# Novel efficient alternatives for sample preparation techniques crucial for functional proteomics

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## Abstract

Good control, efficiency, and reproducibility of protein extraction from cells and tissues are essential aspects for diverse biological and basic research applications. Effective and specific enzymatic digestion of proteins prior to mass spectrometry (MS) analysis is one of the fundamental techniques most commonly used in any proteomics laboratory. However, neither routine procedures for cell and tissue lysis nor for in-solution and in-gel protein digestion have been significantly altered in common practice for over a decade. Here we have tested and optimized several alternative techniques for preparing lysates of mammalian cells, as well as in-solution and in-gel enzymatic digestion compatible with downstream qualitative and quantitative MS-based proteomics applications.

## Introduction

There is a need in both minimizing time of enzymatic protein digestion, which frequently requires up to 24 hours, while maximizing efficiency of digestion without sacrificing results and digestion specificity. For this purpose we have analyzed the role of hydrostatic pressure as an alternative thermodynamic parameter to control enzyme kinetics. We have compared the use of conventional protocols ("standard") and those utilizing pressure-assistance. This work employed alternating hydrostatic pressure (Pressure Cycling Technology, or PCT) providing pressures of up to 35 kpsi shown to be efficient for proteolytic digestion, disruption of cells, micelles and membrane fragments, as well as for efficient protein recovery from cultured cells and tissues [Gross V., et al., J Biomol Tech. 2008, 19(3); and López-Ferrer D, et al., J Proteome Res. 2008;7(8)]. Using high performance LC-MS analysis, we have tested the role of pressurization, organic solvents, chaotropic agents, reducing reagents, enzyme/substrate ratios, temperature, and incubation time on efficiency, selectivity, and throughput of proteolytic digestion. The tests were performed using a mixture of protein standards, and the most effective conditions were applied to HepG2 cell lysates. The PCT was adapted for preparation of HepG2 lysates and protein identification results were compared to those acquired using cell lysis and optimized digestion conducted at atmospheric pressure. Application of PCT resulted in significant improvement of throughput and reproducibility of sample preparation for proteomic analyses. Superior extraction rate for cytosolic, nuclear, mitochondrial, ribosomal, and membrane-associated proteins, as well as for proteins related to vital GO processes (RNA splicing, chromatin assembly, organelle organization among others), were observed in pressure-assisted and organic solvent-assisted sample preparation.

## Methods

### *1. General Sample Preparation and Analysis*

A 1 pmol/μL mixture of standard proteins ("Protein Mix") with varying molecular weights, isoelectric points, and number of amino acid residues [Table 1] was used for examination of various digestion protocols, as shown in Table 2. Each protocol used 5 pmol of protein and was done in triplicate. Sample digestion was done either in an incubated shaker or in a barocycler NEP2320 (Pressure BioSciences, MA). Each sample was analyzed twice using NanoLC-2D HPLC system (Eksigent, CA) and LTQ Orbitrap (ThermoElectron, CA). MS data was analyzed with the SEQUEST-Sorcerer algorithm on the Sorcerer IDA2 (SageN Research, CA). The general workflow is shown in Figure 1.

### *2. Development of an efficient cell lysis procedure for proteomic studies:*

HepG2 cells were grown in MEM supplemented with 10% FBS in three separate 10-cm dishes to 80% confluence. The cells in each plate were washed with 1xPBS and scraped separately. 1,1,1,3,3,3 – hexafluoro-2-propanol (HFIP), an organic solvent, was added to one of the three cell suspensions to the concentration of 30%. In this pilot experiment, three different methods of cell lysate preparation were evaluated. The first method used only sonication to solubilize cellular proteins ("conventional"); the second method involved sonication and membrane disruption / protein solubilization using HFIP ("HFIP-assisted"); the third method used PCT to break up the cells ("PCT-assisted"). Pressure cycling was used to simultaneously homogenize the sample, to facilitate the dissolution of cells, micelles and membrane fragments, and to increase the efficiency of hydrophobic protein recovery. After digestion and analysis by LC/MS, the number and properties of proteins identified in each lysate were determined and compared.

| Protein Description | SwissProt accession # | MW (Da) | PI | AAs |
|---|---|---|---|---|
| Ubiquitin human | P62988 | 9382 | 7.30 | 82 |
| Myoglobin equine | P68082 | 16941 | 7.36 | 154 |
| Cytochrome C bovine | P62894 | 11565 | 9.52 | 104 |
| β -Casein bovine | P02666 | 23568 | 5.13 | 209 |
| Bovine serum albumin | P02769 | 66390 | 5.60 | 583 |
| α1- Casein | P02662 | 22960 | 4.91 | 199 |
| α2- Casein | P02663 | 24333 | 8.34 | 207 |
| κ -Casein | P02668 | 18963 | 5.93 | 169 |

Table 1: **Proteins of "Protein Mix".** Proteins used for method testing and optimization.

## Results and Discussion

### 1. Optimization of Enzymatic Protein Digestion

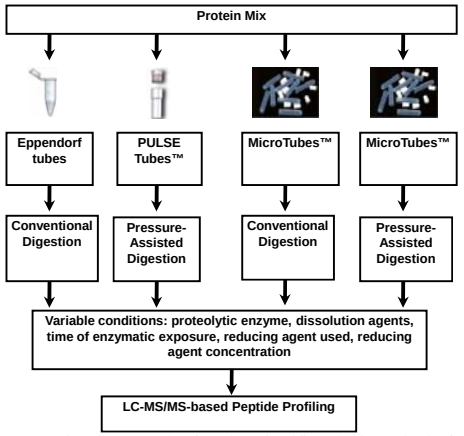



**Figure 1.** Schematic representation of experimental workflow using the Protein Mix of standards. Three types of vessels were employed to test the variables of pressure, solvents, and reduction components in comparison to more conventional methods. Those vessels were 1.4 ml PULSE tubes, 1.5 ml Eppendorfs, and 100 uL MicroTubes.

### 1.1. Optimization of Enzymatic Protein Digestion. Time and Environment.

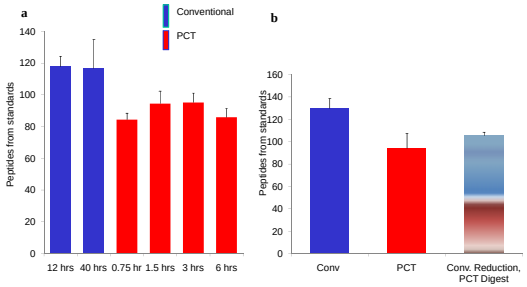



**Figure 2.** Protocol optimization using Protein Mix in Eppendorf and PULSE tubes.
(**A**) **Time of digestion** and peptide profiling results with and without PCT. The number of unique standard peptides detected in LC-MS/MS analyses of digests at different duration of enzymatic digestion. The Protein Mix was digest in Eppendorf tubes (conventional protocols, BLUE) and in 1.4 ml PULSE Tubes™ (PCT protocols, RED). Each experiment was performed in six replicates (n=6).
(**B**) **Reduction environment** in peptide identification yield. Reduction and digestion stages were performed at atmospheric pressure (conventional), using PCT, or a combination (n=6).

### 1.2. Optimization of Protein Digestion. Eppendorf and PULSE Tubes.

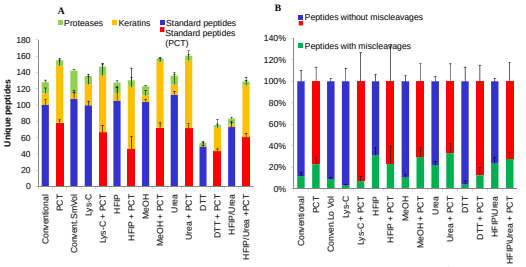



**Figure 3. Protocol optimization using various solubilizing agents in Eppendorf and PULSE tubes.** BLUE – atmospheric pressure, RED - **PCT.** (**A**) Digestion efficiency assessed using the number of identified unique peptides in Protein Mix using Eppendorf and PULSE tubes (n=6).
(**B**) Rate of miscleavages in identification results for unique standard peptides resulted from digestion of Protein Mix using Eppendorf and PULSE tubes (n=6).

### 1.3. Optimization of Protein Digestion. MicroTubes.

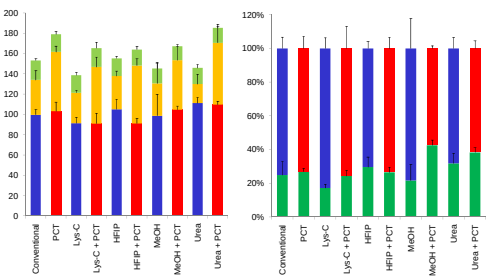



**Figure 4. Protocol optimization using Protein Mix in MicroTubes.** BLUE – atmospheric pressure, RED - **PCT.**
(**A**) Digestion efficiency assessed using the number of identified unique peptides in Protein Mix using Eppendorf and PULSE tubes (n=6).
(**B**) Rate of miscleavages in identification results for unique standard peptides resulted from digestion of Protein Mix using Eppendorf and PULSE tubes (n=6).

### 1.4. Optimization of Enzymatic Protein Digestion. Reproducibility.

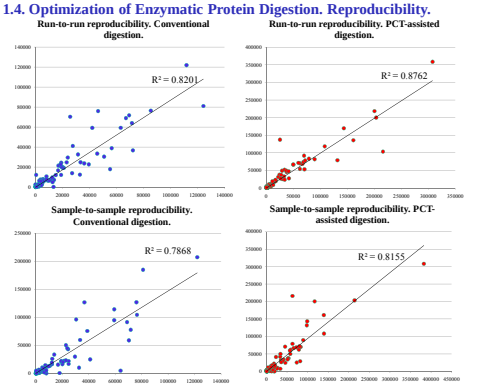



**Figure 5: Run-to-run and sample-to-sample reproducibility for peptide peak volumes in LC-MS analyses of tryptic digests resulted from conventional and PCT-assisted protocols.**

| Run-To-Run, R^2 | LC-MS Runs 1 and 2 | LC-MS Runs 2 and 3 | LC-MS Runs 1 and 3 | Mean | CV |
|---|---|---|---|---|---|
| Conventional Digestion | 0.834 | 0.638 | 0.82 | 0.764 | 14% |
| PCT Digestion | 0.876 | 0.856 | 0.893 | 0.875 | 2% |
| **Sample-To-Sample, R^2** | Samples 1 and 2 | Samples 2 and 3 | Samples 1 and 3 | | |
| Conventional Digestion | 0.736 | 0.824 | 0.786 | 0.782 | 6% |
| PCT Digestion | 0.902 | 0.782 | 0.815 | 0.833 | 7% |

**Table 2: Correlation coefficients for quantitative peptide peak attributes.**

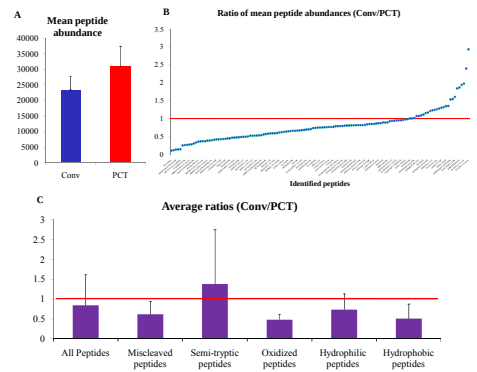
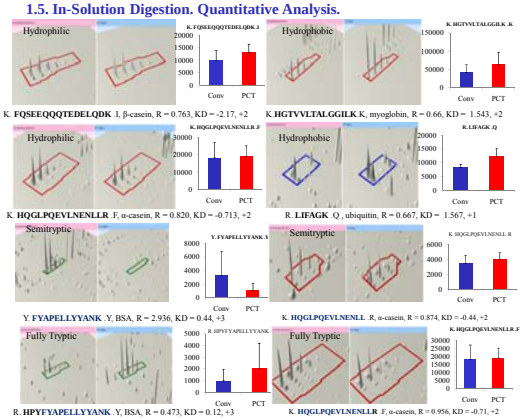



**Figure 6: Differential analysis of peptide abundance by label-free AMT LC-MS.**

### 1.5. In-Solution Digestion. Quantitative Analysis.




K. **FQSEEQQQTEDELQDK** .I, β-casein, R = 0.763, KD = -2.17, +2
K. **HGTVVLTALGGILK** .K, myoglobin, R = 0.66, KD = 1.543, +2
K. **HGQLPQEVLNENLLR** .F, α-casein, R = 0.820, KD = -0.713, +2
R. **LIFAGK** .Q , ubiquitin, R = 0.667, KD = 1.567, +1
Y. **FYAPELLYYANK** .Y, BSA, R = 2.936, KD = 0.44, +3
K. **HQGLPQEVLNENLL** .R, α-casein, R = 0.874, KD = -0.44, +2
R. **HPYFYAPELLYYANK** .Y, BSA, R = 0.473, KD = 0.12, +3
K. **HQGLPQEVLNENLLR** .F, α-casein, R = 0.956, KD = -0.71, +2

**Figure 7: Peptide abundance in protein digests resulted from PCT-assisted and conventional protocols.** Representative examples of hydrophilic and hydrophobic peptides (R-ratio Conv/PCT for mean peptide abundance; KD – Kyte-Doolittle hydrophobicity index; n=6).

### 2. Optimization of Cell Lysis Conditions. Pressure Assistance.

| | | | | | | |
|---|---|---|---|---|---|---|
| **Conventional Lysis** | + | | + | + | + | |
| **PCT-Assisted Lysis** | | + | | | | |
| **Conventional Digestion** | | | + | | + | |
| **PCT-Assisted Digestion** | | | | + | | + |
| **Organic Solvent Assisted Lysis** | | | | | | + |

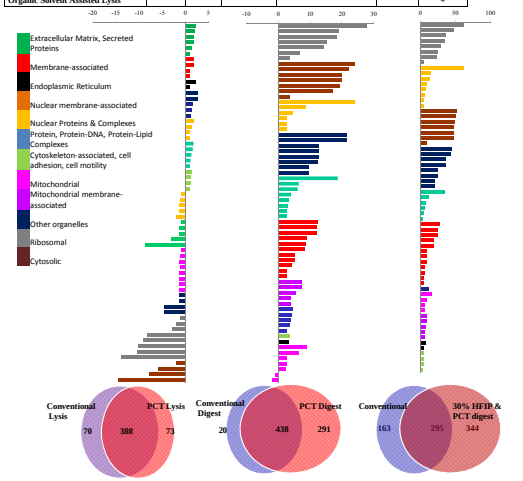



**Figure 9: Differential GO term localization analysis and Venn diagrams for protein LC-MS profiling data resulting from alternative lysis and digestion protocols applied to HepG2 cultured cells.**

## Conclusions:

Application of pressure assistance resulted in significant improvement of cell lysis and digestion procedures prior to proteomic profiling analyses. Specifically, pressure cycling assistance enabled
(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.

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