Streamlined PCT-HD: Alternative Method for Improved Results Using Higher Pressure Trypsin Digestion or N-Propanol

Introduction

The PCT-HD workflow (also known as PCT-SWATH^{Tm} or PCT-ABLE) is a combination of two of PBI's innovative sample preparation tools; the 2320EXT Barocycler and PCT MicroPestles [1-5]. Together they utilize pressure cycling technology (PCT) to extract and digest proteins from biopsy-size tissue samples. The positive effect of PCT on digestion by both trypsin and Lys-C has previously been demonstrated [6-11]. High hydrostatic pressure improves protein digestion via a combination of two mechanisms; one acting on the enzyme and the other acting on the substrates, leading to significantly accelerated and highly reproducible digestion. The method can be adjusted for digestion with trypsin alone, lys-C alone, or a combination of both enzymes. PCT-HD can be carried out using conventional enzymes or boutique enzymes designed for higher temperature methods [3]. Note that additional optimization may be required when using non-conventional proteolytic enzymes.

The PCT-HD workflow starts with small solid tissues, such as biopsies, and ends with peptide samples ready for clean-up and MS, or other analyses (Figure 1). The first step in the PCT-HD method is tissue homogenization by pressure cycling with PCT MicroPestles. This step is described in detail elsewhere [1] and will not be discussed here. The original "Standard" PCT-HD procedure could be performed in about 3.5 hours, and required 5 manual steps; initial filling and capping of the microtubes with tissue sample and lysis buffer (followed by high pressure homogenization for ~30 min), uncapping after homogenization to add reduction and alkylation reagents (followed by a 30min incubation on the bench), uncapping to add the first enzyme (followed by high pressure digestion for ~45min), then uncapping again to add the second enzyme (followed by high pressure digestion for ~90 min), and finally sample recovery from the microtube. Since up to 16 samples are processed simultaneously in the Barocycler, any reduction in the number of required steps can save the user a significant amount of time.

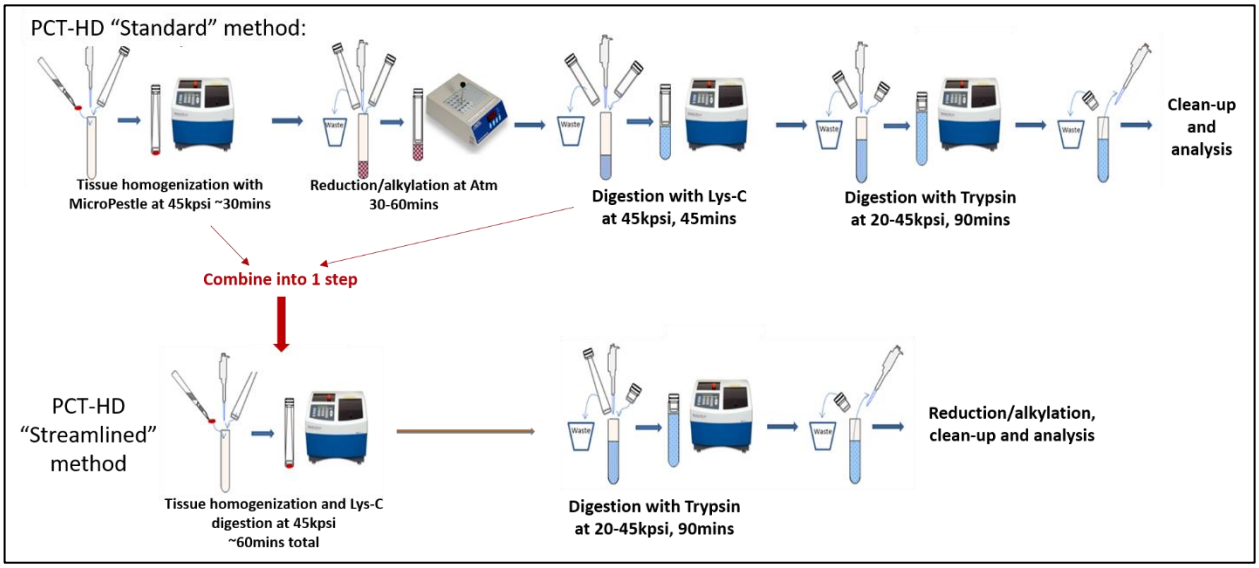

Figure 1. Comparison of PCT-HD Workflows. "Standard" workflow (top panel) and "Streamlined" workflow (bottom panel). The original "Standard" procedure takes 3.5 to 4 hours to complete and requires 5 manual steps. The Streamlined method requires only 3 manual steps and takes only 2.5 to 3 hours to complete.

The current Application Note focuses on alternative conditions for the high-pressure trypsin digestion step in the "Streamlined" PCT-HD method [7], which is designed to minimize both total sample processing time, as well as the number of manipulations required to process the samples. However, the same alternative conditions can be applied to the Standard PCT-HD workflow, at the trypsin digestion step. As shown in Figure 1, combining the Lys-C digestion with the initial tissue homogenization at 45kpsi reduces the number of hands-on steps. In addition, performing the disulfide reduction and alkylation steps after high pressure digestion simplifies the workflow and improves overall results (see below). This Streamlined method requires only 3 manual steps; initial filling and capping of the microtubes with tissue sample, lysis buffer, and Lys-C enzyme (followed by high pressure homogenization for ~30-60 min during which the high pressure-accelerated Lys-C digestion occurs) uncapping to dilute the urea and add trypsin enzyme (followed by high pressure digestion for ~90 min), and finally sample recovery from the microtube.

Results

Samples prepared using conventional digestion (4 hour digestion with Lys-C and 18 hour digestion with trypsin, all at atmospheric pressure) and the Standard PCT-HD protocol (Figure 1, top panel) were compared to the Streamlined PCT-HD protocol (Figure 1, bottom panel) with the following modifications: Trypsin digestion was carried out for 90 minutes at 20kpsi, 35kpsi or 45kpsi, with or without addition of 5%

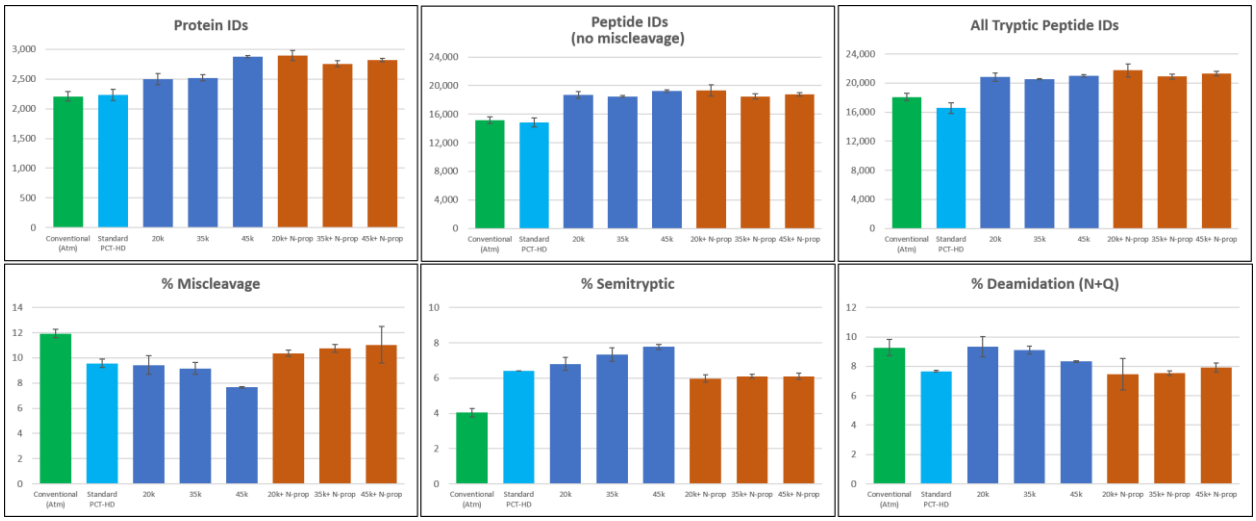

Figure 2. Conventional sample preparation (green bars, n=3) compared to Standard PCT-HD (turquoise bars, n=3) and Streamlined PCT-HD (n=3 each). Samples prepared by Standard or Streamlined PCT-HD are comparable to, or slightly better than, Conventional method as indicated by the total number of protein IDs, as well as the number of peptide IDs. Results for tryptic peptides containing no miscleavages as well as all tryptic peptides (containing up to 2 miscleavages) are shown for comparison. The number of identified peptides containing one or more miscleavages, is presented as percent of total peptide IDs (% Miscleavage). The number of semitryptic and deamidated peptides is also presented as percent of total peptide IDs (% Semitryptic and % Deamidation).
Conventional samples were reduced and alkylated prior to digestion, treated with Lys-C for 4 hours followed by trypsin overnight (all at 37^{0}C at ambient pressure). Standard PCT-HD samples were reduced and alkylated in PCT-MicroTubes after homogenization, prior to digestion, as shown in Figure 1 (top panel), and digested with Lys-C for 45 min at 45kpsi followed by trypsin for 90 min at 20kpsi. Streamlined PCT-HD samples were homogenized and digested with Lys-C in one step at 45kpsi as shown in Figure 1 (lower panel), followed by trypsin digestion for 90 min at 20, 35, or 45kpsi with (red bars) or without (blue bars) addition of 5% N-propanol. After Streamlined PCT-HD, peptides were reduced and alkylated prior to clean-up and analysis. All high-pressure digests were performed at 35^{0}C.
The data suggest that increasing trypsin digestion pressure from 20kpsi to 45kpsi may increase the number of protein IDs slightly. Alternatively, the same effect can be obtained at 20kpsi by adding 5% N-propanol to the reaction. N-propanol appears to improve trypsin specificity at high pressure as indicated by the slight reduction in semitryptic peptide IDs.

N-propanol. Previous tests had indicated that 5-10% N-propanol can accelerate high pressure trypsin digestion [8]. As shown in Figure 2, results obtained using the Streamlined PCT-HD method can be slightly improved by increasing trypsin digestion pressure from 20kpsi to 45kpsi, or by supplementing the reactions at 20kpsi with 5% N-propanol.

Discussion

This Streamlined PCT-HD method can be used "as-is" [7] or as a starting point for further optimization to design alternative protocols for specific needs and sample types.

When designing PCT-enhanced digestion conditions, it should be kept in mind that by forcing substrate proteins into an unfolded/hydrated state, PCT effectively makes them "better" substrates for enzyme digestion. However, since hydrostatic pressure, and the presence of organic solvents, chaotrope, and/or detergents, can also affect the conformation of the enzyme itself, it is generally recommended that digestion be carried out at the lowest pressure, and with the least amount of chemical denaturants (e.g. sodium deoxycholate [11], N-propanol, or urea [9]), at which effective accelerated digestion can be achieved. For this reason, we suggest 20 kpsi as a starting condition for high pressure trypsin digestion. Once good results are obtained at 20kpsi, a comparison to 45kpsi digestion can be performed, if desired, using the same sample material and reagents, to determine whether the higher pressure digestion conditions improve the final result. Similarly, once good results are obtained in conventional buffer, addition of 5-10% (or even 15%) N-propanol can be tested, if desired.

Suggested alternative workflows for Streamlined PCT-HD:

1. Heat 2320 EXT to desired digestion temperature (e.g. 35^{0}C if samples contain urea, or 50^{0}C if no urea is present). Allow unit to reach final temp before proceeding (at least 20-30min when using electric heating. Longer time may be required when using external water bath).
2. Place tissue samples (≤3 mg wet weight) into PCT MicroTubes. Add 30ul lysis reagent such as 4M urea in 50-100mM ammonium bicarbonate supplemented with Lys-C. Enzyme-to-substrate ratio should be ~1:50 but can be adjusted up/down to optimize digestion of different samples. Cap with PCT MicroPestles.
   Note: It is very important that total sample volume not exceed 30-35µl during tissue homogenization with PCT-MicroPestle. Using a larger volume at this step will compromise the effectiveness of homogenization.
   Note: addition of protease inhibitors should be avoided as this will result in poor digestion with trypsin and lys-C. Phosphatase inhibitors may be used, but type and concentration may need to be tested/optimized for high-pressure applications.
3. Place into preheated 2320EXT Barocycler and perform pressure cycling at 45kpsi for 55 cycles (40 sec at high pressure, 10 sec at ambient pressure, per cycle. Total time ~45mins). Remove tubes from Barocycler and discard MicroPestles. Proceed to step 4 or 5.
   (Optional) Place MicroTubes into small centrifuge tubes and centrifuge briefly to bring sample down to the bottom.
4. **Option 1: Trypsin digestion at 45kpsi**.
   Add Trypsin enzyme and 100mM ammonium bicarbonate to bring the total volume to ~145µl (this brings the urea to ~0.8M. Note that higher urea concentrations can interfere with high pressure trypsin digestion and may result in more carbamylation of peptides). If using sodium deoxycholate in place of urea, see [11] for required dilution. Enzyme-to-substrate ratio should be ~1:50, but can be adjusted up/down to optimize digestion of different samples. Seal tubes with short "150µl" MicroCaps. Perform trypsin digestion in heated Barocycler for 60-90 cycles at 45kpsi (50 sec at high pressure, 10 sec at ambient pressure, per cycle. ~60-90min total).
5. **Option 2: Trypsin digestion at 20kpsi with 5-10% N-propanol**
   Add Trypsin enzyme, 7-14ul 100% N-propanol, and 100mM ammonium bicarbonate to bring the total volume to ~145µl (this brings the urea to ~0.8M, and N-Propanol to 5-10%. Note that higher

urea concentrations can interfere with high pressure trypsin digestion and may result in more carbamylation of peptides). If using sodium deoxycholate in place of urea, see [11] for required dilution. Enzyme-to-substrate ratio should be ~1:50, but can be adjusted up/down to optimize digestion of different samples. Seal tubes with short "150µl" MicroCaps. Perform trypsin digestion in heated Barocycler for 60-90 cycles at 20kpsi (50 sec at high pressure, 10 sec at ambient pressure, per cycle. ~60-90min total).

6. Proceed to sample reduction, alkylation (if required) and clean-up using standard methods.

Materials and Methods

Trypsin (Promega, sequencing grade) and Lys-C (Wako), were prepared at 0.2mg/ml per manufacturer's instructions and stored frozen in aliquots. Rat liver was purchased from Pel-Freeze and stored at -80^{0}C. To reduce biological sample-to-sample variability, a single stock of liver lysate in 4M urea/50mM ammonium bicarbonate was prepared and used for all samples (protein concentration ~2.5 mg/ml by Bradford assay). 30µl lysate aliquots (approx. 75µg protein per sample) in PCT Microtubes were processed using the Standard or Streamlined methods (as shown in Figure 1). High pressure incubations were carried out in a Barocycler 2320EXT.

For Standard PCT-HD the sample tubes were capped with PCT MicroPestles and treated as for tissue homogenization at 45kpsi, room temp, 60 cycles (25 sec at high pressure, 10 sec at low pressure, per cycle). After homogenization, samples were reduced and alkylated by addition of 10mM TCEP and 30mM iodoacetamide to each MicroTube (30 min at ambient pressure at room temp, protected from light). After disulfide reduction, samples were adjusted to 40µl total volume by addition of 100mM ammonium bicarbonate and 2µg Lys-C (bringing final urea concentration to 3M). Lys-C digestion was carried out at 35^{0}C, for 45min at 45kpsi (45 cycles, 50 sec at 45kpsi, 10 sec at Atm, per cycle). After Ly-C digestion samples were adjusted to 145µl total volume by addition of 100mM ammonium bicarbonate and 2µg trypsin (bringing final urea concentration to 0.8M). Trypsin digestion was carried out at 35^{0}C, for 90min at 20kpsi (90 cycles, 50 sec at 20kpsi, 10 sec at Atm, per cycle). Reactions were stopped by addition of formic acid and stored frozen until ready for clean-up and analysis.

For Streamlined PCT-HD, 2µg Lys-C enzyme was added directly to the samples in MicroTubes (in 4M urea). Tubes were capped with PCT MicroPestles and treated at 45kpsi, 35^{0}C for 55 cycles (40 sec at 45kpsi, 10 sec at Atm, per cycle). After Lys-C digestion samples were adjusted to 145µl total volume by addition of 100mM ammonium bicarbonate and 2µg trypsin with or without 5% N-Propanol (bringing final urea concentration to 0.8M). Trypsin digestion was carried out at 35^{0}C, for 90min at the indicated pressure (90 cycles, 50 sec at high pressure, 10 sec at Atm, per cycle). Reactions were stopped by addition of formic acid and stored frozen until ready for clean-up and analysis. Reduction/alkylation was carried out after thawing, using 10mM TCEP and 30mM iodoacetamide for 30 min, prior to standard peptide clean-up.

Conventional sample processing was carried out in 0.65ml polypropylene Eppendorf-style tubes. 30µl of tissue homogenate was reduced and alkylated by addition of 10mM TCEP and 30mM iodoacetamide (30 min at ambient pressure at room temp, protected from light). After disulfide reduction samples were adjusted to 40µl total volume by addition of 100mM ammonium bicarbonate and 2µg Lys-C (bringing final urea concentration to 3M). Lys-C digestion was carried out at 37^{0}C for 4 hours with shaking. Samples were then adjusted to 145µl total volume by addition of 100mM ammonium bicarbonate and 2µg trypsin (bringing final urea concentration to 0.8M). Trypsin digestion was carried out at 37^{0}C overnight (~18-20 hours) with shaking. Reactions were stopped by addition of formic acid and stored frozen until ready for clean-up and analysis.

Peptide clean-up (and reduction/alkylation of Streamlined PCT-HD samples), mass spectrometry and data analyses were carried out at the Children's Medical Research Institute (CMRI) in Westmead NSW, Australia. Peptides were cleaned up using C18 SPE columns (Empore C18, Sigma) per manufacturer's instructions, and 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 (37,596 proteins) with Cys-CAM and urea denaturation specified in the search settings. The FDR was set at 1%. All data are expressed as average (n=3 biological replicates) ± standard deviation.

References

1. Fast and Efficient Protein Extraction in MicroTubes Using PCT µPestles. https://www.pressurebiosciences.com/component/edocman/fast-and-efficient-protein-extraction-in-microtubes-using-pct-pestles/download?Itemid=
2. Minimal sample requirement for highly multiplexed protein quantification in cell lines and tissues by PCT-SWATH mass spectrometry. Shao et al., Proteomics 2015. 15(21):3711-3721.
3. Accelerated Barocycler Lysis and Extraction Sample Preparation for Clinical Proteomics by Mass Spectrometry. Lucas, et al., J. Prot. Res. 2018.18(1):399-405.
4. Rapid mass spectrometric conversion of tissue biopsy samples into permanent quantitative digital proteome maps. Guo et al. Nature Medicine 21:407-413 (2015) doi:10.1038/nm.3807
5. Application Note-PCT-HD: Homogenization, Extraction and Digestion of Small Tissue Samples for Proteomic Applications https://www.pressurebiosciences.com/component/edocman/appication-note-pct-hd-homogenization-extraction-and-digestion-of-small-tissue-samples-for-proteomic-applications/download?Itemid=
6. Modified PCT-HD Workflow: Simplified Protocol with Fewer Steps and Less Hands-on Time.
7. Streamlined PCT-HD Workflow: Simplified Protocol with Fewer Steps and Less Hands-on Time II.
8. Streamlining PCT-HD for Tissue Biopsy Samples: Better Digestion in a Shorter Time. Poster presented at ASMS 2017. https://www.pressurebiosciences.com/documents/scientific-meetings/asms-2017/streamlining-pct-hd-for-tissue-biopsy-samples-better-digestion-in-a-shorter-time
9. Proteolysis-PrEP: Optimization of Pressure Cycling Conditions for Protein Digestion with Trypsin in Solution. https://www.pressurebiosciences.com/component/edocman/trypsin-prep-91815-v1-pdf/download?Itemid=
10.Pressure-Accelerated Protein Digestion with Endoproteinase Lys-C. https://www.pressurebiosciences.com/component/edocman/publications-by-type/pressure-enhanced-enzymes/pressure-accelerated-protein-digestion-with-endoproteinase-lys-c
11. Compatibility of Deoxycholate Detergent with Pressure-enhanced Digestion by Trypsin and Lys-C: Alternative PCT-HD Workflow https://www.pressurebiosciences.com/component/edocman/compatibility-of-deoxycholate-detergent-with-pressure-enhanced-digestion-by-trypsin-and-lys-c-alternative-pct-hd-workflow/download?Itemid=