# Proteolysis-PrEP: Pressure-accelerated Protein Digestion with Endoproteinase Lys-C

## Introduction

Pressure Cycling Technology (PCT) has been proven to accelerate enzymatic protein digestion. The positive effect of PCT on trypsin digestion is well established [1-4] for improved sequence coverage, higher recovery and significantly reduced digestion times. Not only has PCT been shown to accelerate and improve digestion in solution, but it can also accelerate in-gel trypsin digestion [4, 5]. Additionally, the enhancing effect of PCT on the activity of several other enzymes, including Proteinase K, PNGase F, chymotrypsin and lysozyme has been reported [6-10].

Pressure induces protein denaturation, but the pressure-perturbed proteins assume conformational forms that are different from those caused by thermal or chemical treatment [11]. Pressure-induced denaturation of the substrate proteins leads to better access of the enzyme to previously inaccessible target sites. This, in turn, results in improved and accelerated digestion, as long as the level of pressure that is applied is below the level at which the enzyme itself is denatured and inactivated. In addition, under certain conditions, hydrostatic pressure can have a positive effect on enzyme activity, independent of substrate conformation.

Here we report that PCT increases Lys-C activity and accelerates the digestion of model proteins in solution. In addition, we investigate the effect of various conditions and reagents on enzyme activity and digestion efficiency of protein substrates. The goal of this work is to provide the user with the best set of *starting conditions* for pressure-enhanced Lys-C digestion.

## PCT Sample Preparation System (PCT SPS)

The Pressure Cycling Technology Sample Preparation System (PCT SPS) uses cycles of hydrostatic pressure between ambient and very high levels to control biomolecular interactions and to accelerate certain enzymatic reactions. The PCT SPS uses small, semi-automated bench-top Barocycler instruments in combination with PCT MicroTubes or FT500-ND PULSE Tubes. The specially designed PCT MicroTubes are single-use sample processing containers designed to hold 50-150 µL, while the FT500-ND PULSE Tubes are suitable for larger sample volumes up to 1.4 mL. Samples in MicroTubes can be pressure cycled in sets of up to 12-48 (depending on Barocycler model), to allow batch-mode processing of multiple samples.

## Materials and Methods

Lys-C (mass spectrometry grade) was purchased from Wako. Equine myoglobin was purchased from Sigma. Chromogenic substrate (N-p-Tosyl-Gly-Pro-Lys-pNA) was purchased from Sigma. PCT was performed either in an NEP2320-Enhanced Barocycler or in an SW16 pressure chamber attached to a HUB 440 Barocycler. Both units were equipped with built-in temperature control (via electric heating blankets). Reactions were carried out in 100mM ammonium bicarbonate (ambic) unless indicated otherwise.

Lys-C activity was measured at $35^0$ C using the chromogenic substrate in 100 mM ambic supplemented with urea or other reagents as indicated in **Figure 1**. All reactions were carried out using 150 µl of reaction per MicroTube, in triplicate. Pressure cycling was performed in PCT MicroTubes using one minute cycling parameters (50 seconds at high pressure and 10 seconds at ambient pressure, per cycle). Control reactions were incubated in MicroTubes at the same temperature but without pressure. OD405 was measured after 30 minutes.

Model protein digestion by Lys-C was assayed using native myoglobin as the substrate. All reactions were carried out in 100mM ambic supplemented with urea or other reagents as indicated in text. Pressure cycling was performed in PCT MicroTubes, 45-50 µl of reaction per MicroTube, using one minute cycling parameters (50 seconds at high pressure and 10 seconds at ambient pressure per cycle) unless indicated otherwise. Control reactions were incubated in MicroTubes at the same temperature but without pressure. All digests were performed at 35°C, unless indicated otherwise. Enzyme-to-substrate ratio was ~1:50. SDS-PAGE was carried out using Criterion gels (Bio-Rad). Proteins were visualized by staining with coomasie blue (Bloo Moose Staining Solution, Kerafast). Protein digestion was assayed by SDS-PAGE as the disappearance of the intact parent protein band.

## Results and Discussion

*PCT-Enhanced Lys-C Activity*

To assay the effect of pressure on the enzyme, rather than on the substrate proteins, tests were carried out using a small synthetic substrate (which is unlikely to be directly affected by pressure.) Proteolytic cleavage of the substrate leads to an increase in optical density at 405nm (OD 405). Increase or decrease in enzyme activity was assayed by comparing the OD405 of the treated samples to the OD405 of control samples incubated at ambient pressure (pressure treatment of the substrate alone was also performed to rule out the possibility that the increase in OD405 was due to non-specific pressure-induced breakdown of the chromogenic substrate).

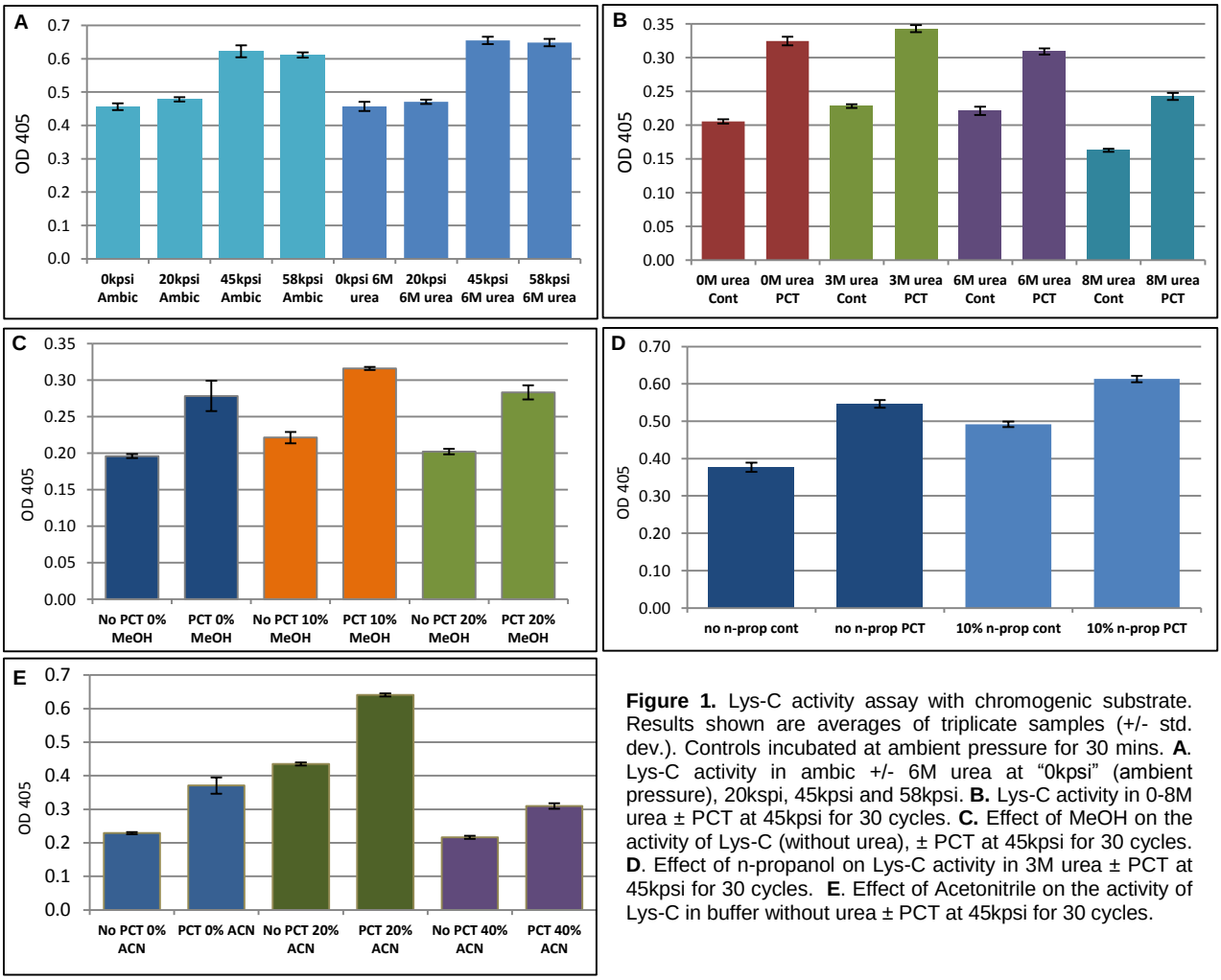



**Figure 1.** Lys-C activity assay with chromogenic substrate. Results shown are averages of triplicate samples (+/- std. dev.). Controls incubated at ambient pressure for 30 mins. **A**. Lys-C activity in ambic +/- 6M urea at "0kpsi" (ambient pressure), 20kspi, 45kpsi and 58kpsi. **B.** Lys-C activity in 0-8M urea ± PCT at 45kpsi for 30 cycles. **C.** Effect of MeOH on the activity of Lys-C (without urea), ± PCT at 45kpsi for 30 cycles. **D**. Effect of n-propanol on Lys-C activity in 3M urea ± PCT at 45kpsi for 30 cycles. **E**. Effect of Acetonitrile on the activity of Lys-C in buffer without urea ± PCT at 45kpsi for 30 cycles.

The effect of PCT on Lys-C enzyme activity is shown in **Figure 1**. Since Lys-C is a protein, it was predicted that pressure levels above a certain point would induce denaturation of the enzyme and lead to reduction or loss of enzymatic activity. Unexpectedly, Lys-C remained very active at 45kpsi, and even at 58kpsi. PCT at 45kpsi significantly increased OD405, both in ammonium bicarbonate alone, and in 6M urea (**Fig 1A**), while PCT at 20kpsi resulted in little, if any, improvement in activity above the control. These results suggest that the enzyme is quite stable at high pressure and is not significantly denatured even at 58kpsi in the presence of 6M urea. This result is in contrast to previous results obtained with trypsin at high pressure, which showed that in the presence of urea, trypsin activity at high pressure is severely reduced [12]. These results suggest that 1) this enzyme is extraordinarily pressure-stable and 2) pressures above 45kpsi may be useful for Lys-C digestion of very-difficult-to-digest targets, where more extensive denaturation of the target protein may improve enzyme access to cleavage sites.

Lys-C activity at elevated pressure was also assayed in the presence of several commonly used denaturants. It was predicted that the synergistic effect of pressure and chemistry could result in changes in Lys-C enzyme

activity, similar to those observed with trypsin under pressure (e.g., the reduction of trypsin activity at pressure in the presence of methanol, or the increase in activity in n-propanol) [12].

**Figure 1B** shows the effect of different concentrations of urea on the pressure-induced acceleration of Lys-C activity. As can be clearly seen from these results, does pressure not contribute to enzyme inactivation in the presence of urea, but even at the highest concentration tested (8M), the enzyme activity is higher under pressure compared to the control incubated in the same urea concentration. Overall, the best concentration of urea for maximal Lys-C activity appears to be 3M, although higher concentrations may be used, with little loss of activity, in order to improve denaturation of very recalcitrant target proteins.

**Figure 1C** shows the effect of different concentrations of methanol on the pressure-induced acceleration of Lys-C activity in 100mM ambic. These data indicate that the addition of methanol has little effect on the activity of the enzyme, and that the addition of 10-20% methanol to the pressure-treated samples does not lead to loss of enzyme activity. Therefore, while methanol does neither helps nor harms the enzyme itself, it may be beneficial in some applications, and its use is not expected to interfere with pressure-accelerated Lys-C digestions.

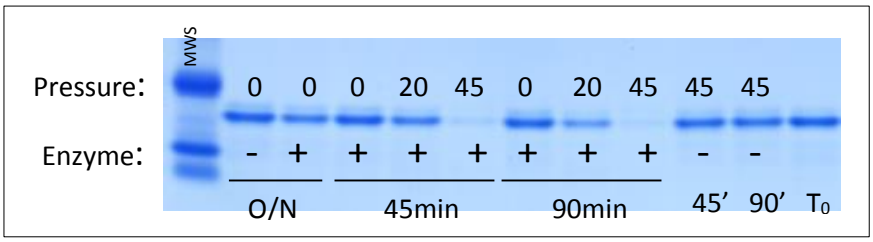
**Figure 1D** shows the effect of adding 10% n-propanol on the pressure-induced acceleration of Lys-C activity in 3M urea. Previously it was demonstrated that addition of 10% n-propanol improved protein digestion by trypsin under pressure. Therefore, we tested whether n-propanol could have a similar beneficial effect on Lys-C activity. Our results indicate that the addition of n-propanol can increase Lys-C activity, which is then further increased by PCT.

**Figure 1E** shows the effect of different concentrations of acetonitrile on the pressure-induced acceleration of Lys-C activity in 100mM ambic. These results demonstrate that the addition of 20% acetonitrile has a pronounced positive effect on the activity of the enzyme, which is further increased when combined with PCT. However, increasing the concentration to 40% is detrimental to the enzyme both at atmospheric and at high pressure.

*PCT-Enhanced Lys-C Digestion of Model Protein*

PCT-enhanced protein digestion was investigated using native horse heart myoglobin as the substrate. Since myoglobin is poorly digested by Lys-C under standard conditions, thus it is a good model of a "difficult-to-digest" protein. Digests performed with or without PCT demonstrated significant improvement in Lys-C digestion of myoglobin at high pressure (**Figures 2 and 3**). This proteolysis occurs more rapidly at 45kpsi than at 20kpsi, although, even at 20kpsi, significant improvement in digestion efficiency is observed at 90 minutes relative to the ambient pressure controls. Furthermore, the loss of intact protein from the gel we confirmed that the loss of intact protein from the gel is due to proteolysis by Lys-C, since the controls incubated at 45kpsi without enzyme show no loss of the parent protein band. These protein digest results agree with the colorimetric activity assays (**Figure 1A and B**), and confirm that PCT at 20kpsi has a slight positive effect on the digestion rate, but that at 45kpsi, the reaction is significantly accelerated. Furthermore, the loss of myoglobin in Lys-C digests could be prevented by the addition of protease inhibitors, further confirming the specificity of the digestion reaction (data not shown).

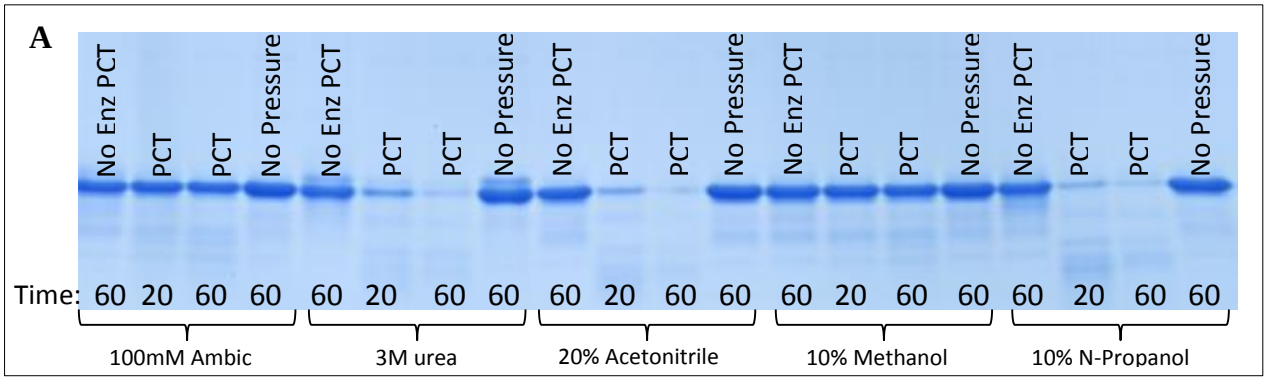
**Figure 2**. Effect of PCT on the digestion of native myoglobin by Lys-C. Digests performed for 45 or 90 mins with PCT at 20 or 45 kpsi were compared to controls digested without pressure. All reactions were carried out at 35$^0$ C in 100mM ambic with 3M urea. Negative controls were pressure cycled at 45kpsi without enzyme. Overnight control digests (O/N) were also performed (with or without enzyme) as a comparison to standard method of digestion. The $T_0$ untreated control is also shown.

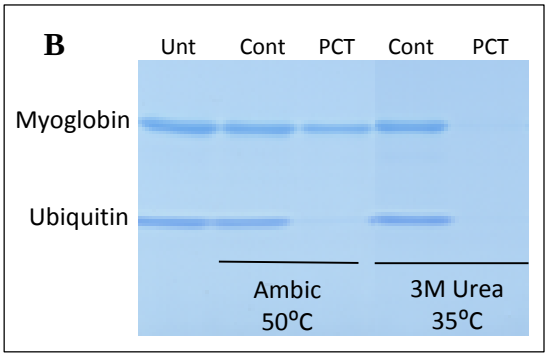



Protein digestion by Lys-C was also assayed in the presence of denaturants, to test whether results obtained with the synthetic substrate (**Figure 1, B-D**) were applicable to real protein digests. **Figure 3A** shows myoglobin digestion by Lys-C at 45kpsi in 100mM ambic with and without various denaturants. Overall, these results agree well with those obtained using the colorimetric assay. Addition of either 20% acetonitrile or 10% n-propanol significantly improved proteolysis by Lys-C at pressure, while addition of 10% methanol had only a marginal effect. One unexpected result was the poor digestion of the protein in 100mM ambic alone, compared to ambic supplemented with 3M urea. This was not predicted by the colorimetric enzyme activity assay, and it is likely due to the tight folding of the native myoglobin, which requires urea for efficient unfolding (even at

45kpsi) to allow Lys-C access to its recognition sites. This conclusion is supported by the results shown in **Figure 3B**. Both myoglobin and ubiquitin are poorly digested by Lys-C at ambient pressure, whether the reaction is supplemented with 3M urea or not. When subjected to PCT at 45kpsi, the ubiquitin is readily digested even in the absence of urea, which agrees with the increased enzyme activity observed in the enzyme assays (**Figure 1**), but the myoglobin requires both urea and pressure for efficient digestion. This result highlights the need for optimization of digestion conditions using real protein samples, not just activity assays.

**Figure 3**. Effect of PCT and chemical denaturants on the digestion of native protein by Lys-C. **Panel A**. Digests of native myoglobin performed for 20 or 60 mins with or without PCT at 45 kpsi. Negative controls were pressure cycled at 45kpsi without enzyme (No Enz PCT) to demonstrate that loss of the parent protein band is not due to non-specific effects of high pressure. All reactions were carried out at 35$^0$ C in 100mM ambic supplemented with either 3M urea, 20% acetonitrile, 10% methanol, or 10% n-propanol. **Panel B**. A mixture of native myoglobin and ubiquitin was digested with Lys-C in either 100mM ambic or in 3M urea with or without PCT at 45 kpsi for 45 minutes. Reactions in ambic were carried out at 50$^0$ C; reactions in 3M urea were carried out at 35$^0$ C. Unt is the untreated control.

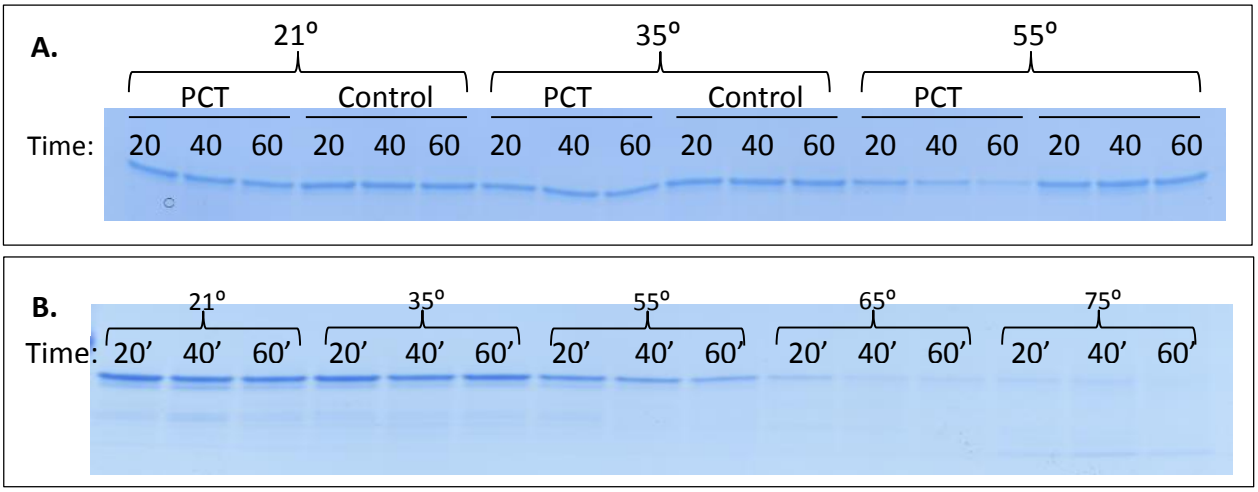



Pressure-accelerated protein digestion by Lys-C was also assayed at different temperatures to test whether higher temperatures could be used in place of denaturants (especially urea) to improve digestion. Digests performed in the presence of urea must be kept at or below 37$^0$ C to avoid protein carbamylation. However, in the absence of urea, higher temperatures can be used as long as inactivation of the enzyme itself does not occur. We therefore tested Lys-C digestion of myoglobin at different temperatures with and without PCT (**Figure 4**). Results presented in **Figure 4A** show that, in the absence of urea or other denaturants, the synergistic effect of heat and pressure leads to significantly improved digestion, compared to heat or to pressure alone. At 55$^0$ C, the pressure-treated samples show significantly more digestion compared to samples incubated either at 55$^0$ C without pressure, or those incubated with pressure at 35$^0$ C or at ambient temperature (~21$^0$ C). **Figure 4B** shows a similar experiment performed using PCT with a wider range of temperatures. This demonstrates that the optimum temperature for Lys-C digestion of myoglobin in ambic (without additional denaturants) is ~55-60$^0$ C. At higher temperatures (65$^0$ C and 75$^0$ C), the enzyme appears to be less efficient, with some accumulation of lower molecular weight fragments detectable below the myoglobin band.

For applications where sample heating may be undesirable an addition of 10% n-propanol was tested to evaluate effectiveness of pressure-accelerated digestion at lower temperatures. **Figure 5** shows Lys-C digestion of myoglobin in 100mM ambic (no urea) supplemented with 10% n-propanol, at room temperature with and without PCT. The gel shows that the addition of n-propanol, although it has little effect on the control digest (in the short timeframe tested), leads to a significant improvement in digestion of the pressure-treated samples. This suggests that the combination of PCT with reagents such as n-propanol may be used to accelerate digestion even at ambient temperature (lower temperatures have not been tested).

**Figure 4**. Effect of PCT at different temperatures on the digestion of native myoglobin protein by Lys-C. All samples in 100mM ambic (no urea or other denaturants). **Panel A**. Digests performed for 20, 40 or 60 mins with PCT and without PCT (Control). **Panel B.** Digests performed for 20, 40 or 60 mins with PCT at 45 kpsi.



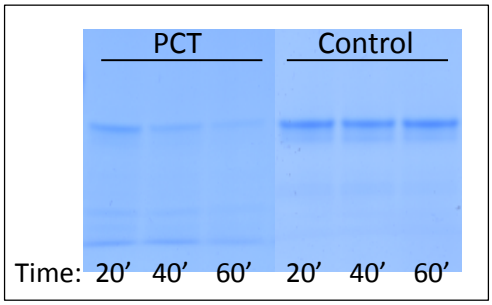




**Figure 5**. Lys-C digestion of myoglobin at room temperature (~21° C) with 10% n-propanol (no urea). Samples were incubated for 20, 40 and 60 minutes with PCT at 45kpsi and without pressure (Control).

## Summary

Here we summarize our optimized PCT parameters and PCT-compatible chemistries (**Table 1**) that produce efficient pressure-accelerated Lys-C digestion. Suggested conditions to generate pressure-enhanced protocols (**Table 2**) can be used "as-is" or as a starting point for further optimization by users for their specific needs and sample types.

**Table1. Compatibility of Various Commonly Used Denaturants with PCT-Enhanced Lys-C Digestion**

| Denaturant | Recommended concentration | Notes |
|---|---|---|
| rea | 3M | Can be used at higher concentrations, up to 6M, with little or no loss of activity. |
| Methanol | Up to 20% | Higher concentrations have not been tested. |
| Acetonitrile | 20% | Higher concentrations (40% and above) are likely to be detrimental to enzyme activity. |
| n-Propanol | 10% | Higher concentrations have not been tested. N-propanol can be combined with 3M urea. |

**Table 2. Suggested Starting Conditions for PCT-enhanced Lys-C Digestion Protocol Optimization**

| | |
|---|---|
| Pressure | 45 kpsi in NEP2320-Enhanced or 35 kpsi in NEP2320. |
| Cycle profile | 50 sec at high pressure/ 10 sec at ambient, per cycle |
| Temperature | 55°C without urea or 35°C for urea-containing samples. |
| Buffer | Ammonium bicarbonate (50-100mM) |
| Denaturant | 3M urea ±10% n-propanol, **or** 20% acetonitrile |
| Time | ~1 hour, depending on target, temperature, and reaction buffer composition |

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