

Tissue Fractionation by Hydrostatic Pressure Cycling: The Unified Sample Preparation Technique for Systems Biology Studies

Vera Gross¹, Greta Carlson¹, Gary Smejkal¹, Ada Kwan¹, Emily Freeman², Alexander R. Ivanov² and Alexander Lazarev¹

¹ Pressure BioSciences, Inc., West Bridgewater, MA ² Harvard School of Public Health, Boston, MA

Corresponding author: Vera Gross, Ph.D. Pressure BioSciences, Inc. 6 Gill St., Woburn, MA 01801; vgross@pressurebiosciences.com

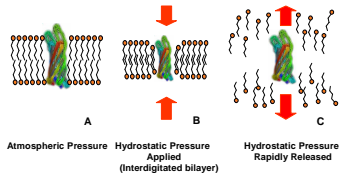


Introduction

Systems biology studies are gaining momentum driven by success in genomics, transcription profiling, proteomics, and rapidly emerging metabolomic technologies. While powerful and sensitive methods are available for the analysis of nucleic acids, proteins and small molecules, major bottlenecks arise from the limitations of current sample preparation techniques. We have developed a detergent-free sample preparation approach which allows concurrent isolation and fractionation of protein, DNA, RNA and lipids from cells and tissues. This novel method relies on a synergistic combination of cell disruption by alternating hydrostatic pressure (Pressure Cycling Technology, or PCT) and optimized reagents that dissolve and partition distinct classes of molecules into separate fractions. Previously we reported the performance of the PCT-driven detergent-free protein isolation system (1), which laid the foundation for the development of the ProteoSolve_{RS} reagent kit. In the current study we present the expansion of the ProteoSolve_{RS} strategy for rapid simultaneous isolation of DNA, RNA, proteins and lipids from individual samples. Gel electrophoresis and real-time RT-PCR confirm that nearly quantitative recovery of intact genomic DNA and high yields of intact RNA are obtained using this novel technique. Additionally, high yields of proteins and lipids are obtained from the same sample for proteomic analysis. The results have been highly reproducible and several protein species more efficiently extracted by the new method have been identified by in-gel tryptic digestion and LC-MS/MS. We also report direct analysis of the lipid fractions using MALDI-TOF mass spectrometry.

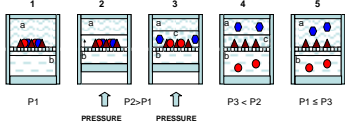
Pressure-Mediated Extraction: Principle and Mechanism

PCT-mediated extraction relies on the use of rapidly alternating levels of hydrostatic pressure from atmospheric to high (up to 35,000 psi, or 240 MPa) and back to ambient to destabilize molecular interactions in samples of cells and tissues. High hydrostatic pressure acts only on the compressible constituents of the sample.

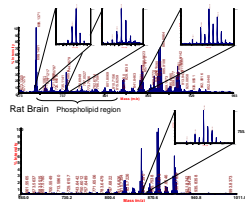
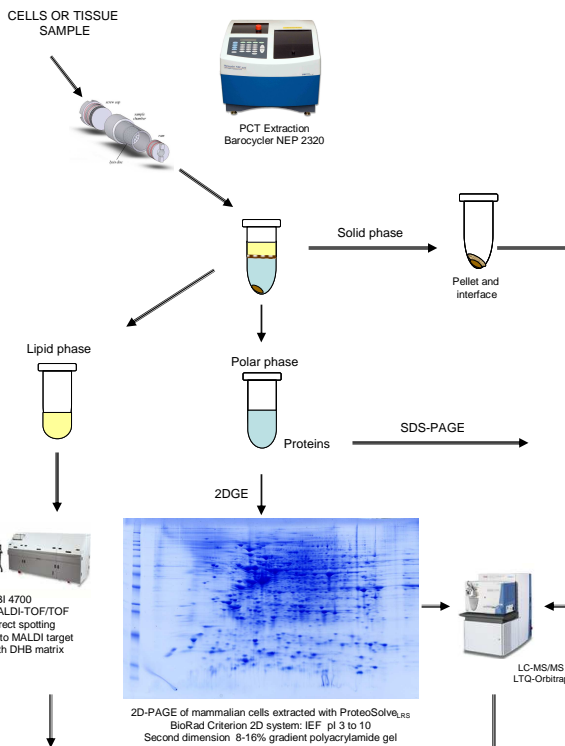


Lipids, the most compressible sample components, are affected most, and dissociate upon depressurization. Thus, selective energy distribution results in destabilization of molecular interactions in the lipid bilayers and other cellular components, but not in the breakage of covalent bonds.

Proteins and lipids are solubilized under pressure and are maintained in solution by amphiphatic organic solvents, such as fluorinated alcohols (Hexafluoroisopropanol is a key ingredient of Reagent A of the ProteoSolve_{RS} kit), while nucleic acids tend to precipitate upon depressurization.

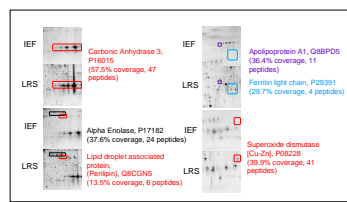


PCT-assisted liquid-liquid extraction (patent pending) uses high hydrostatic pressure to alter solvation energy and solubility of various compounds. Properly selected poorly miscible phases interact under pressure such that the phase boundary presents less of a barrier for partitioning of molecules between solvent phases. As a result, partitioning occurs in the entire volume of the vessel, rather than at the interface between solvent phases.



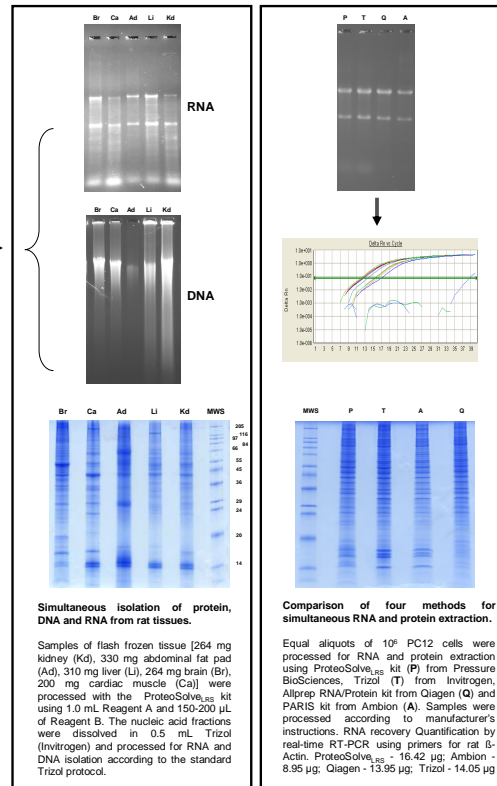
Phospholipid and triglyceride profiles of lipid phase by MALDI-TOF.

Aliquots of lipid phase (0.5 µL) were spotted directly onto a wet 2 µL droplet of 0.5M 2,5-dihydroxybenzoic acid (DHB) matrix solution on the MALDI target. Data collected in positive ionization mode on an ABI 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA)



Differentially extracted murine adipose tissue proteins identified by LC-MS/MS. IEF – extraction with 7M urea 2M thiourea 4% CHAPS. LRS – Extraction using ProteoSolve_{RS} reagents.

Tryptic protein digests were separated on a 12 cm C₁₈ capillary column and analyzed on LTO-Orbitrap (Thermo Fisher Scientific). Data were analyzed on a Sage-N Research Sorcerer System. Identification results were filtered and validated using Protein Prophet and Peptide Prophet platforms.



Simultaneous isolation of protein, DNA and RNA from rat tissues.

Samples of flash frozen tissue [264 mg kidney (Kd), 330 mg abdominal fat pad (Ad), 310 mg liver (Li), 264 mg brain (Br), 200 mg cardiac muscle (Ca)] were processed with the ProteoSolve_{RS} kit using 1.0 mL Reagent A and 150-200 µL of Reagent B. The nucleic acid fractions were dissolved in 0.5 mL Trizol (Invitrogen) and processed for RNA and DNA isolation according to the standard Trizol protocol.

Comparison of four methods for simultaneous RNA and protein extraction.

Equal aliquots of 10⁶ PC12 cells were processed for RNA and protein extraction using ProteoSolve_{RS} kit (P) from Pressure BioSciences, Trizol (T) from Invitrogen, Allprep RNA/Protein kit from Qiagen (Q) and PARIS kit from Ambion (A). Samples were processed according to manufacturer's instructions. RNA recovery Quantification by real-time RT-PCR using primers for rat β-Actin. ProteoSolve_{RS} - 16.42 µg; Ambion - 8.95 µg; Qiagen - 13.95 µg; Trizol - 14.05 µg

Materials and Methods

Cell and tissue disruption by pressure cycling technology (PCT):

Twenty pressure cycles were applied to each sample using a Barocycler (model NEP3229 or NEP2320 Pressure BioSciences, West Bridgewater, MA). Each pressure cycle consisted of 20 seconds at high pressure (35,000 psi) followed by 20 seconds at low (atmospheric) pressure.

Protein, lipid, RNA and DNA extraction using the ProteoSolve_{RS} kit: 50-300 mg of tissue were combined in a PULSE Tube with 0.9-1.1 mL ProteoSolve_{RS} reagent A and brought to 1.4 mL using Reagent B. For cell culture, 1-5 x 10⁶ pelleted cells were suspended in 0.9-1.0 mL ProteoSolve_{RS} Reagent A, transferred to PULSE Tubes and brought up to 1.4 mL with Reagent B. All samples were vortexed briefly before and after pressure cycling.

After pressure cycling, samples were transferred to centrifuge tubes and centrifuged for 15 minutes to separate the phases and pellet the nucleic acid fraction. The upper phase containing lipids was transferred to a clean tube for subsequent lipid analysis by MALDI-TOF. The protein-containing lower organic phase was transferred to a clean tube for subsequent protein analysis by SDS-PAGE or 2D PAGE. The pellet and any solid interface layer, containing the bulk of the sample's DNA and RNA, were then processed for nucleic acid extraction.

Results and Discussion

Pressure-mediated extraction in combination with the unique chemistry of the ProteoSolve_{RS} kit has been demonstrated to be an efficient method to extract proteins from lipid-rich samples such as adipose and brain tissue (1). Here we expand the utility of this sample preparation system, by demonstrating that four major components of biological samples; proteins, lipids, RNA and DNA, can all be easily and efficiently isolated from a single sample of cultured cells or tissue. In addition we confirm that the RNA recovery is comparable to, or better than, that achieved using three currently available kits and reagents. Examination of the individual fractions after pressure mediated extraction with the ProteoSolve_{RS} kit has demonstrated the following: **A)** The lipid fraction contains no additional extractable protein. **B)** The polar solvent phase contains the bulk of the sample proteins, and only traces of RNA and DNA. **C)** The pellet and the interface contain the bulk of the RNA and DNA, with the pellet containing ~70% and the interface containing ~20%. Additionally, preliminary results indicate that the RNA in the pellet/interface fraction is stable for extended periods of time at -20°.

The new expanded application of the ProteoSolve_{RS} kit provides efficient simultaneous extraction of proteins, lipids and nucleic acids from samples that are precious or scarce, such as human or wild animal biopsy tissue or samples that are difficult to duplicate, such as small cell populations like early stem cell cultures. Another advantage is in more accurate analysis of non-homogenous samples. Since splitting samples for separate protein, lipid and nucleic acid analyses is not necessary, artifacts due to uneven distribution of components in the sample are avoided.

The new method is advantageous not only for small and precious samples, but also for larger samples where a single convenient method for purification of multiple components is desired. Since the ProteoSolve_{RS} kit is easily scalable for larger samples, it has many advantages over other currently available methods. In many applications, ProteoSolve_{RS} can be used with sample-to-solvent ratios as high as 300 mg per mL, and possibly higher in some cases. After ProteoSolve_{RS} extraction of the bulk of the sample's proteins and lipids, the nucleic acid-enriched fraction can be brought up in a relatively small volume of reagent such as Trizol, thus allowing for more efficient RNA precipitation from large samples that contain little RNA (like soil, yogurt, skin, etc).

ProteoSolve_{RS} is compatible with most common downstream applications. Since proteins can be dried or precipitated out of the detergent-free solvent, no extensive clean-up, washing or concentration of proteins is necessary. Protein pellets can be dissolved directly in a buffer of choice and subjected to further analyses.

The lipid fractions are available for direct profiling by mass spectrometry or for separation and enzymatic digestion for structural analysis.

The DNA/RNA fraction is compatible with many commonly used reagents and kits for isolation of DNA and/or RNA, such as Trizol and the Qiagen DNeasy and RNeasy kits.

Summary

The combination of sample disruption by PCT and extraction in ProteoSolve_{RS} can be used to efficiently and easily extract lipids, proteins, RNA and DNA from many types of samples without the need for multiple replicates, tissue homogenizers or sonicators, liquid nitrogen grinding, or other inconvenient and inconsistent sample disruption methods.

Reference

1. Lazarev A., et al. Proteomic Analysis of Adipose Tissue Using Detergent-Free Protein Extraction by Pressure Cycling Technology (PCT) and High Resolution Tandem Mass Spectrometry. (2007) Proceedings of the 55th ASMS Conference on Mass Spectrometry and Allied Topics, Indianapolis, IN. Journal of The American Society for Mass Spectrometry, 18.(9).