Streamlining PCT-HD for Tissue Biopsy Samples: Better Digestion in a Shorter Time.

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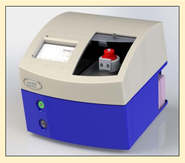
Introduction

High–throughput proteomic analysis of small tissue biopsies for precision medicine presents particular sample preparation challenges with respect to individual sample handling, protein extraction, and digestion. The Pressure Cycling Technology (PCT) sample preparation approach employs PCT MicroPestle-based preparation of tissue samples (Shao et al. 2016 JPR 15(6):1821-9). The hands-off homogenization of up to 16 samples at a time, followed by pressure enhanced digestion, can be carried out using a single-tube protocol that minimizes sample loss and the possibility of cross-contamination (Guo et al. 2015 Nat. Med. 21:407-13). The goal of the current study was to streamline and optimize this protocol in order to minimize manual sample handling steps and total preparation time, while maximizing quantitative recovery of proteotypic peptides.

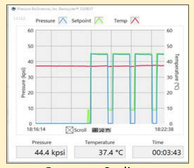
Here we show that tissue samples prepared using the ~3 hour PCT-HD protocol yield comparable or superior results to samples prepared using a conventional method that requires ~20 hours from start to finish. The PCT-HD protocol resulted in more protein and peptide IDs, more consistent protein digestion, and improved sequence coverage, without significant loss of enzyme specificity or increase in protein modifications. We further show that several steps of the PCT-HD protocol can be modified or combined, in order to further streamline the method and reduce sample processing time to ~2 hours per batch (up to 16 samples per batch).



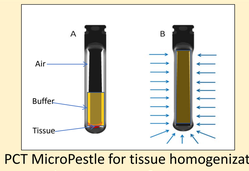
Barocycler 2320EXT

PCT MicroTubes

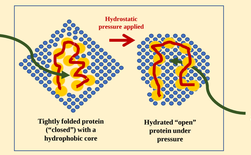
MicroTube Cartridge



Pressure Cycling



PCT MicroPestle for tissue homogenization
A: at atmosphere, B: at pressure.

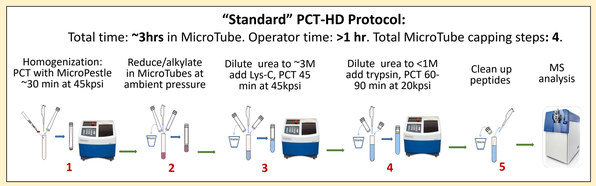


Effect of Pressure Cycling on proteins and proteolytic digestion

Preliminary PCT-HD Optimization and Streamlining.

Figure 1. Options to Streamline the "Standard" PCT-HD Protocol:

- Omit step 3 (Lys-C digest). Save ~45mins and 1 tube capping step.
- Omit step 2 (Digest unreduced proteins, reduce and alkylate peptides prior to MS). Save ~30mins and 1 tube capping step.
- Combine steps 1 and 2 (Homogenization and reduction/alkylation of disulfide bonds). Save ~30min and 1 tube capping step.
- Combine steps 1 and 3 (Homogenization and Lys-C digest). Save ~45min and 1 tube capping step.
- Combine steps 3 and 4 (simultaneous digestion with trypsin and lys-C). Save ~45min and 1 tube capping step.
- Combine steps 1 and 3 **and** omit step 2 (Homogenization and Lys-C digest together, reduce and alkylate peptides prior to MS). Save ~75min and 2 tube capping steps.



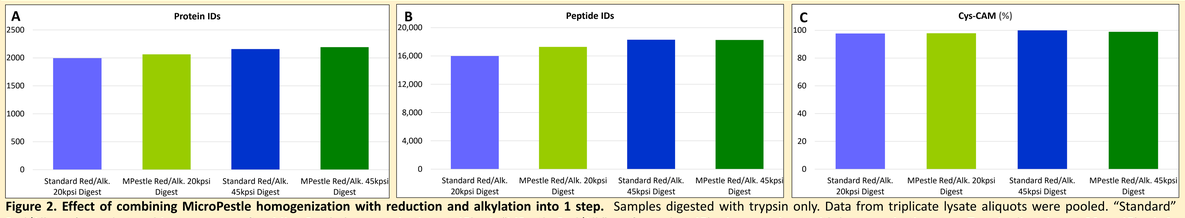


Figure 2. Effect of combining MicroPestle homogenization with reduction and alkylation into 1 step. Samples digested with trypsin only. Data from triplicate lysate aliquots were pooled. "Standard" Red/Alk performed at ambient pressure after MicroPestle homogenization at 45kpsi. "MPestle Red/Alk" performed at 45kpsi concomitant with homogenization step. Trypsin digestion at 20 or 45kpsi. **A**. Total protein IDs. **B**. Total peptide IDs. **C**. Cysteine Carbamidomethylation was used as an indicator of the completeness of the alkylation reaction.
Conclusion: Combining the reduction/alkylation with the MicroPestle step has no negative effect on subsequent trypsin digestion and the number of protein and peptide IDs. Increasing pressure to 45kpsi appears to increase the number of protein and peptide IDs slightly.

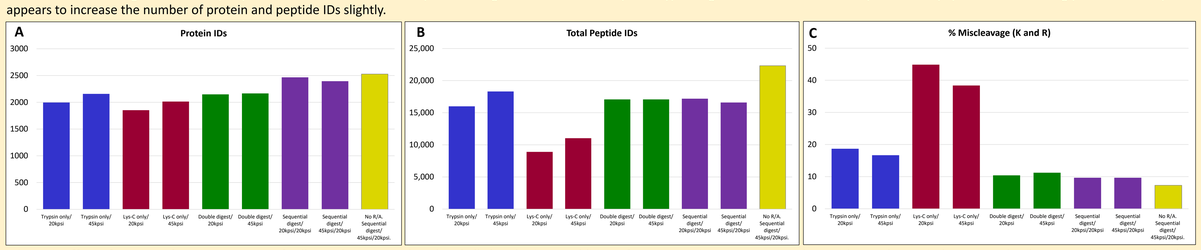


Figure 3. Effect of Lys-C on the PCT-HD protocol. All samples were reduced and alkylated prior to addition of enzymes. Digests were carried out either with trypsin (at 20 or 45kpsi), or Lys-C (at 20 or 45kpsi), or double digested with lys-C and trypsin together (at 20 or 45kpsi), or sequentially with lys-C (at 20 or 45kpsi) followed by trypsin at 20kpsi. Additionally, non-reduced/alkylated samples were digested with lys-C (45kpsi) followed by trypsin (20kpsi), with peptide red/alk carried out prior to MS (yellow bars). Data from triplicate lysate aliquots were pooled. **A**. Total protein IDs. **B.** Total peptide IDs. **C.** Miscleavage rate (% of peptides containing one or more lysines or argenines. Note that since lys-C does not cleave at argenines, the apparent miscleavage rate is not reflective of this enzyme's specificity in this assay).
Conclusion: Incorporating a lys-C step marginally increases protein IDs and significantly reduces miscleavage, compared to digestion with trypsin alone. Interestingly, digestion with lys-c alone, resulted in almost as many protein IDs as with trypsin. Overall, best results were obtained with sequential digestion by the two enzymes. Further improvement in the number of protein and peptide IDs, and miscleavage rate, was observed in samples where disulfide bond reduction and alkylation was performed after the digests were complete (during peptide clean-up).

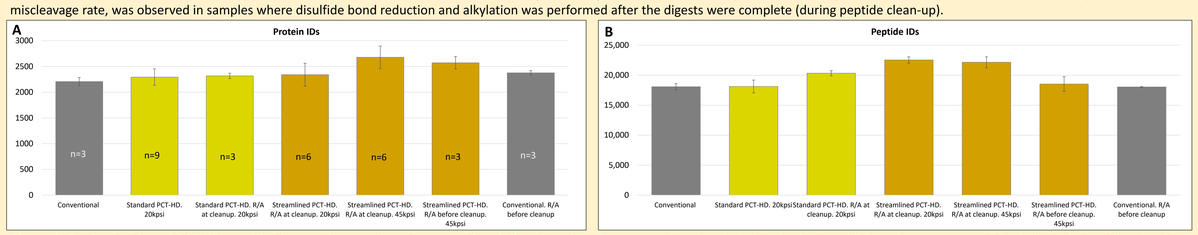


Figure 4. Further testing of PCT-HD modifications and comparison to conventional digestion. Samples were reduced and alkylated prior to addition of enzymes, or at the indicated step (directly after digestion/before peptide clean-up, or during the peptide clean-up step). Lys-C digestion was carried out at ambient pressure for 4hrs (Conventional) or at 45kpsi for 45 mins (all PCT-HD samples). Trypsin digestion was carried out overnight at ambient pressure (Conventional) or at the indicated pressure for 90 mins. Data from 3-9 replicates (avg ± SD). Standard PCT-HD shown in **Figure 1**. Streamlined PCT-HD shown in **Figure 8**. **A**. Total protein IDs. **B.** Total peptide IDs.
Conclusion: Streamlining the PCT-HD workflow and moving reduction/alkylation to the end of the protocol can improve results, save time, and reduce hands-on manipulations.

PCT-HD Optimization and Comparison to Conventional Sample Preparation.

Method (n=3)	Protein extraction method	Reduction/ Alkylation	Lys-C digestion at 37°C	Trypsin digestion at 37°C	Total Protein IDs	Total Peptide IDs	Fully Tryptic Peptide IDs (0 MC)	Tissue mass (mg)
Conventional method control (no PCT, o/n digest)	Manual homogenization	After homogenization	4 hours at ambient pressure	Overnight at ambient pressure	1,887 ± 34	16,701 ± 870	10,329 ± 1,261	2.1 ± 0.7
"Standard" PCT-HD	PCT MicroPestle 45kpsi	After homogenization	45 mins at 45kpsi	90 mins at 20kpsi	**2,446 ± 44**	19,196 ± 110	**14,079 ± 492**	1.9 ± 0.5
"Modified" PCT-HD	PCT MicroPestle 45kpsi	Concomitant with homogenization at 45kpsi	45 mins at 45kpsi	90 mins at 20kpsi	**2,349 ± 32**	20,024 ± 341	**15,163 ± 188**	2.1 ± 0.1
"Streamlined" PCT-HD	PCT MicroPestle 45kpsi	After digestion	Concomitant with homogenization. 45 mins at 45kpsi	90 mins at 20kpsi	2,227 ± 43	17,585 ± 124	12,059 ± 483	1.7 ± 0.6
"Streamlined" PCT-HD (45kpsi)	PCT MicroPestle 45kpsi	After digestion	Concomitant with homogenization. 45 mins at 45kpsi	90 mins at 45kpsi	2,133 ± 77	16,185 ± 1,001	11,803 ± 576	1.5 ± 0.2
PCT-HD negative control	PCT MicroPestle 45kpsi	After homogenization	45 mins at ambient pressure	90 mins at ambient pressure	1,870 ± 62	14,485 ± 496	10,354 ± 258	1.9 ± 0.2

Table 1. PCT-HD Permutations and Comparison to Conventional Sample Preparation. Values are average ± SD of biological triplicates.
Conclusion: Sample digestion with pressure cycling results in more protein and peptide IDs compared to digestion at ambient pressure whether carried out for ~18 hrs or for 90 minutes.

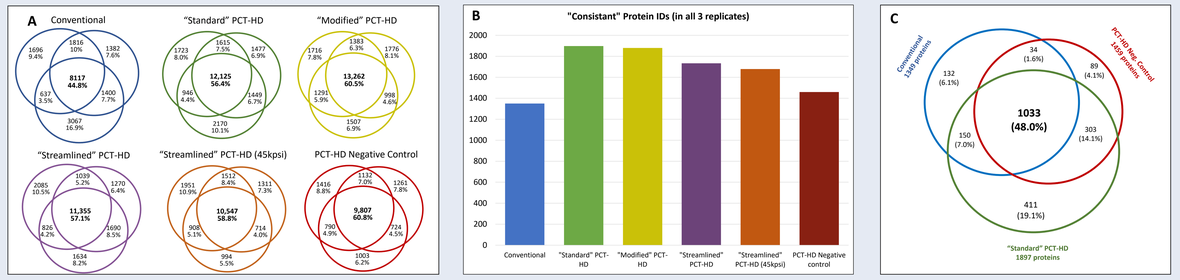


Figure 5. Reproducibility and Signal Intensity. A. Peptides consistently identified in all 3 biological replicates. Venn diagrams show overlap of peptide IDs in biological triplicates. Peptide IDs include unique sequences only, regardless of presence/absence of modifications or miscleavage. (Similar Venn diagrams were used to generate the protein values shown in **B**). **B.** Number of proteins consistently identified in all 3 biological replicates. Conclusion: Samples extracted with PCT MicroPestle (all PCT-HD, including negative control) show much higher reproducibility compared to the Conventional method. Overall signal intensity in all pressure-treated samples is as good as, or better, than in the conventional method control. **C**. More unique proteins are consistently identified in PCT-HD than in either control group, suggesting that more reproducible extraction and better digestion in the PCT-HD method, lead to improved results as well as significant time savings

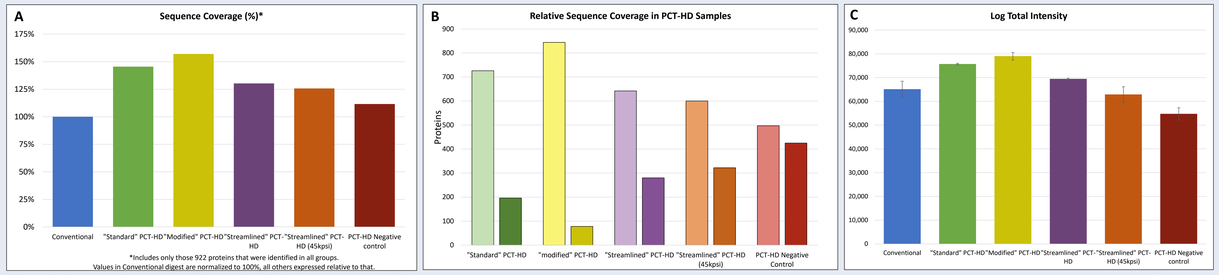


Figure 6. Sequence Coverage and Intensity. A. Sequence coverage of each of 922 individual proteins was compared between all groups. For each protein, sequence coverage in the Conventional digest (average of n=3) was calculated and set as 100%. The sequence coverage of each of these protein in all the treatment groups (average of n=3 per group) was calculated and expressed relative to the Conventional value. **B.** For each treatment group, proteins were separated into those whose sequence coverage increased (light bars) or decreased/remained unchanged (dark bars), relative to Conventional method. In a random distribution, the number of proteins showing increased and decreased coverage should be ~50/50, as seen in the PCT-HD Negative control which was digested without pressure (note: sum of light and dark bars is 922 in all cases). Samples digested with pressure cycling show a significant increase in the number of proteins with increased coverage (>100%) **C**. Total signal intensity (average ± std dev, n=3).
Conclusion: Overall sequence coverage is greater in PCT-HD methods compared Conventional sample preparation without pressure, as seen in **A**. Careful examination of the data also reveal that while the "Streamlined" PCT-HD methods save more time, and do increase sequence coverage significantly, the "Standard" and "Modified" PCT-HD methods yield the best improvement in sequence coverage.

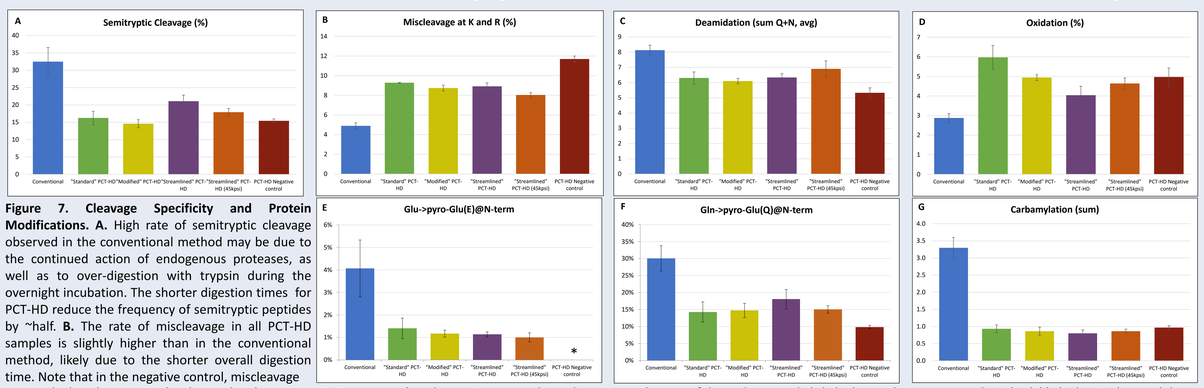


Figure 7. Cleavage Specificity and Protein Modifications. A. High rate of semityptic cleavage observed in the conventional method may be due to the continued action of endogenous proteases, as well as to over-digestion with trypsin during the overnight incubation. The shorter digestion times for PCT-HD reduce the frequency of semityptic peptides by ~half. **B.** The rate of miscleavage in all PCT-HD samples is slightly higher than in the conventional method, likely due to the shorter overall digestion time. Note that in the negative control, miscleavage is ~30% higher than in samples digested with pressure. **C**. Pressure cycling does not increase deamidation rate. The rate of deamidation is slightly higher in the conventional method, likely due to the much longer sample processing time (~20 hours). **D**. Oxidation is known to increase slightly under pressure, as air becomes dissolved in the sample under pressure. Oxidation is highest in the "Standard" PCT-HD method, where samples are exposed to hydrostatic pressure for the longest total time (~2hr 45min). **E and F**. Other chemical modifications, such as those on glutamic acid and glycine, are a result of long incubation time and are decreased in samples processed by PCT-HD. * This modification was not detected in the PCT-HD negative control. **G**. Carbamylation is a result of exposure to urea. The shorter overall processing time by PCT-HD, reduces the frequency of carbamylation significantly.

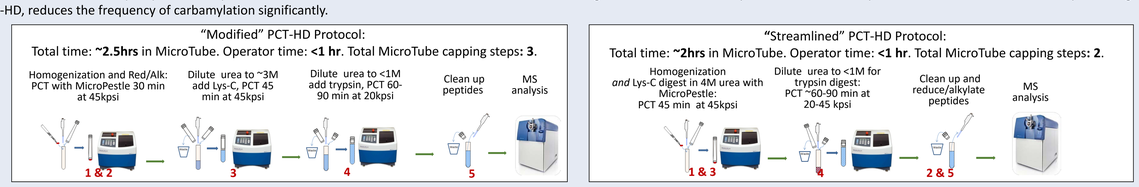


Figure 8. PCT-HD Workflows. A. The "Modified" PCT-HD Workflow combines homogenization and reduction/alkylation into 1 step, saving ~30mins and one MicroTube capping step. **B.** The "Streamlined" PCT-HD Workflow combines homogenization and Lys-C digestion into 1 step and moves the reduction and alkylation reactions to the end of the workflow. This saves ~1 hour and two MicroTube capping steps. Steps (red) correspond to the sequence shown in Figure 1.

Materials and Methods

Preliminary data (Figures 2-4) were generated using aliquots of a single lysate (rat liver) to reduce biological sample-to-sample variability. Lysate was made in 8M urea and diluted to 4M. Aliquots in PCT Microtubes were processed essentially as for solid tissue. Subsequent optimization (Figures 5-7) was performed using fresh-frozen rat liver tissue pieces, individually cut and weighed just prior to use (3 biological replicates per group). Samples were extracted in 4M urea/ 50mM ammonium bicarbonate. Reduction and alkylation with 10mM TCEP and 30mM iodoacetamide was carried out at the indicated step. Trypsin (Promega, sequencing grade) and Lys-C (Wako), were each used at ~1:50 enzyme-to-substrate ratio for preliminary lysate digests (Figures 2-4), and at ~1:100 for tissue digests (Figures 5-7). Digest reactions were carried out at 35-37°C. Peptides were cleaned up using C18 SPE columns (Empore C18, Sigma) per manufacturer's instructions. Samples were run on a Sciex 6600 equipped with a Turbo V source. Peptides were separated over a 90 min run time using an SGE C18G 250 mm x 300 µm, 3µm column after trapping on an SGE C18G trap column 10 mm x 300 µm, at a flow rate of 5µL/min. The top 60 peptides were analysed using dynamic exclusion. The data were searched using ProteinPilot 5.0 (thorough search with FDR calculation) against all rat sequences extracted from UniProt (37596 proteins) with Cys-CAM and urea denaturation specified in the search settings. The FDR was set at 1%. Percent sequence coverage (Figure 6) is based on 922 individual proteins identified in all samples. Sequence coverage for each protein in the groups was compared to the sequence coverage of the same protein in the conventional samples (using average values from 3 biological replicates). Values in the conventional samples were normalized to 100%, all others are expressed relative to that.
Venn diagrams were generated from the original protein lists using Venny (*Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams.* http://bioinfogp.cnb.csic.es/tools/venny/index.html), and redrawn for scale and color.