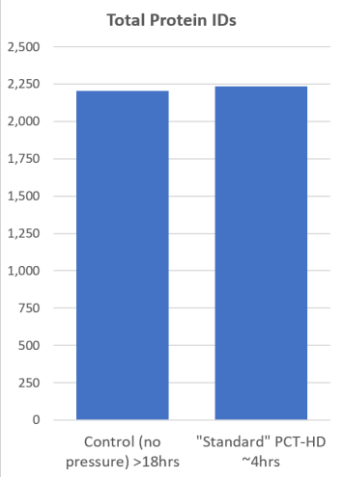
Modified PCT-HD Workflow: Simplified Protocol with Fewer Steps and Less Hands-on Time

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 [1-4]. Together they utilize pressure cycling technology (PCT) to extract and digest proteins from biopsy-size tissue samples. PCT has been proven to accelerate enzymatic protein digestion; the positive effect of PCT on digestion by both trypsin and Lys-C has previously been demonstrated [2-9]. 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 (Figure 1). The method can be adjusted for digestion with trypsin alone, lys-C alone, or a combination of both enzymes. Additional optimization may be required when using non-conventional proteolytic enzymes. PCT-HD can be carried out using conventional enzymes or boutique enzymes designed for higher temperature methods [4].



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. High Pressure Accelerated Digestion; Conventional sample processing compared to PCT-HD. Conventional samples digested with Lys-C for 4 hours followed by trypsin digestion overnight (all at 37^{0}C at ambient pressure). PCT-HD samples digested with Lys-C for 45 mins at 45kpsi followed by trypsin digestion for 90 mins at 20kpsi (all at 35^{0}C).
PCT-HD is significantly faster than conventional method and yields essentially the same number of total protein IDs. Pooled data from 6 MS runs (3 biological replicates and 2 technical replicates).

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. This Application note focuses on reduction

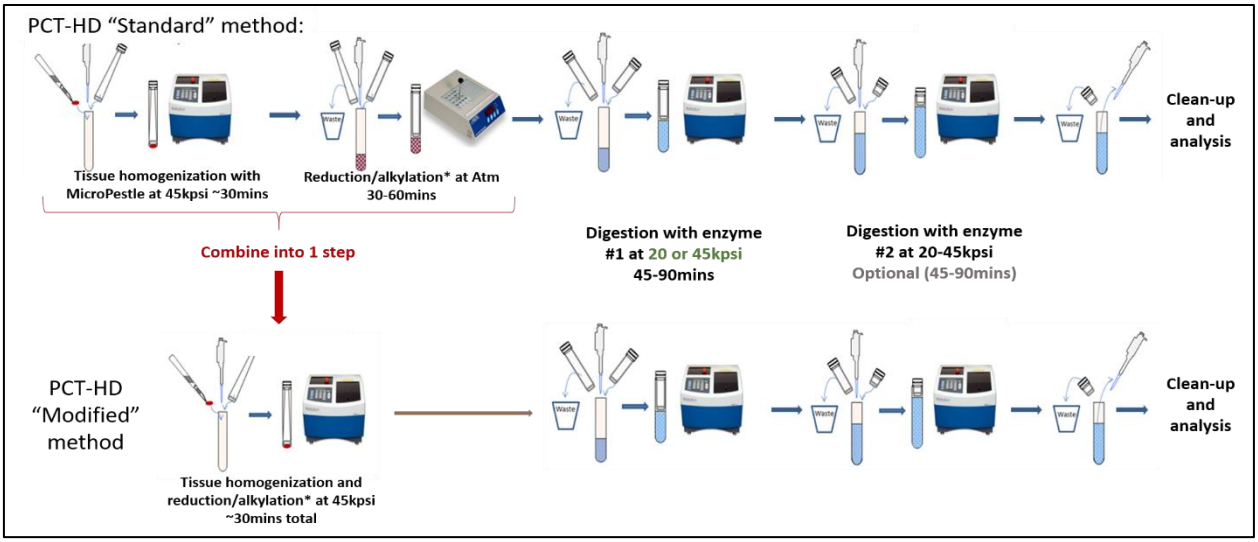


Figure 2. Comparison of PCT-HD Workflows. "Standard" workflow (top panel) and "Modified" workflow (bottom panel). The original "Standard" procedure takes 3.5 to 4 hours and required 5 manual steps. The Modified method requires only 4 manual steps and takes ~3 hours to complete. Both panels show sequential digestion with 2 enzymes (usually Lys-C followed by Trypsin). However, excellent results can be obtained with a single enzyme digest (data generated with trypsin alone are presented in Figures 3 and 4), which would reduce both workflows by ~45mins and eliminate one hands-on step.

and alkylation of protein disulfide bonds during tissue homogenization, and on the conditions used for trypsin digestion.

The original "Standard" procedure could be performed in 3-4 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. The Modified method described here requires only 4 manual steps; initial filling and capping of the microtubes with tissue sample, lysis buffer, and the reduction/Alkylation (Red/Alk) reagents (followed by high pressure homogenization for ~30 min during which the disulfide bonds are reduced and alkylated), 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 (Figure 2).

Note that sequential digestion with two enzymes (usually Lys-C followed by Trypsin) is optional in both the Original and Modified workflows. PCT-HD can be carried out with trypsin alone, which saves one manual step and ~45min of processing time [9]. Alternatively, high pressure digestion with Lys-C and Trypsin can be combined into a single step, if desired (additional optimization by the user may be required when performing combined enzyme digestion).

Results

Combining the reduction/alkylation step with the high-pressure tissue homogenization step saves time and has no negative effect on subsequent digestion and the number of protein and peptide IDs (Figure 3). The data indicate that the shorter Modified method yields results that are as good as, or better than, the Original method, and that there is essentially no difference between sample alkylated at ambient pressure and at

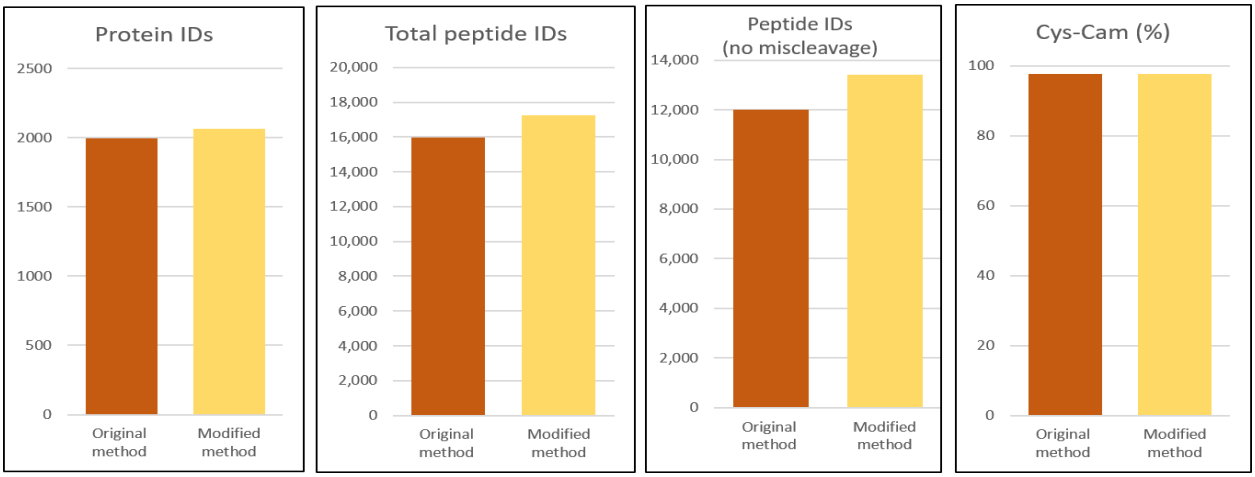


Figure 3. Trypsin digestion using the Original and Modified PCT-HD methods. Original method: Red/Alk was performed at ambient pressure after MicroPestle homogenization at 45kpsi. Modified method: Red/Alk was performed at 45kpsi concomitant with the high-pressure homogenization step. Trypsin digestion was carried out for 90 cycles (~90 minutes) at 20kpsi. Graphs show pooled data from 3 biological replicates and 2 technical replicates. The data indicate that the shorter Modified method yields results that are as good as, or better than, the Original method, based on the number of protein and peptide IDs. Cysteine carbamidomethylation (Cys-Cam) was used as an indicator of the completeness of the alkylation reaction. There is essentially no difference between sample alkylated at ambient pressure and at 45kpsi, indicating that combining high pressure tissue homogenization with the Red/Alk step does not negatively impact the efficiency of the alkylation reaction and can be used to save time and reduce the number of hands-on steps required for PCT-HD.

45kpsi. Therefore, combining high pressure tissue homogenization with the Red/Alk step does not negatively impact the efficiency of the alkylation reaction and can be used to save time and reduce the number of hands-on steps required for PCT-HD.

Further improvement can be achieved by increasing trypsin digestion pressure from 20kpsi to 45kpsi (Figure 4). The number of total protein and peptide IDs, as well as the number of fully tryptic peptides containing no miscleavages, is as high, or higher, in samples digested at 45kpsi compared to those digested at 20kpsi. Digestion at the higher pressure does not cause an increase in semitryptic cleavage, indicating that higher pressure does not significantly modify trypsin specificity.

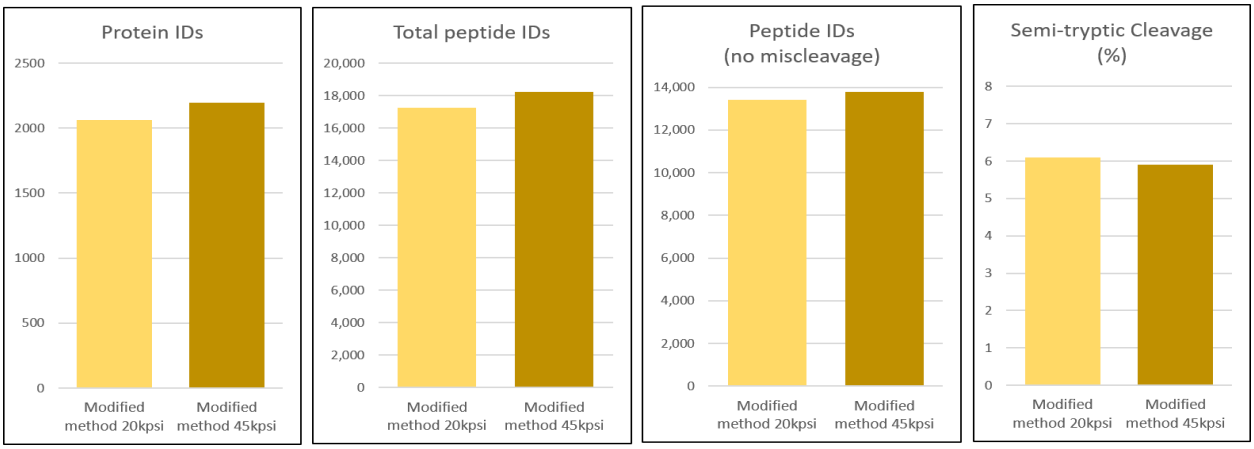


Figure 4. Effect of pressure level on trypsin digestion. Trypsin digestion using the modified PCT-HD method was carried out for 90 cycles (~90 minutes) at either 20kpsi or 45kpsi. Graphs show pooled data from 3 biological replicates and 2 technical replicates. The data indicate that increasing trypsin digestion pressure from 20kpsi to 45kpsi results in more protein IDs. The number of total peptide IDs, as well as the number of fully tryptic peptides containing no miscleavages, is as high, or higher, in samples digested at 45kpsi. Digestion at the higher pressure does not cause an increase in semitryptic cleavage, indicating that higher pressure does not negatively impact trypsin specificity.

Discussion

Based on the results described here, the PCT-HD method can be modified to shorten the workflow and reduce hands-on time by combining the tissue homogenization step with the disulfide reduction/alkylation step. This Modified protocol can be used “as-is” (see below) or as a starting point for further optimization, to design pressure-enhanced 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 chaotropes 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 detergent (e.g. sodium deoxycholate [8]) or urea [5] 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 buffers, to determine whether the higher pressure digestion conditions improve the final result (Figure 4). Conversely, Lys-C has been shown to be much more pressure-stable than trypsin. Lys-C activity assays [6] suggest that this enzyme requires higher pressure levels to significantly accelerate digestion. Therefore, for digestion with Lys-C, we recommend 45kpsi as the optimum pressure level.

Suggested workflow for Modified PCT-HD:

1. Place tissue samples (≤3 mg wet weight) into PCT MicroTubes. Add 30µl lysis reagent such as 4M urea in 50mM ammonium bicarbonate supplemented with reducing and alkylating reagents such as 10mM TCEP and 30mM iodoacetamide (ensure that the reagents used are compatible with single step processing. If using other reagents, such as DTT, it may be necessary to separate the reduction and alkylation into separate steps). Cap with PCT MicroPestles.
2. Place into 2320EXT Barocycler and perform pressure cycling at 45kpsi for 60 cycles at room temperature (20 sec at high pressure, 10 sec at ambient pressure, per cycle). Remove tubes from Barocycler and discard MicroPestles.
3. 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 20min).
4. (Optional) Place MicroTubes into small centrifuge tubes and centrifuge briefly to bring sample down to the bottom. If not performing Lys-C digestion, proceed directly to step 6.
5. (Optional) Add Lys-C enzyme and 100mM ammonium bicarbonate to bring the total volume to ~40 µl so that urea concentration is reduced to 3M. Enzyme-to-substrate ratio should be ~1:50 but can be adjusted up/down to optimize digestion of different samples. Seal with "50 µl" MicroCap. Perform Lys-C digestion in heated Barocycler, using 45kpsi for 45 cycles (50 sec at high pressure, 10 sec at ambient pressure, per cycle). Withdraw and discard used MicroCaps. Tubes can be centrifuged briefly, if desired.
   Note: If performing combined Lys-C/trypsin digestion, skip step 5 and add Lys-C along with trypsin at step 6.
6. 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). If using sodium deoxycholate in place of urea, see [8] 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 90 cycles at 20 or 45kpsi (50 sec at high pressure, 10 sec at ambient pressure, per cycle).
7. Proceed to sample 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. 75 µg protein per sample) in PCT Microtubes were processed using the Standard or Modified 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 Red/Alk 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 Modified PCT-HD, 10mM TCEP and 30mM iodoacetamide were added directly to the samples in MicroTubes (in 4M urea). Tubes were capped with PCT MicroPestles and treated at 45kpsi for 60 cycles (25 sec at 45kpsi, 10 sec at Atm, per cycle). 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). The 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 35^{0}C, for 90min at 20kpsi or at 45kpsi (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.

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, 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. 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 (37,596 proteins) with Cys-CAM and urea denaturation specified in the search settings. The FDR was set at 1%. All data are pooled from 3 to 9 biological replicates (2 technical replicates per sample).

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