Hands-free Sample Homogenization and Protein Extraction from Small Tissue Biopsy Samples using Pressure Cycling Technology and PCT µPestle

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PBI Pressure BioSciences Inc.

Abstract

Efficient homogenization of small amount of samples, such as biopsy-level tissues, and reproducible protein extraction from these samples, remain outstanding technical challenges for protein analyses in clinical research, particularly in the coming era of precision medicine. We have recently presented an integrated method based on pressure cycling technology and pressure-resistant MicroTubes to efficiently perform tissue homogenization and protein extraction with minimal manual operation, assisted by the Barocycler.

In order to further enhance the efficiency of this method, here we present an improved extraction method which utilizes the PCT µPestle, a disposable mechanical tissue homogenizer that is incorporated directly into the MicroTube sample container. The container walls collapse around the insert under repeated pulses of high hydrostatic pressure, resulting in a homogenizer-like effect. Pressure cycling also acts directly on the sample, resulting in more efficient tissue lysis.

We optimized the pressure cycling conditions for µPestle, and further evaluated the performance of the optimized µPestle method using our conventional MicroCap method as a benchmark. Comparison of extraction yields from the two methods using mouse liver, heart, and brain tissues of different amounts (ranging from 0.5 mg to 13 mg) led to the same conclusion, that µPestle extracted significantly more proteins than the MicroCap method.

We further integrated the µPestle method into the PCT-based protein digestion and SWATH-MS analysis workflow [1], and found the optimized µPestle method produced 20-40% more peptides for various tissues and identified more biologically informative proteins after SWATH analysis when compared to the MicroCap method.

In conclusion, we have developed a hands-free method for efficient and reproducible tissue homogenization and protein extraction, and integrated it into the PCT-SWATH workflow which rapidly converts small amount of samples into a digital map representing the MS-measurable proteome.

Materials and Methods

Optimization of µPestle extraction conditions

Rat liver and heart muscle tissue (Pel-freeze) was washed in PBS to remove surface blood. Tissues pieces (0.5-1.5mg each) were blotted to remove buffer, individually weighed and homogenized in 30ul of lysis buffer (8M urea, 0.1M ammonium bicarbonate, with protease inhibitor cocktail). Pressure cycling was performed at the indicated pressure for the indicated number of cycles. Total protein concentration in the clarified homogenate was measured by BCA assay.

High pressure extraction and accelerated digestion

Tissue homogenization (mouse liver, heart, brain and human kidney) and extraction was carried out by pressure cycling at 45,000psi for 60 cycles in 8M urea, with brief sonication, using either the PCT µPestle or the "MicroCap" method [1]. Samples were then reduced/alkylated, and diluted for sequential digestion with Lys-c followed by Trypsin at 20,000 psi. Duplicate biological replicates and duplicate technical replicates were used.

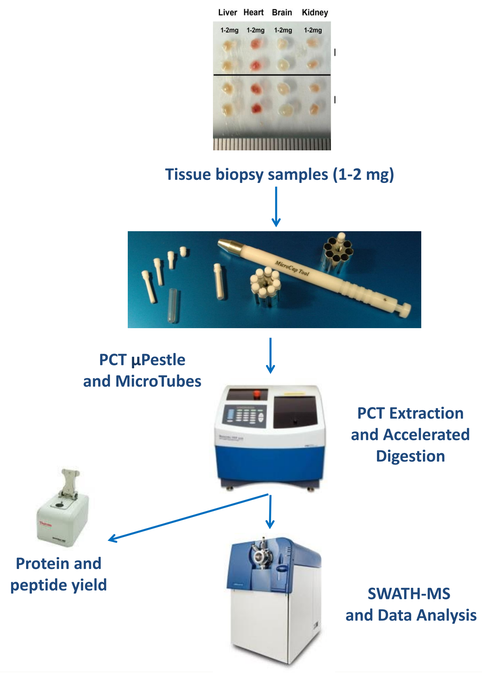
SWATH-MS and data analysis

0.6 µg of clean peptides were injected and then separated by an Eksigent 1D+ Nano liquid chromatography (LC) systems. SWATH data were acquired in a 5600 Triple-TOF mass spectrometer (AB Sciex, Concord, Canada) with an isolation width of 26 Th. Human and mouse libraries used in this study were reported by Guo et al [1] and Shao et al [2]. SWATH data was analyzed using OpenSWATH and the computation was performed using Brutus Cluster, the central resource for high-performance/high-throughput scientific computation at ETH Zurich.

Conclusions

Pressure cycling with PCT µPestle is a convenient, hands-off method for homogenization and extraction of very small tissue samples such as biopsies. The PCT MicroTubes offer an opportunity to integrate the homogenization and lysis of small tissue samples, with subsequent steps of the proteomic workflow. Reduction/alkylation and pressure-accelerated enzymatic digestion can be carried out in the same sample container, by replacing the µPestle with a regular MicroCap (accommodating 50, 100 or 150µl of sample volume) and diluting the sample to achieve the desired final concentration of proteins and urea. Moreover, sequential pressure-enhanced digestion using Lys-C and trypsin produces highly reproducible peptide profiles in a significantly shorter period of time compared to conventional protocols. By integrating this hands-free sample preparation approach into SWATH-MS, we have designed a new workflow that can be utilized in high-throughput and reproducible proteomic analyses for biomarker discovery and Precision medicine.

Pressure-Enhanced Protein Digestion Workflow

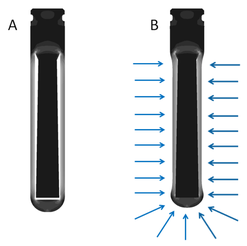


µPestle Design and Mechanism of Action

MicroCap vs µPestle.
Appearance of PCT µPestle and MicroCap inserted into MicroTubes. The sides of each MicroTube are cut away for clarity.

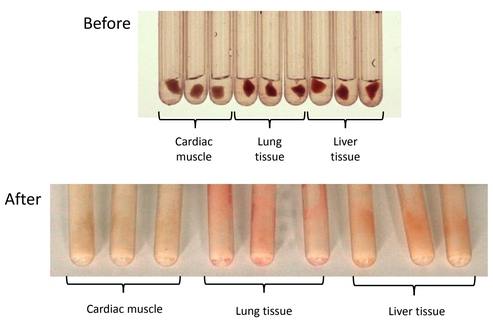
The MicroCap was designed for liquid and suspension samples. Pressure cycling with the MicroCap exposes the sample to alternating cycles of ultra high pressure, which causes cell lysis, but does not allow for the extensive homogenization of solid tissue pieces that is needed for efficient extraction.

The µPestle is designed to form a tight fit between the pestle and the inner walls and bottom of the tube. This homogenizer-like MicroTube/µPestle arrangement can accommodate up to 30µl of extraction reagent.



Mechanism of action of PCT µPestle system.
A. Tight fit of µPestle traps the small tissue sample at the tube bottom. **B**. Hydrostatic pressure compresses the tube walls against the insert, causing the tube to collapse and shorten. Tissue sample is thus extruded from the bottom of the tube through the tight annular gap between the incompressible pestle and the compressed tube walls. Repeated pressure cycles lead to efficient homogenization of soft tissue samples. Up to 48 samples can be processed at once, depending on the Barocycler being used.

Appearance of tissues in MicroTubes (side view), before µPestle is inserted, and after PCT at 35,000 psi.



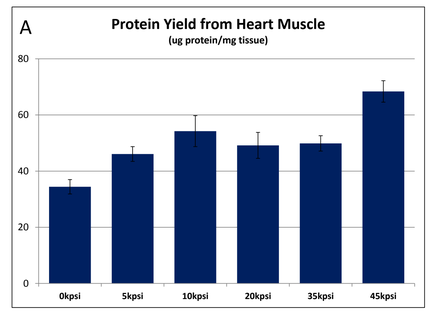
Optimization of PCT Conditions for µPestle Tissue Homogenization

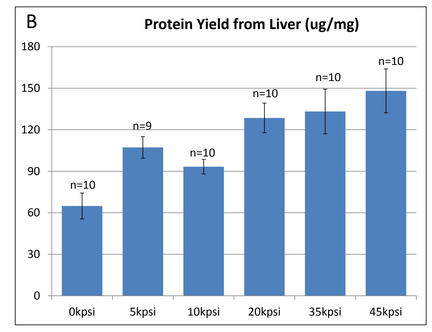
Optimization of pressure cycling parameters to maximize protein and peptide yield from biopsy-size samples.

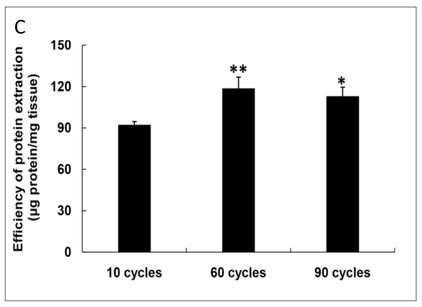
The best overall results were obtained when µPestle extraction was performed at 45 kpsi for 60 cycles, using 20 seconds at high pressure and 10 seconds at ambient pressure, per cycle.

A and **B**. Effect of pressure level on protein yield. Rat heart muscle (A) and liver tissue (B) extraction with µPestle at different pressures. PCT was carried out for 30 cycles at 33˚C in 8M urea (in 0.1M Ambic with protease inhibitor cocktail). The number of cycles was reduced from 60 to 30, to help accentuate any differences between more- and less-effective homogenization at different pressures. Bars show average protein yield (n=10) and standard error of the mean (SEM).

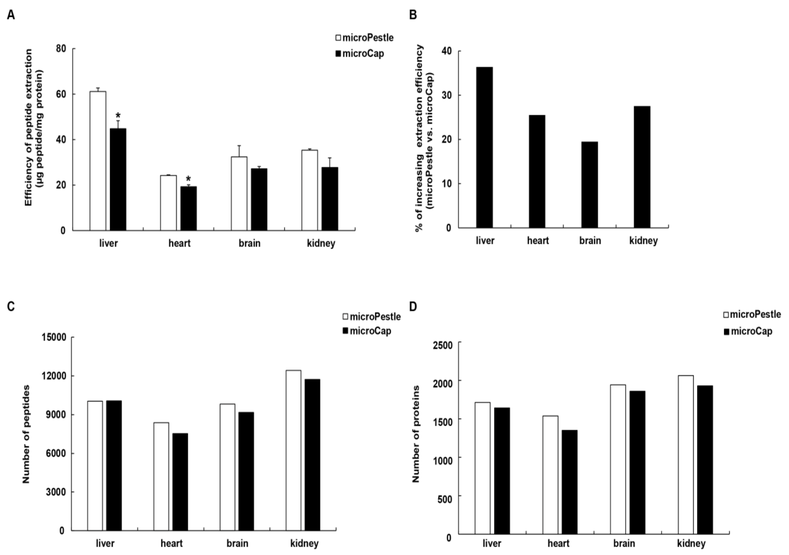
C. Effect of number of pressure cycles on protein yield. Mouse liver tissue extraction with µPestle at 45,000psi in 8M urea. Bars show average (n=3) and standard error of the mean (SEM). Tests with other tissues (not shown), also indicate that 60 pressure cycles is the best condition for high protein yield from a broad range of tissue types.

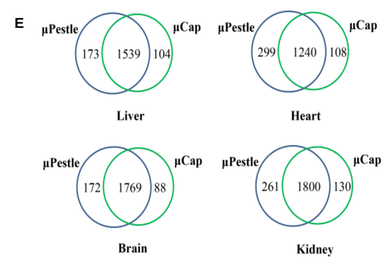






PCT µPestle vs MicroCap: Protein and Peptide Yields.

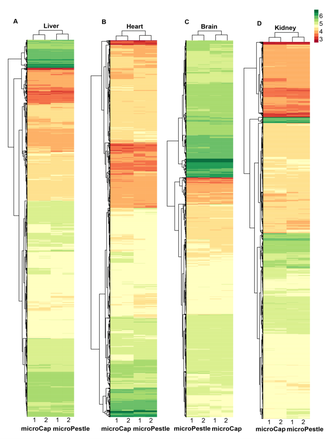




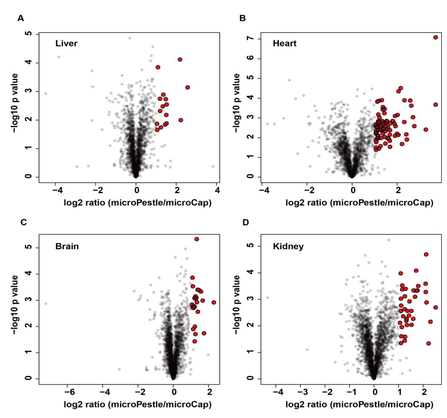
Comparison between samples extracted with PCT µPestle and MicroCap control method.

A. Peptide concentration measured by Nanodrop. Yield expressed as µg peptide per mg of tissue. Data presented are the mean ± SEM of three independent experiments.
B. The percentage of increasing extraction yield when PCT µPestle was compared to microCap. **C** and **D**. 0.6µg total peptide mass was subject to SWATH-MS analysis in duplicate. The bar plots show the number of peptides (C) and proteins (D) detected. **E**. Venn Diagram plots showing number of identified proteins .

PCT µPestle vs MicroCap: Protein Profiling and Quantification.



Hierarchical clustering analysis of proteins from mouse (liver, heart, brain) and human (kidney) tissue samples in duplicate. The relative abundance of these proteins across samples is shown in the heatmap.
To analyze the correlation of the proteome patterns between µPestle- and MicroCap-extracted samples, an unsupervised hierarchical clustering analysis was performed. We observed that the overall proteomes from µPestle were consistent with those from MicroCap in all four tissues tested.



Volcano plots of proteins quantified in mouse liver (A), heart (B), brain (C), and human kidney tissue samples (D). Proteins showing notable difference between µPestle and MicroCap methods were defined as fold change higher than 2 when the p value was lower than 0.05. Red dots indicate proteins that are at higher abundance in samples extracted by PCT µPestle compared to MicroCap. There are 16, 85, 24, and 39 proteins with higher intensity in liver, heart, brain, and kidney tissues respectively, when compared to MicroCap-processed samples.
All samples processed in duplicate.

References cited

1. Guo T, et al. Rapid mass spectrometric conversion of tissue biopsy samples into permanent quantitative digital proteome maps. Nat Med. 2015 Mar 2.
2. Shao S, et al. Hands-free Sample Homogenization and Protein Extraction from Small Tissue Samples using Pressure Cycling Technology and µPestle. *Manuscript in preperation.*

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