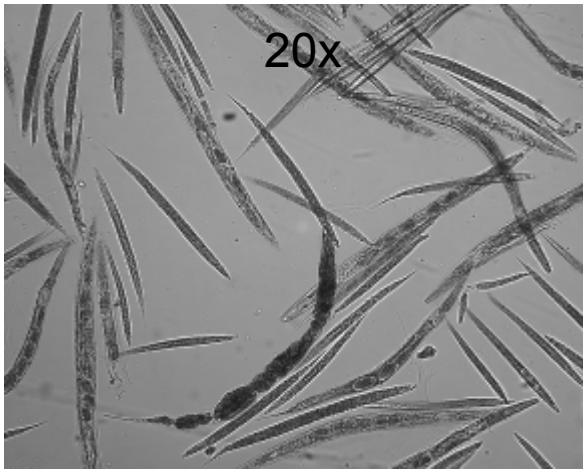




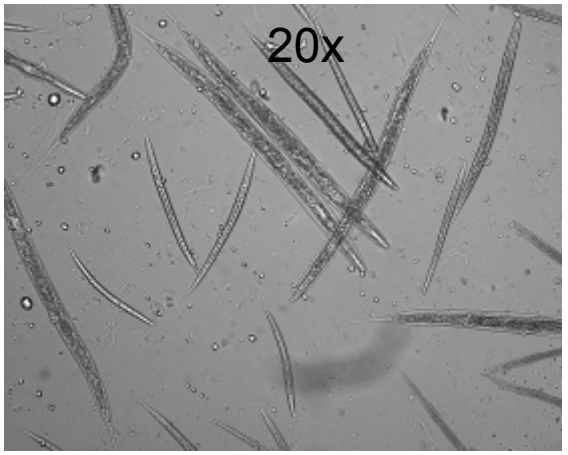
PBI Pressure BioSciences Inc.

Caenorhabditis elegans extraction by various methods

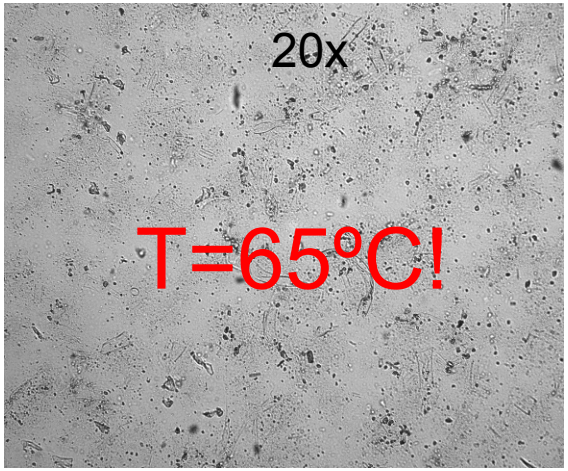
Freeze-thaw



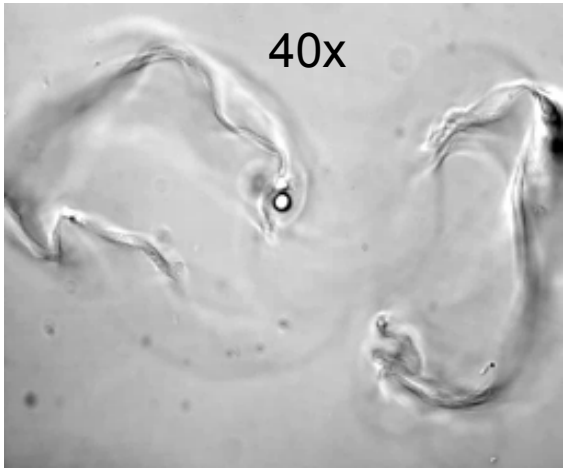
Bead Beater, 4x20s



Sonication 3x20s

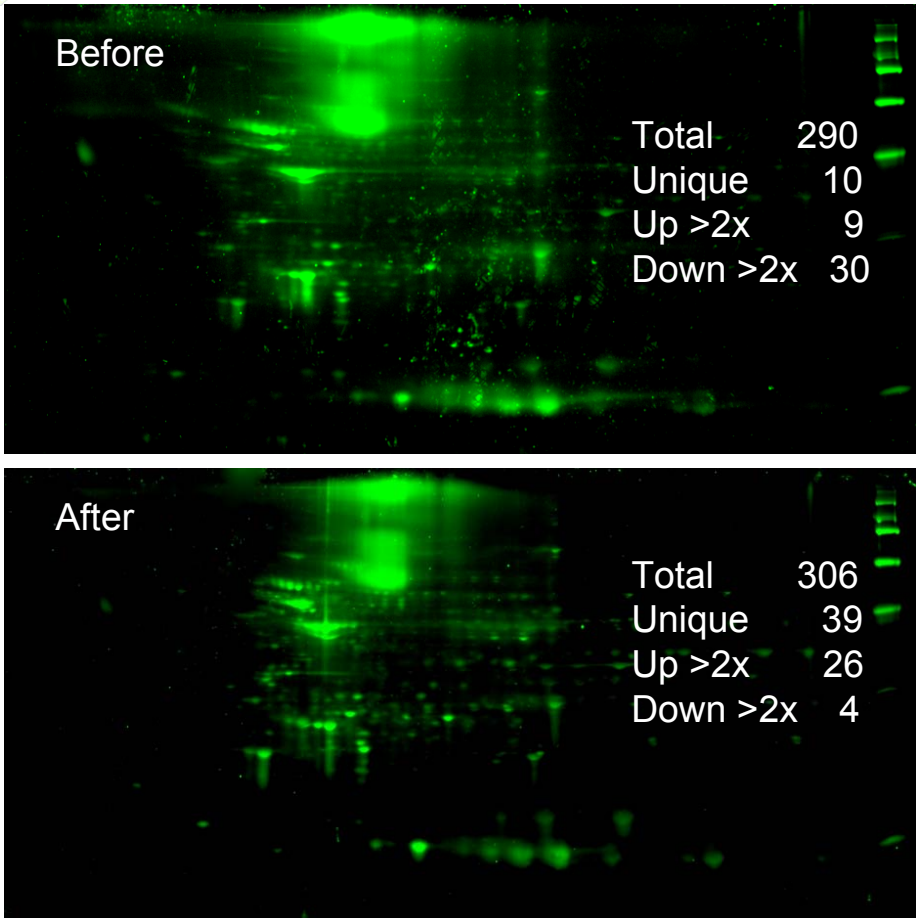


PCT – 5 cycles



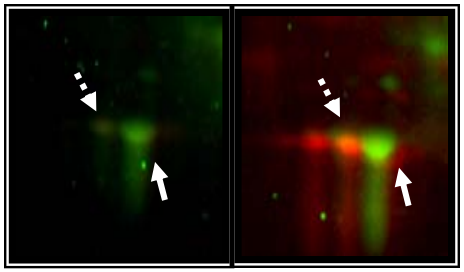
PBI Pressure BioSciences Inc.

Human atrium samples before and after cardiac surgery



2D: 3-10 pH gradient, 8-20% SDS-PAGE

- cardioplegia and cardiopulmonary bypass were used
- representative blots form one patient
- **100 mg** of atrial tissue
- SYPRO Ruby and ProQ-Diamond stains



Pre-CP/CPB **Post-CP/CPB**

MLC2a Protein

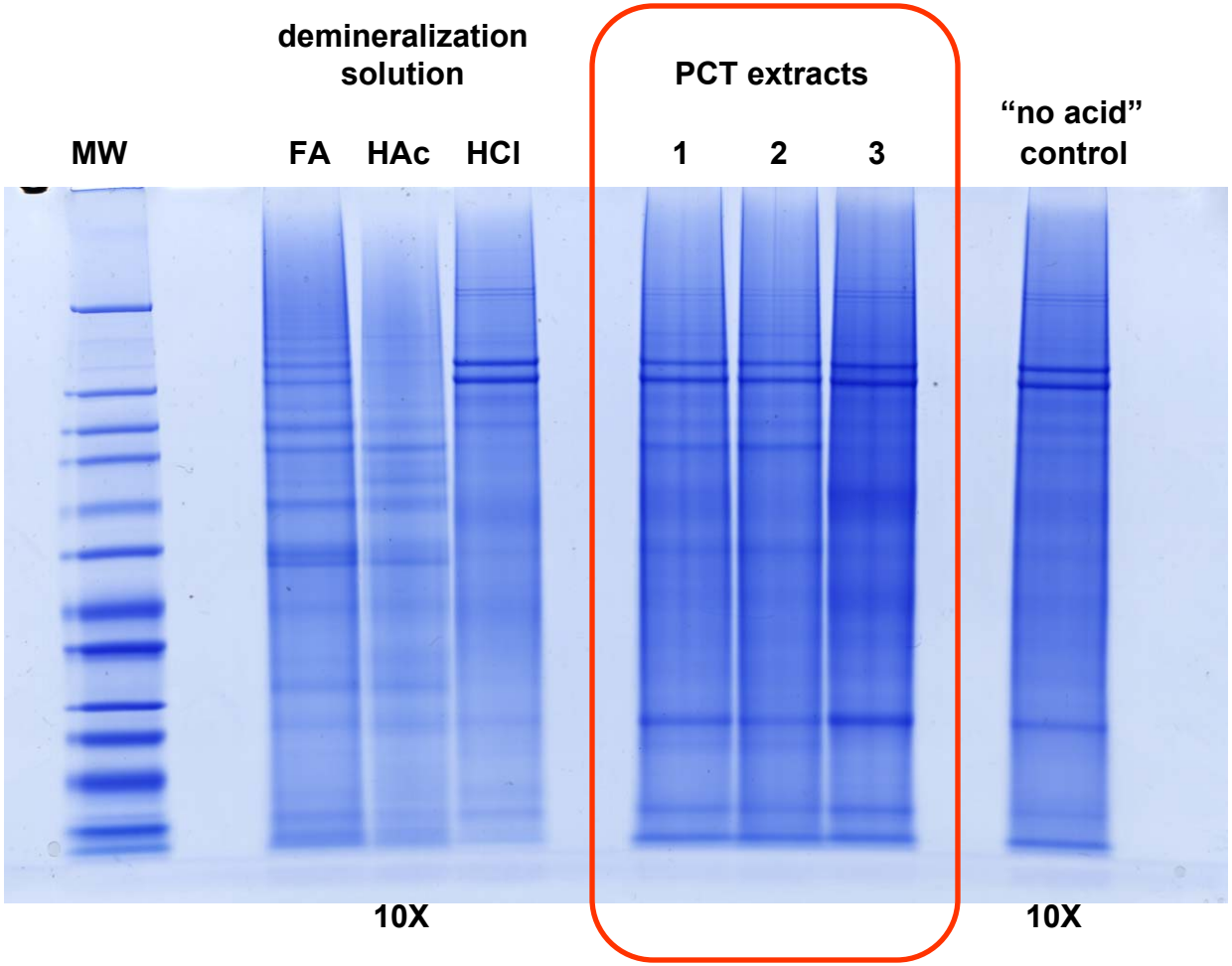
Phosphorylated MLC2a protein

R. Clements et al, AHA 2007 poster presentation

PBI Pressure BioSciences Inc.

Protein extraction from cortical bone

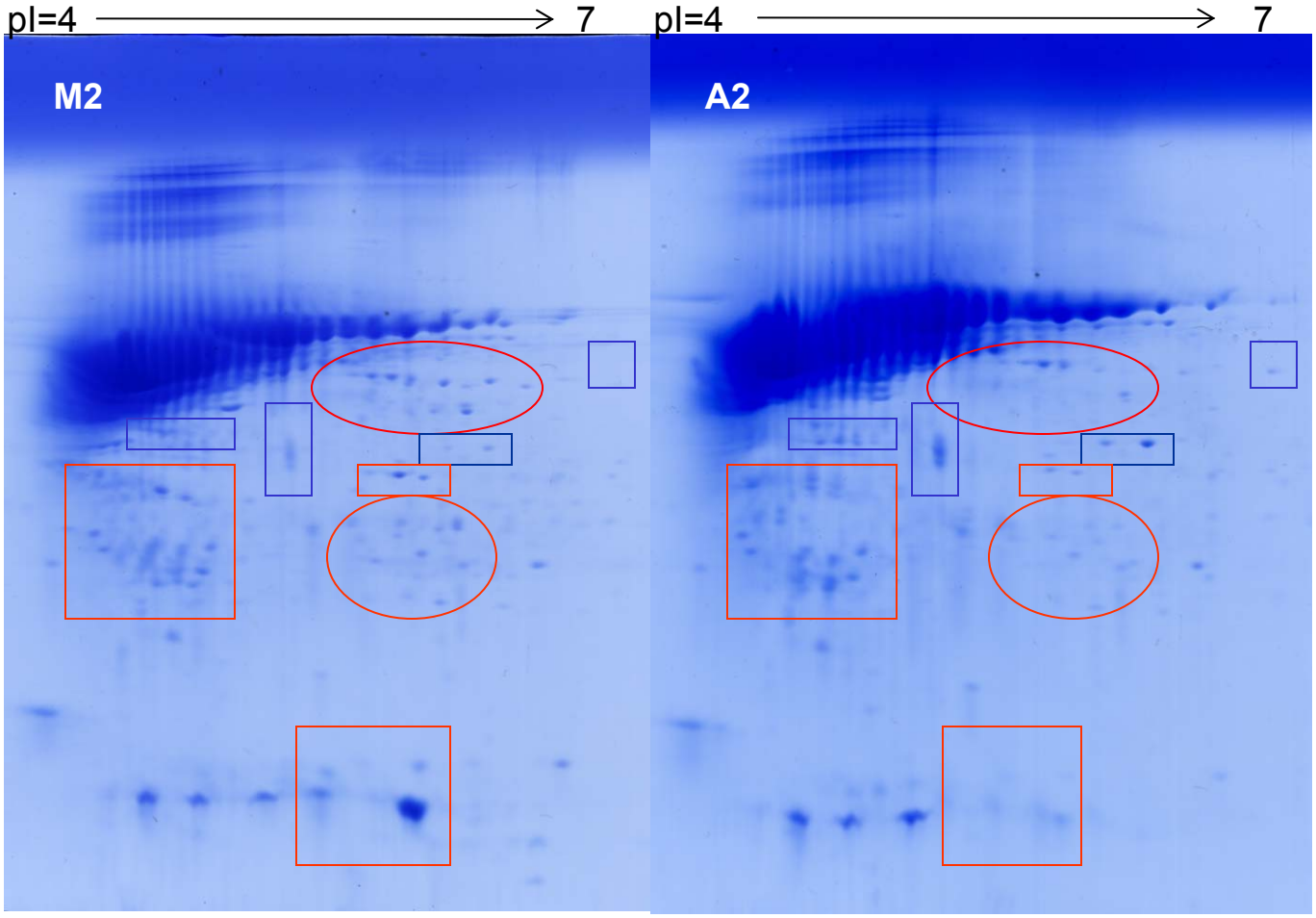
PCT releases proteins from bone without de-mineralization



1DGE of aged ostrich bone following acid demineralization, PCT, and Norgen column for removal of Ca and PO4

Human *Stratum corneum* proteome

skin samples of two healthy volunteers



Cells consecutively collected on 10 1” adhesive disks from the same location
and processed in two PULSE Tubes in IEF Buffer (7M urea, 2M thiourea, 4% CHAPS)

Problems with traditional methods of protein extraction from sample with high lipid content

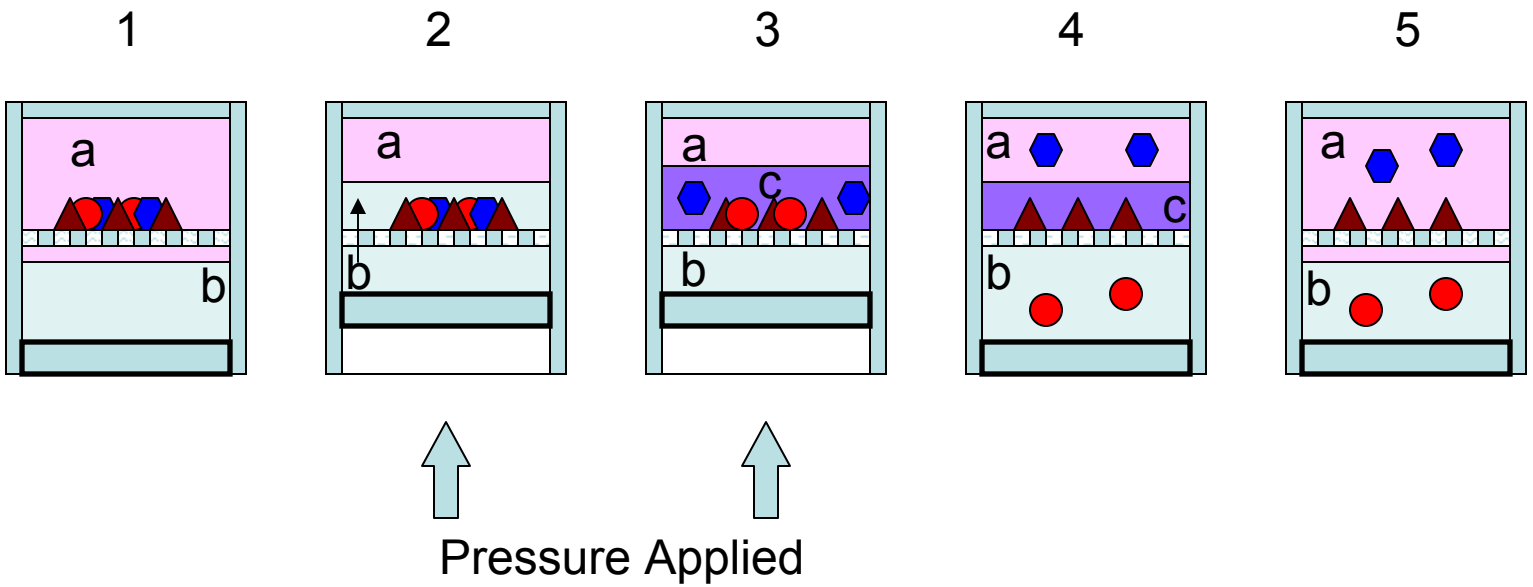
- Proteomic investigations of adipose tissue may lead to discovery of new biomarkers of diabetes, obesity, other metabolic disorders and cancer.

- Adipocytes may contain up to 70% lipids by weight
- Small amount of detergent (1-5%) is sequestered into micelles
- Membrane proteins are captured by micelles or remaining lipid phase

- Cavitation and shearing homogenizers promote formation of emulsion
- Dounce homogenizers, bead beaters: sample loss on the surfaces

PCT-mediated liquid-liquid extraction

(*patent pending*)



ProteoSolve$_{LRS}$

PCT Dependent Detergent-Free Extraction of Proteins from Lipid-Rich Samples

A Dissolution Reagent

B Partitioning Reagent

C Precipitation Reagent

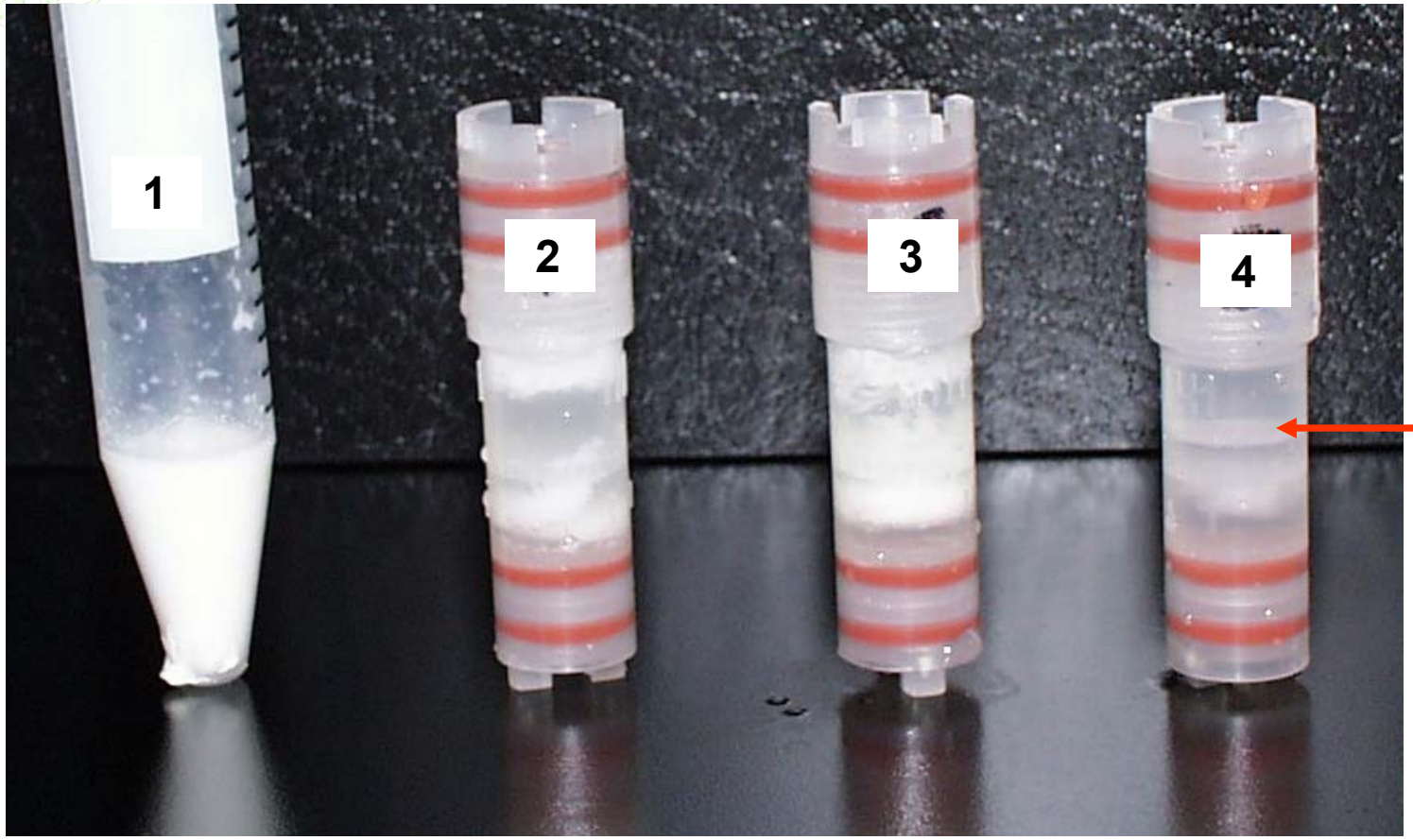
12 PULSE Tubes™

2007 Frost and Sullivan Innovative Product Award

PBI Pressure BioSciences Inc.

ProteoSolve$_{LRS}$ kit performance

200 mg of porcine adipose tissue per tube



Sonication 2% SDS

PBS

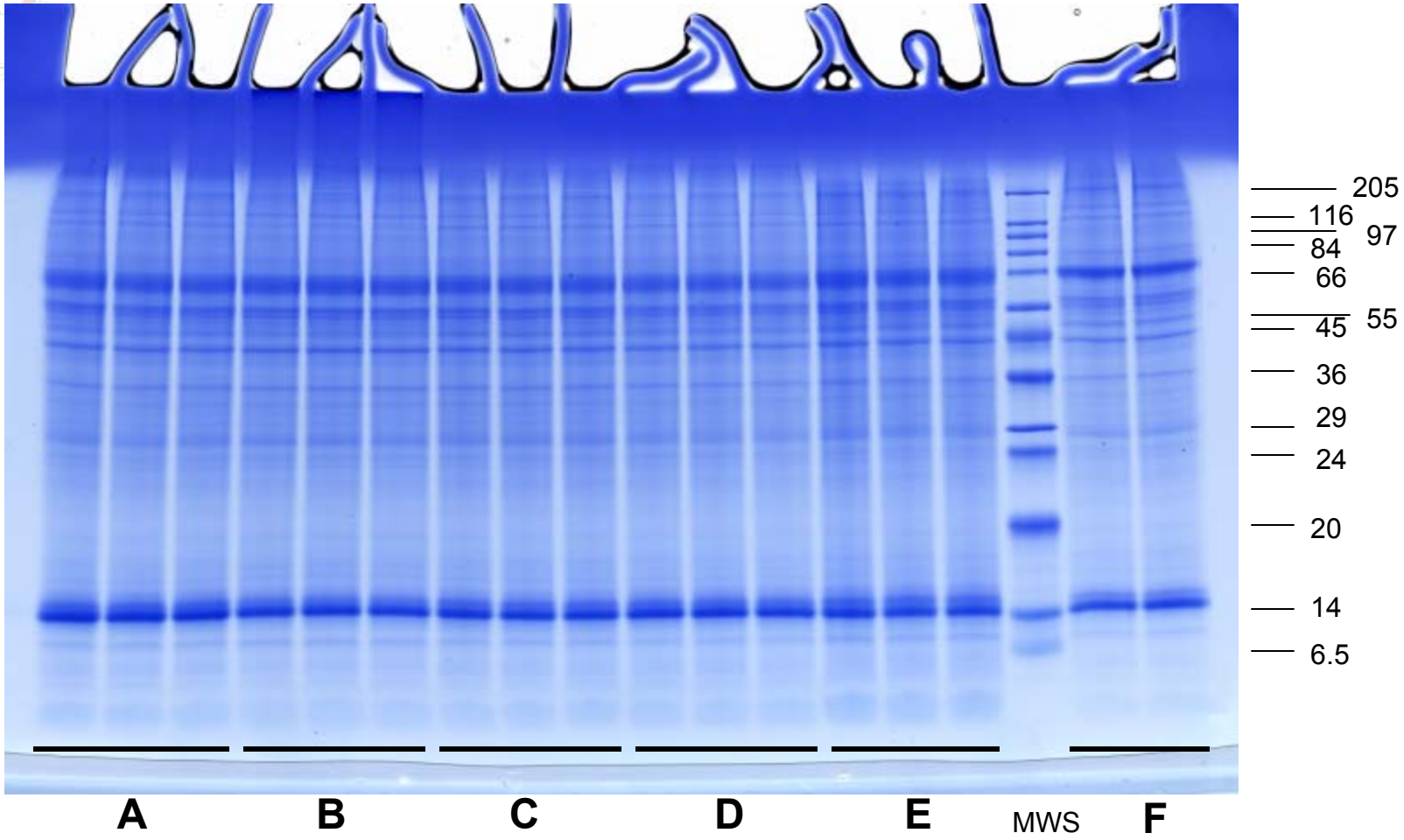
2% SDS

ProteoSolve$_{LRS}$

PBI Pressure BioSciences Inc.

ProteoSolve$_{LRS}$ kit reproducibility

Murine Abdominal Fat Pad Protein Extraction



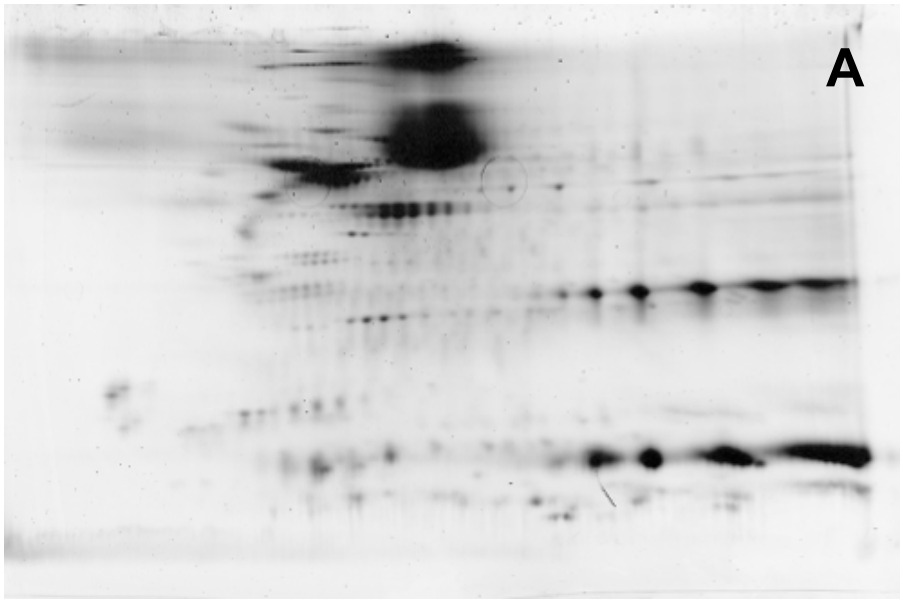
Six 200 mg pieces of tissue (A-F above) were processed in parallel, and loaded in triplicate onto 8-16% gradient SDS-PAGE gel.

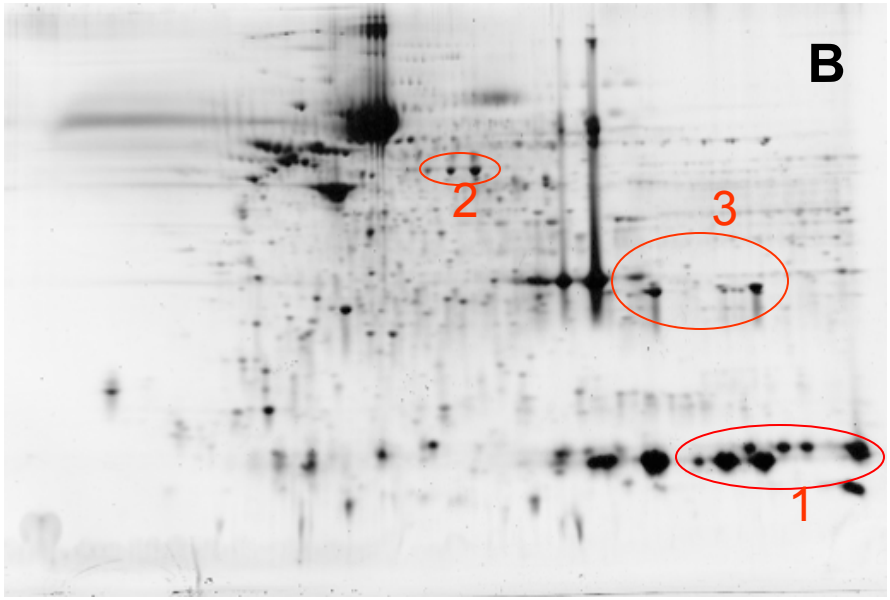
PBI Pressure BioSciences Inc.

Extraction of protein from adipose tissue

IEF sample buffer

ProteoSolve$_{LRS}$





Proteins from the identical samples of murine adipose tissue extracted by PCT using a traditional detergent-based buffer (9M urea, 4% CHAPS, pH=8) *versus* a detergent-free ProteoSolve LRS kit based on a combination of organic solvents [B].

Proposed ProteoSolve SB workflow

Place sample into PULSE Tube. Add Reagent A +/- Reagent B.
Vortex 10-20 seconds

↓

Place into Barocycler and run program

↓

Remove from Barocycler and Vortex 10-20 seconds.

↓

Transfer entire contents of PULSE tube to centrifuge tube and spin at ~12,000g for 10 min.

Nucleic Acids

Solid material contains RNA, DNA and some DNA-associated proteins.

↓

Dissolve pellet material in appropriate reagent for DNA or RNA isolation.

↓

Vortex sample and proceed with isolation according to manufacturer's protocol. No liquid nitrogen grinding or other tissue disruption is necessary.

Lipids

Upper non-polar phase contains extracted lipids.

↓

GC-MS,
LC-MS,
MALDI/TOF
TLC, NMR

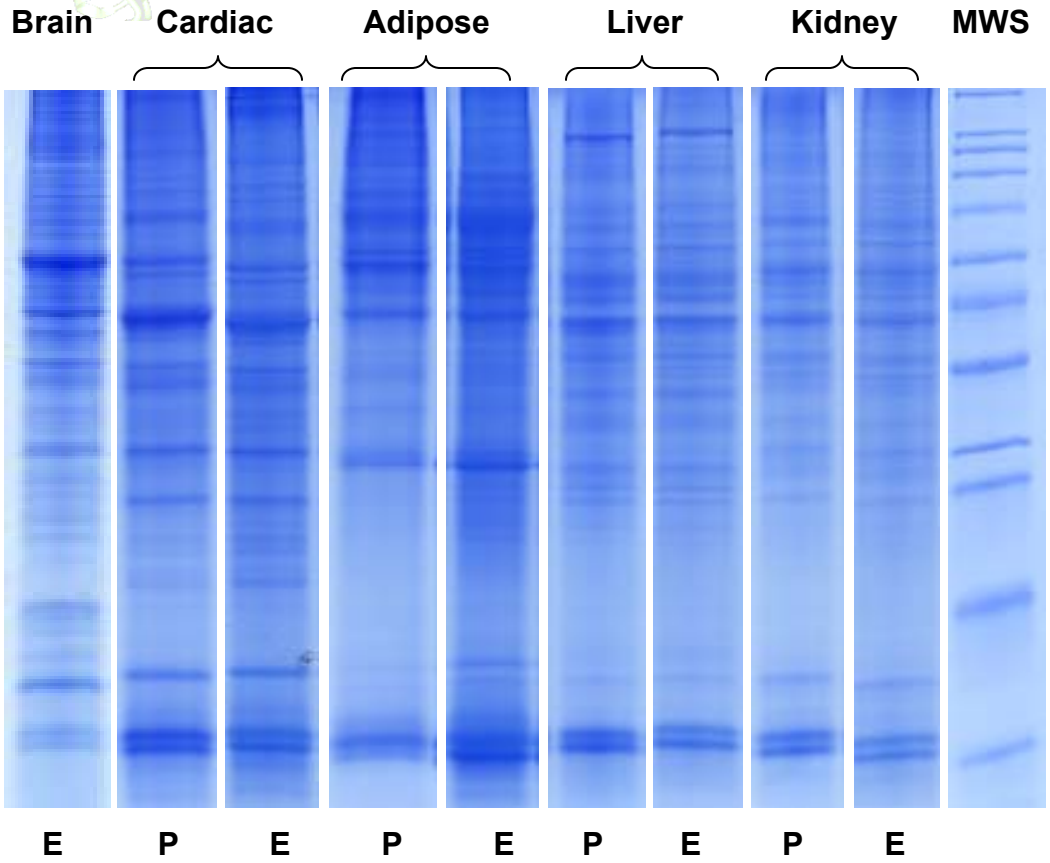
Proteins

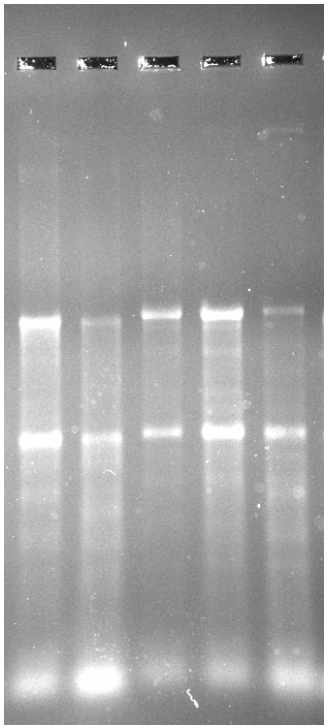
Lower liquid phase contains dissolved sample proteins. Remove solvent by evaporation or precipitation.

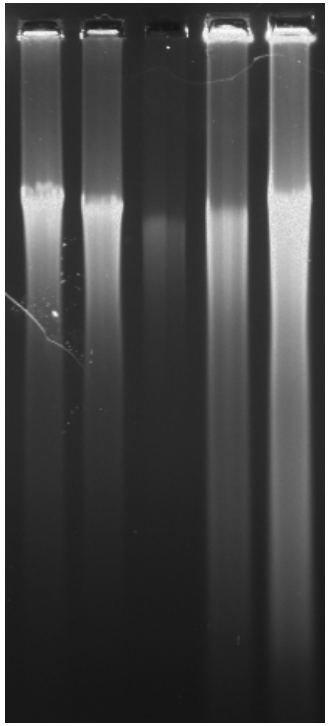
↓

SDS-PAGE, 2D PAGE,
Western blotting, LC-MS/MS,
MALDI-TOF, etc...

Protein, RNA and DNA Recovery From Rat Tissues



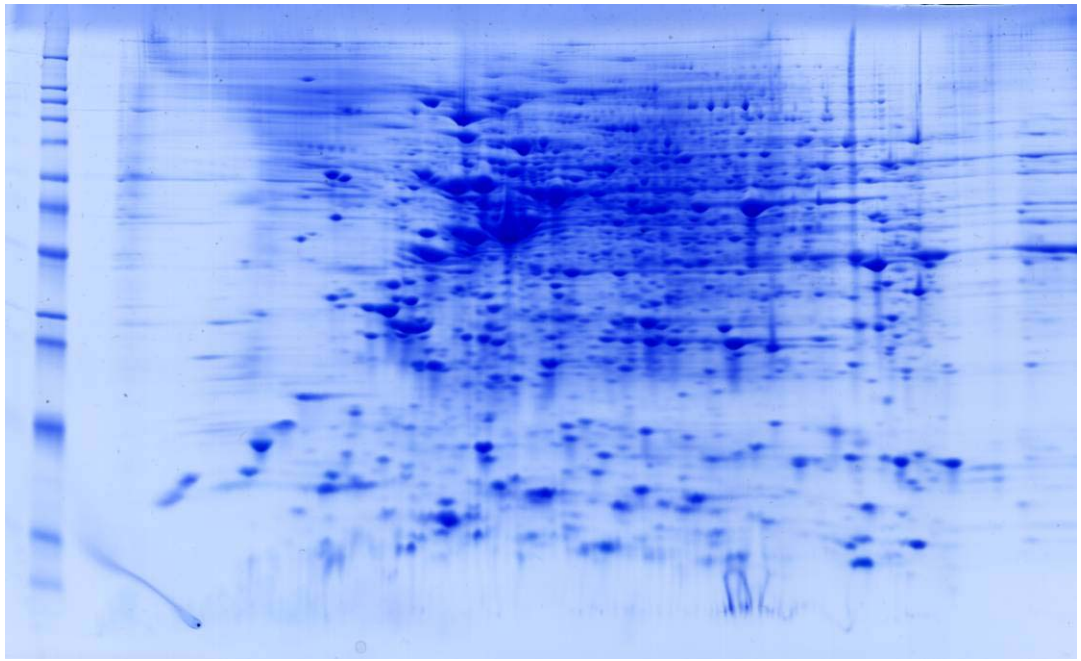




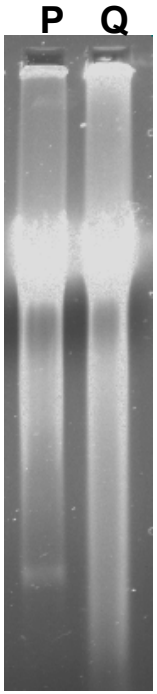
Solvent removed by: E – evaporation; P - precipitation

PBI Pressure BioSciences Inc.

Protein and DNA Recovery, PC12 cells

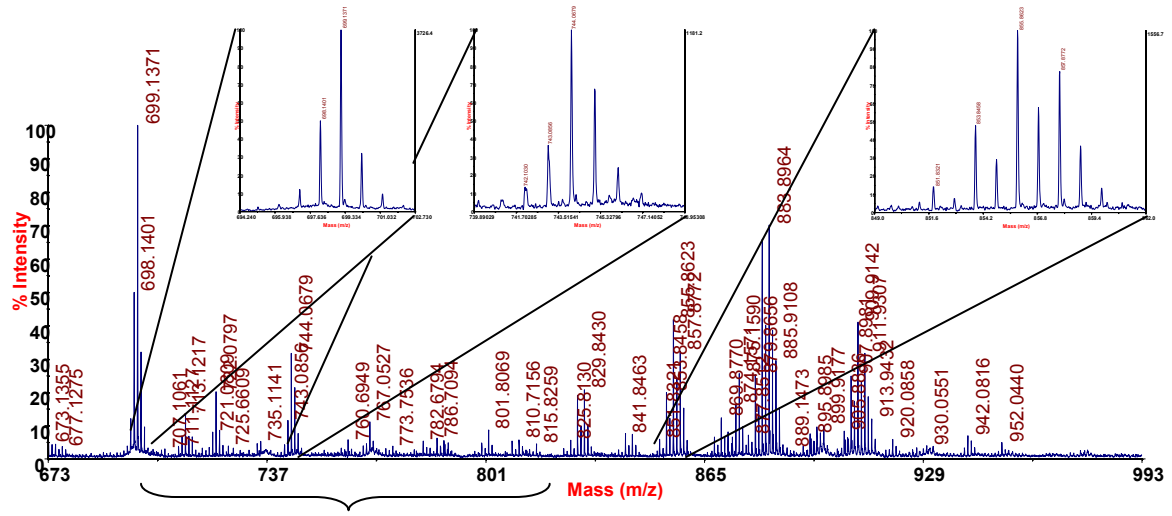


Protein

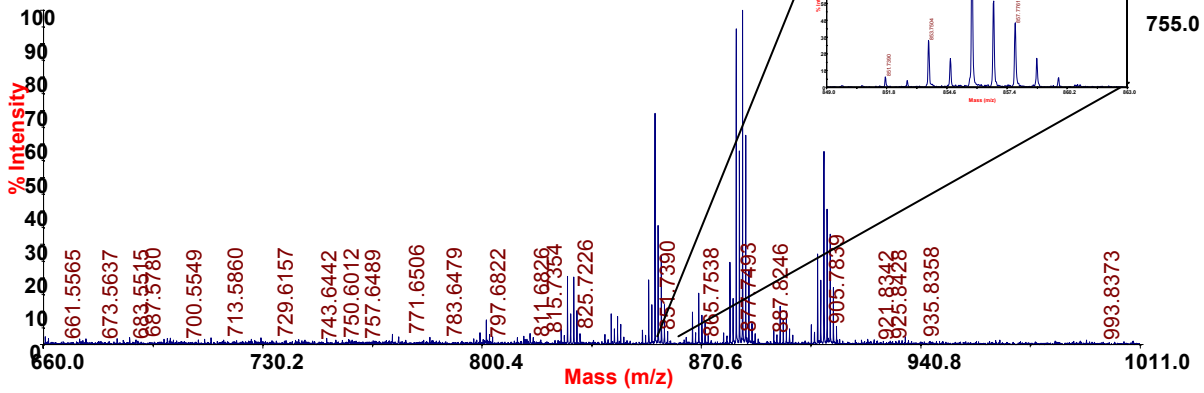


DNA

Direct Lipid Profiling by MALDI-TOF MS



Rat Brain Phospholipids

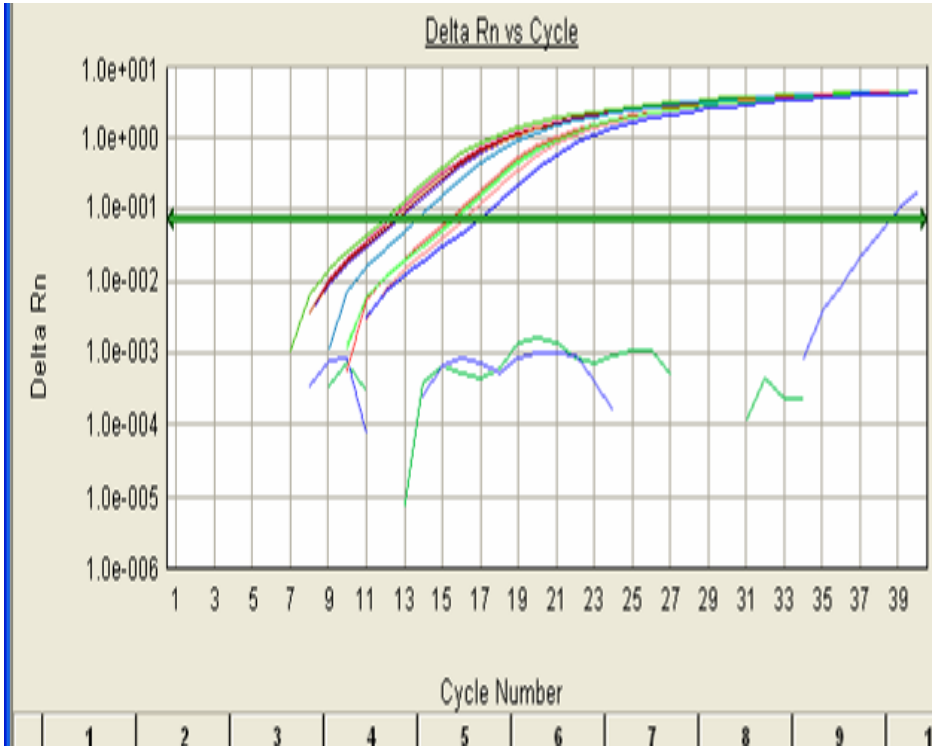


Beef pericardial fat

Triacylglycerides

PBI Pressure BioSciences Inc.

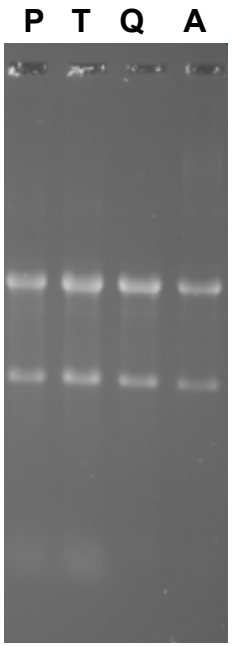
RNA extracted in ProteoSolve$_{LRS}$ is compatible with RT-PCR amplification

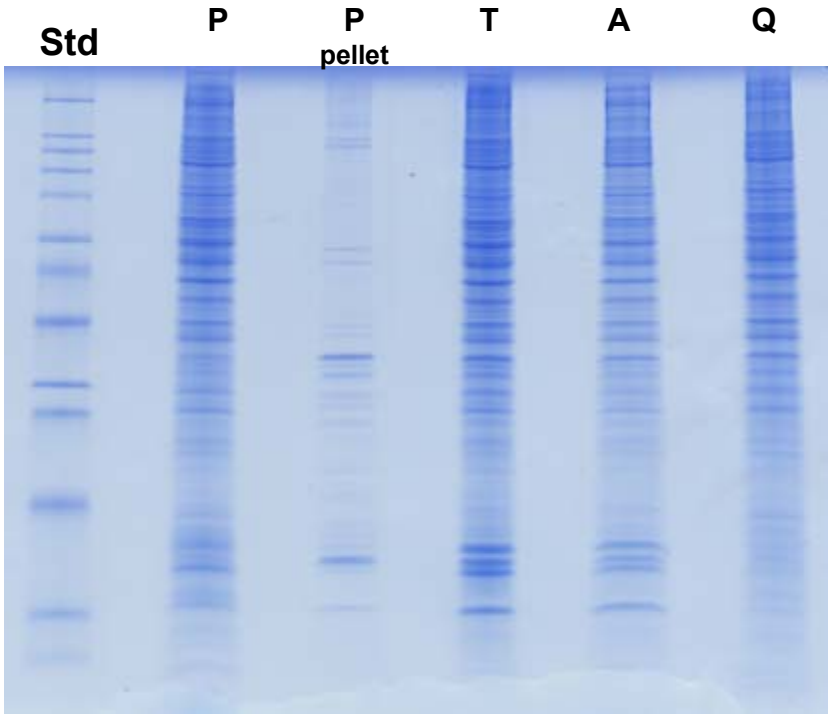


RNA recovery from 10^6 PC12 cells- Quantification by real-time RT-PCR using primers for rat ß-Actin.

Protein and RNA Recovery, PC12 cells

Comparison of 4 commercially available kits.



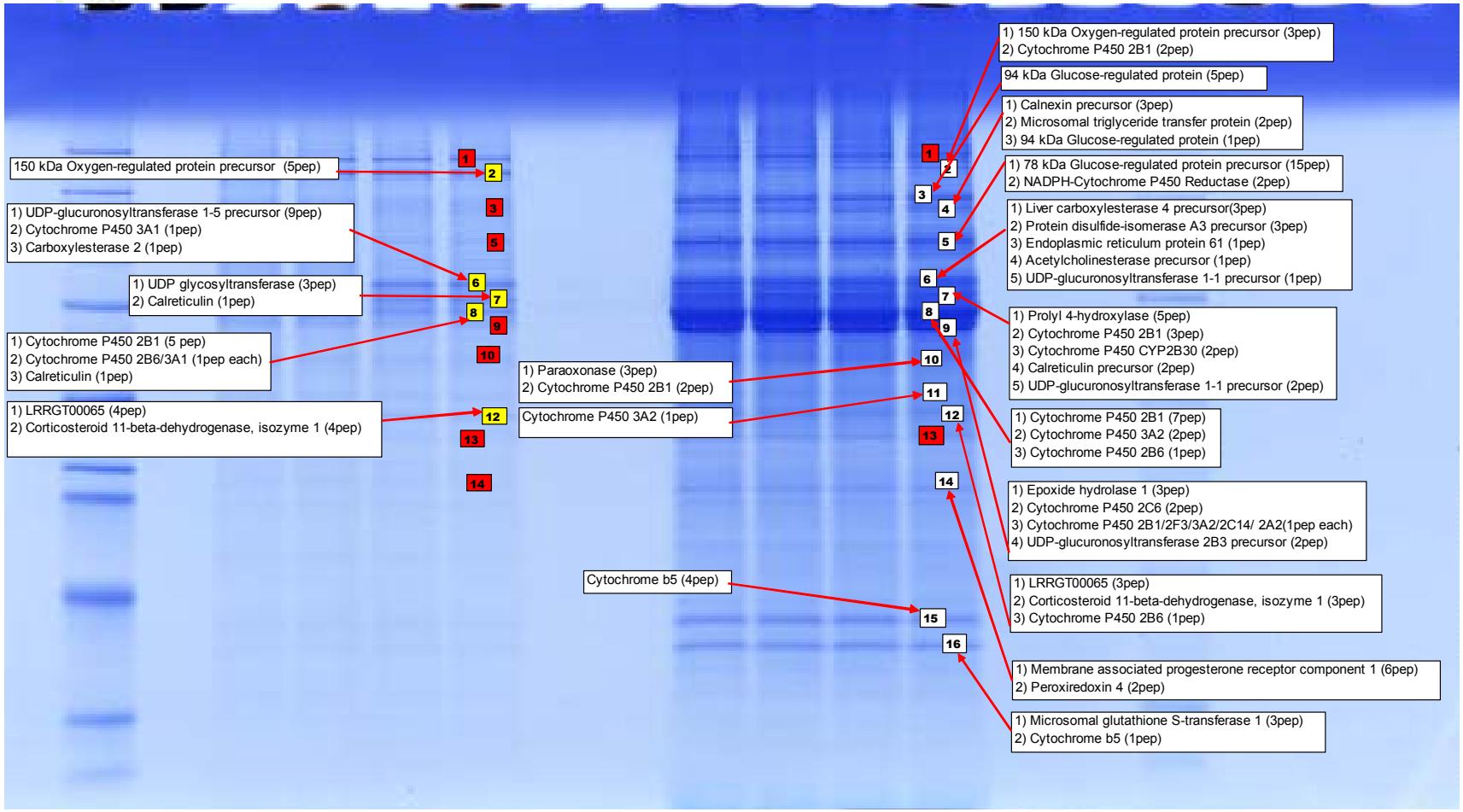


RNA recovery:
ProteoSolve$_{LRS}$: 11.9 µg total RNA
Ambion: 5.3 µg total RNA
Qiagen: 11.4 µg total RNA
Trizol: 16.2 µg total RNA

A: Ambion PARIS kit
Q: Qiagen Allprep kit
T: Trizol
P: ProteoSolve$_{LRS}$

Staring material for each sample was 10^6 PC12 cells.

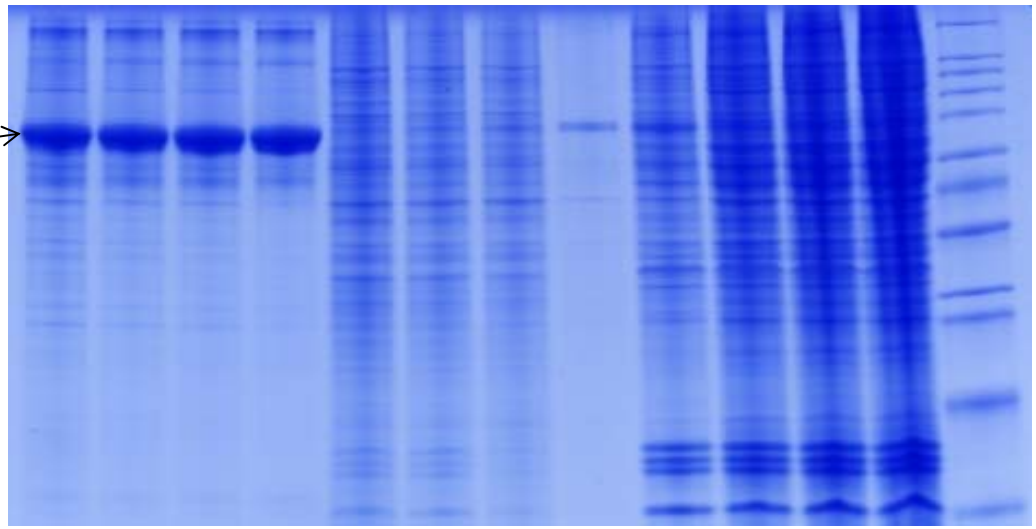
Fractionation of Rat Microsomal Proteins using Proteosolve$_{LRS}$



Data courtesy Dr. Michail Alterman, CBER, FDA

PBI Pressure BioSciences Inc.

Extraction of Mitochondria from PC12 Cells. Optimization of PCT Conditions.

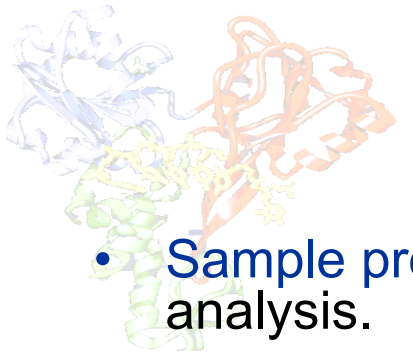


25 15 5 0 25 15 5 0 25 15 5 0

Cytosolic fraction | Mitochondrial fraction | First fraction

PCT at indicated pressure, 15 cycles (30 sec up, 20 sec down)
MIB (HEPES, BSA, Sucrose), differential centrifugation

PBI Pressure BioSciences Inc.



Conclusions:

- Sample preparation frequently present a bottleneck in biomarker analysis.
- Pressure cycling technology is applicable to a wide variety of applications, including initial steps of sample preparation for genomics and proteomics.
- PCT should be considered as an orthogonal extraction technique, not just homogenization or cell disruption method.
- PCT acts directly on the lipid bilayers, micelles and emulsions, promoting phase separation in poorly miscible solvent systems.
- ProteoSolve$_{LRS}$ kit combined with PCT allows nearly complete tissue dissolution and fractionation of tissue-derived molecules.
- Barocycler system provides several advantages over conventional extraction methods, including reproducibility, safety, convenience, speed, automation and precise control over the process.

Acknowledgements:

HSPS

Gökhan Hotamisligil
Alexander Ivanov

BIDMC

Cesario Bianchi
Rick Clements

Barbara Corkey
Catherine Costello

Jennifer Van Eyk

HSDM

Winston Kuo
Cortino Sukotjo

Ric Schumacher
Nathan Lawrence
Edmund Ting
Vera Gross
Greta Carlson
Gary Smejkal
Ada Kwan
Jim Behnke
Feng Tao
Chunqin Li

James Willett
Emmanuel Petricoin
Lance Liotta
Valerie Calvert

Bruce Kristal

Lou Tisa
Kelley Thomas
Deena Small