Barocycler Applications Training

Presentation for Scientists and Non-scientists

June 17, 2024

Part 1
General Overview of Barocycler Applications:

Lysis/extraction/MicroPestle

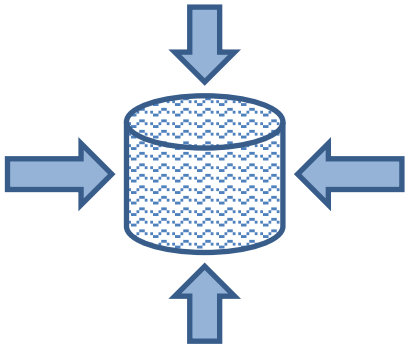
Enzymes/Digestion

Solubility/Folding (Barofold)

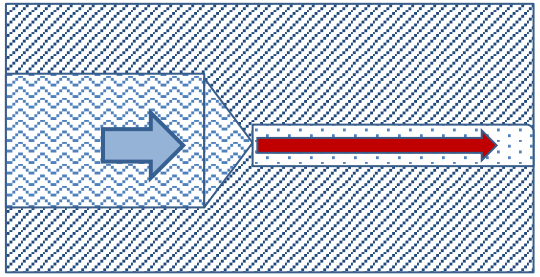
Laser Capture Microdissection (LCM)

Gentle Hydrostatic Pressure vs Disruptive Hydrodynamic Pressure

Hydrostatic pressure



Hydrodynamic pressure



Hydrostatic pressure is gentle, uniform and is associated with only small temperature changes and essentially no shearing force. Hydrostatic pressure is very effective for gently unfolding proteins. This is the basis of both the accelerated enzyme digestion and Barofold applications.

In order to break up solid tissues to extract proteins, nucleic acids, etc, a mechanical component, such as the MicroPestle, is required for most types of samples. The MicroPestle combines hydrostatic pressure and mechanical disruption into a single step.

Hydrodynamic pressure is directional and transforms kinetic energy into mechanical shearing and heat. It is a very powerful tool for lysing yeast or bacterial cells, shearing DNA, denaturing proteins, emulsification, etc. It is not good for accelerated enzyme digestion or protein folding applications.

Tubes and Caps for use in Barocyclers

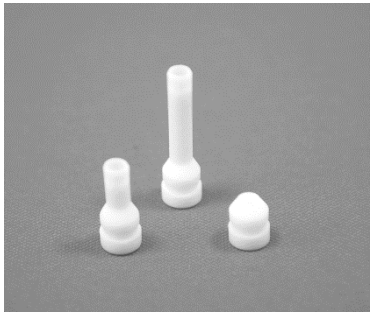
Note that ordinary laboratory sample tubes should not be placed into the Barocycler

MicroTubes can hold up to 150µl of liquid or suspension

-Up to 16 tubes per run can be processed in the 2320EXT Barocycler

MicroCaps are available in three sizes available to accommodate different sample volumes in MicroTubes

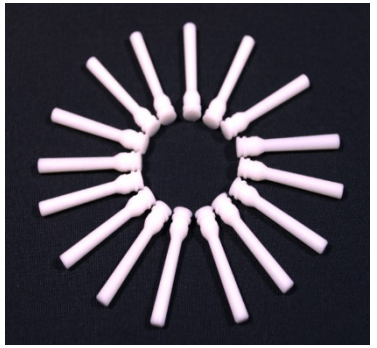
- Longer caps are used to displace excess air when processing 50 or 100µl per tube
- The shortest caps allow the tubes to be filled with a full 150µl



MicroPestles are long, tapered inserts used in place of MicroCaps for Homogenization of **small** (up to ~3mg) solid tissue samples

-Combine the *mechanical* effect of PCT to compress the microTube around the microPestle and the *thermodynamic* effect of PCT on sample extraction.

-MicroPestles can be used for homogenization of samples for extraction of various analytes (lysis buffer determines extraction target).



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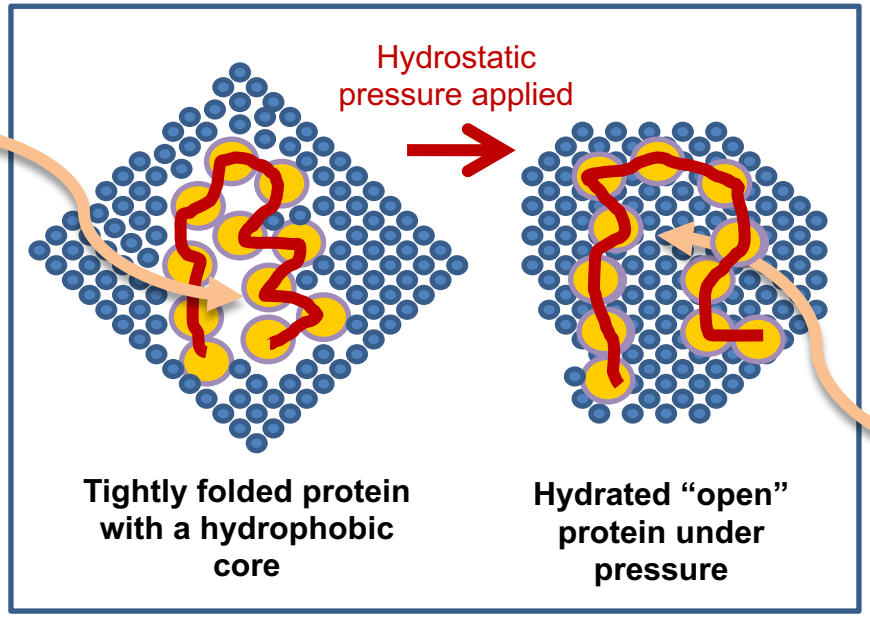
Introduction to Hydrostatic Pressure and its Effects on Proteins

Think of the effect of pressure on proteins as forcing water into a dry sponge, which will soak up the water and swell under pressure.

Folded protein or aggregate. Protein interior not accessible to water or enzymes.



Unfolded proteins will disaggregate. Unfolded protein interior now accessible to water and enzymes.

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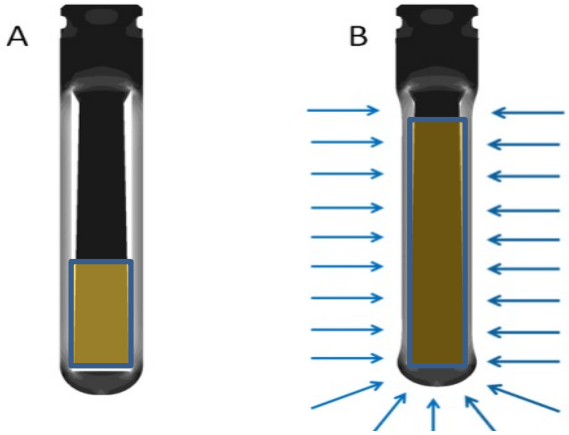
Lysis and Extraction using Barocycler

- Hydrostatic Pressure can be used to lyse cells
  - Mammalian cell cultures or other cell-containing liquids (e.g., blood)
  - Generally, hydrostatic pressure is too gentle for lysis of non-mammalian cells such as yeast, bacteria, etc. Barocyclers are not recommended for lysing samples that have cell walls or other tough coats/cuticles.
- For efficient extraction from solid tissues, the samples must be homogenized/disaggregated/disrupted prior to pressure cycling. Pressure alone will not break down cellulose, collagen, or other matrices in solid samples
  - MicroPestle for very small samples
  - Other types of samples may need to be pulverized/pureed/pounded or otherwise treated to break down insoluble matrix prior to high-pressure processing
- Extraction of various cellular components
  - Proteins, Metabolites, Lipids
    - Improved extraction of poorly-soluble proteins (e.g., membrane proteins)
    - Improved extraction of certain lipids (depending on extraction chemistry)

PCT MicroPestles

The MicroPestle fits into the same MicroTube as used for other PCT applications, and acts as a tiny, disposable tissue homogenizer. Tissue samples up to ~3 milligrams per tube can be processed under identical mechanical and thermodynamic conditions, ensuring convenient, hands-off, and highly reproducible preparation of up to 16 samples at a time (note: Some customers confuse micropestles with the similar-looking long microcaps. Using the caps in place of pestles will result in poor homogenization and low yield)



PCT-MicroPestle: Design and Dechanism of Action

A. Appearance of PCT-MicroPestle and microTube at ambient pressure (MicroPestle shown in black, MicroTube shown in gray). White area represents the highly compressible volume of air. The yellow area is the volume of buffer and tissue.
B. Under high hydrostatic pressure, the air dissolves and the MicroTube is compressed, causing it to narrow and shorten. This mechanical tissue disruption, repeated for ~60 cycles, results in effective tissue homogenization. The high pressure also significantly improves extraction, especially of poorly soluble components such as membrane proteins.

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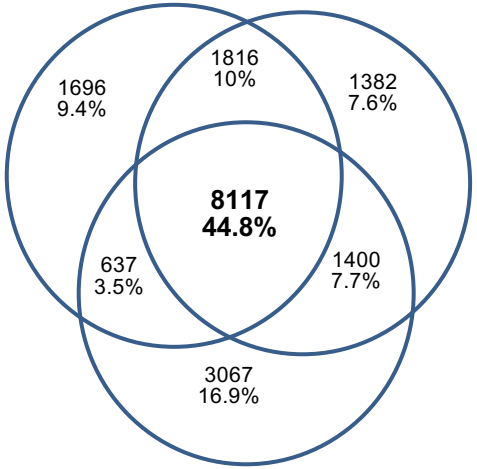
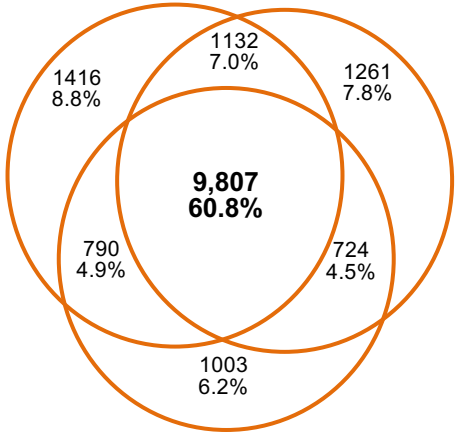
MicroPestle vs Conventional Tissue Processing for Protein Extraction:

Better Sample-to-sample Reproducibility than Conventional Method

Conventional manual tissue homogenization



MicroPestle Extraction
60 pressure cycles at 45kpsi



Extraction with MicroPestle improves reproducibility between replicates

Individual data for triplicate biological samples (# of peptide IDs).

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Enzymes Under Pressure

Accelerated digestion for mass spectrometry and other proteomic and Biopharma applications with enzymes such as

- **Trypsin**
- **Lys-C**
- Glu-C
- Chymotrypsin
- Pepsin
- Thermolysin
- Lysozyme
- Proteinase K
- **PNGase F**

Note that high pressure does not accelerate DNases or RNases

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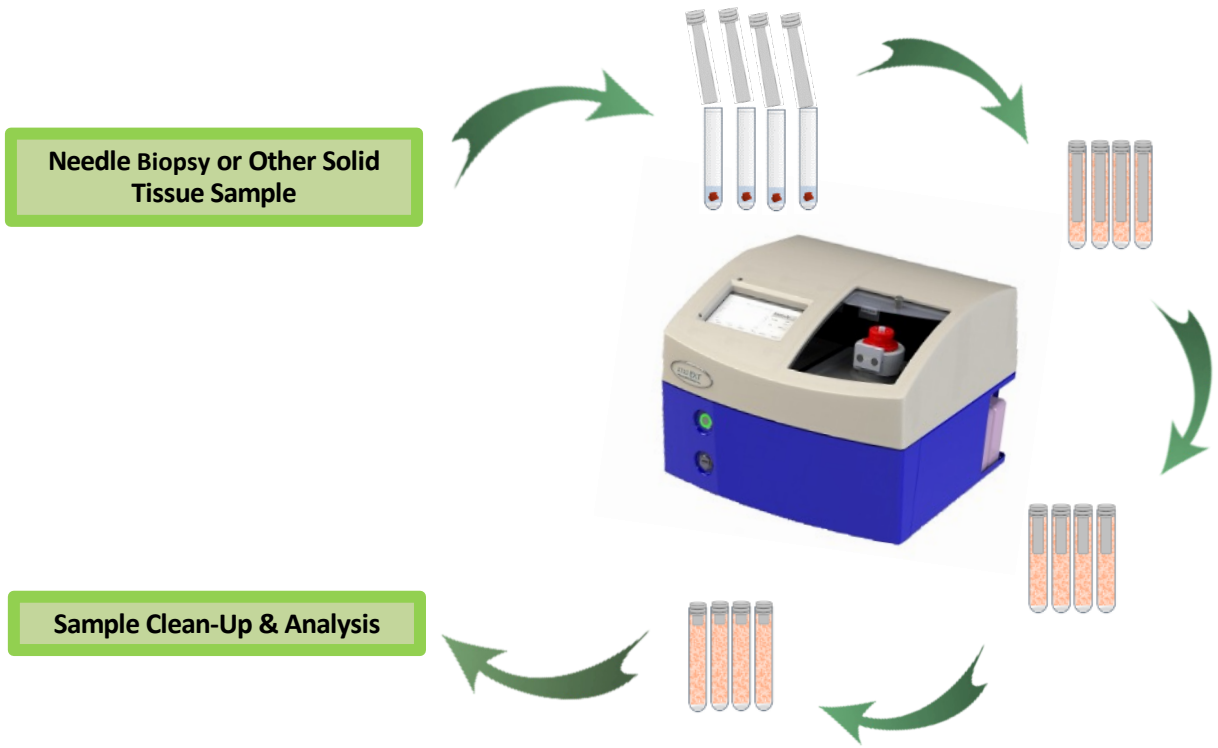
Benefits of Pressure for Digestion in Proteomic Applications

- Better digestion of difficult-to-digest proteins such as
  - Disulfide crosslinked proteins (“unreduced” proteins)
  - Membrane-derived, or other poorly soluble proteins.
- We have data showing that there is no significant increase in non-specific cleavage (where an enzyme starts to clip at wrong or random sites)
- A slight increase in missed cleavages is normal in high-pressure digestion (where an enzyme skips a site it should have clipped). We don’t know why.
- We have observed no significant increase in peptide modifications like oxidation and deamidation. However, customers who focus on these types of modifications and have more sensitive assays may observe some changes that we have not.

For customers asking about protein modifications during high-pressure digestion, we have an application note and a poster on our website

PCT-HD
(also known as “PCT-SWATH” or “PCT-ABLE”)

Sequential single-tube tissue* protein extraction and digestion for proteomics or other applications



*Refers to soft or semi-soft animal tissues such as liver, kidney, brain, muscle, tumor, etc. Does not apply to bone, cartilage, or plant tissues.

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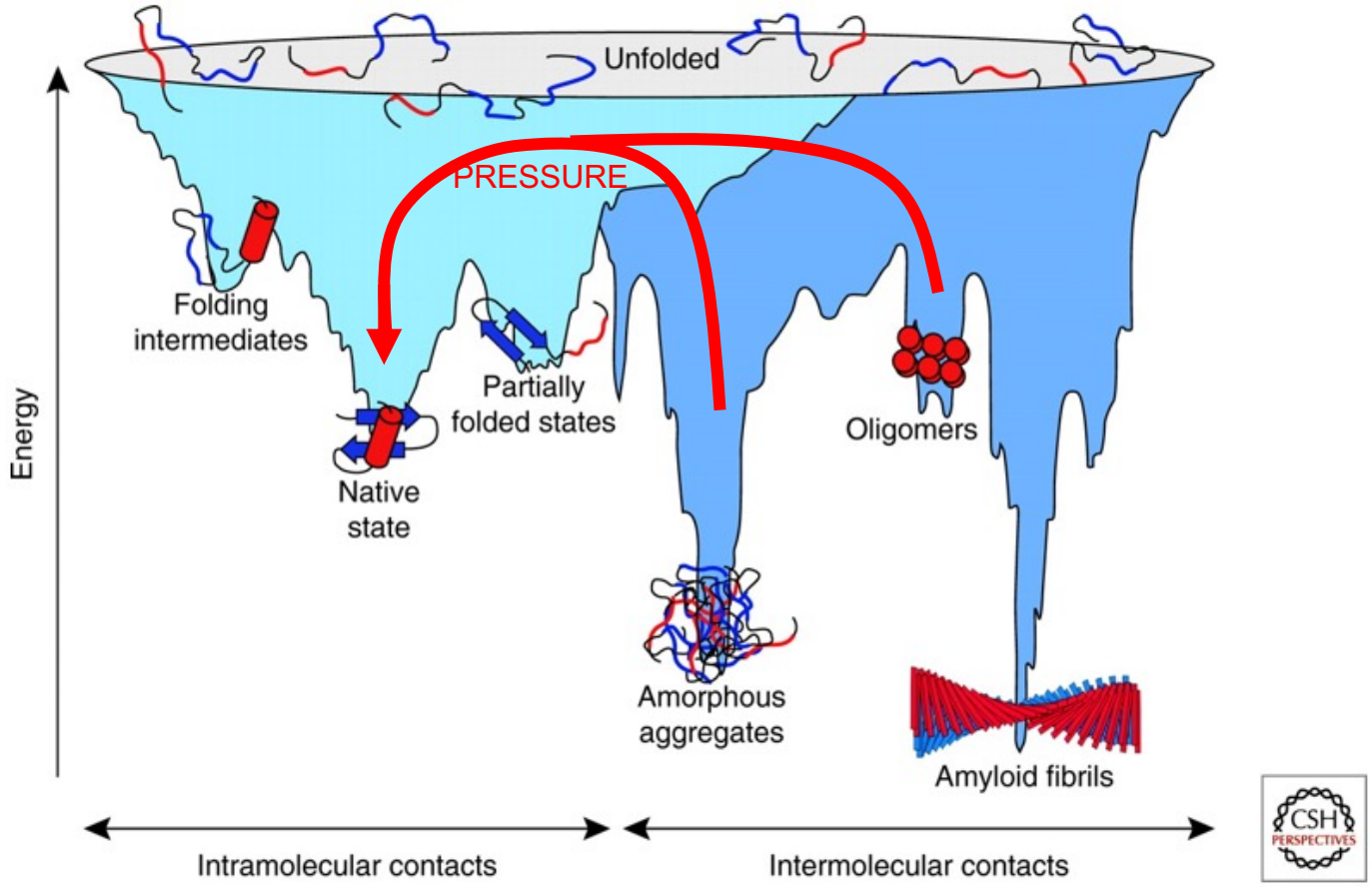
Barofold: Protein Solubility and Folding

Energy landscape of protein folding/unfolding and aggregation



R. Martin Vabulas et al. Cold Spring Harb Perspect Biol 2010;2:a004390

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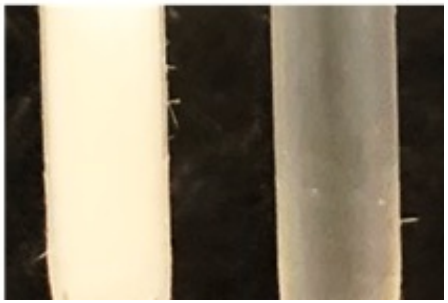
Soluble Protein Can be Extracted from Inclusion Bodies by Brief Incubation at High Pressure

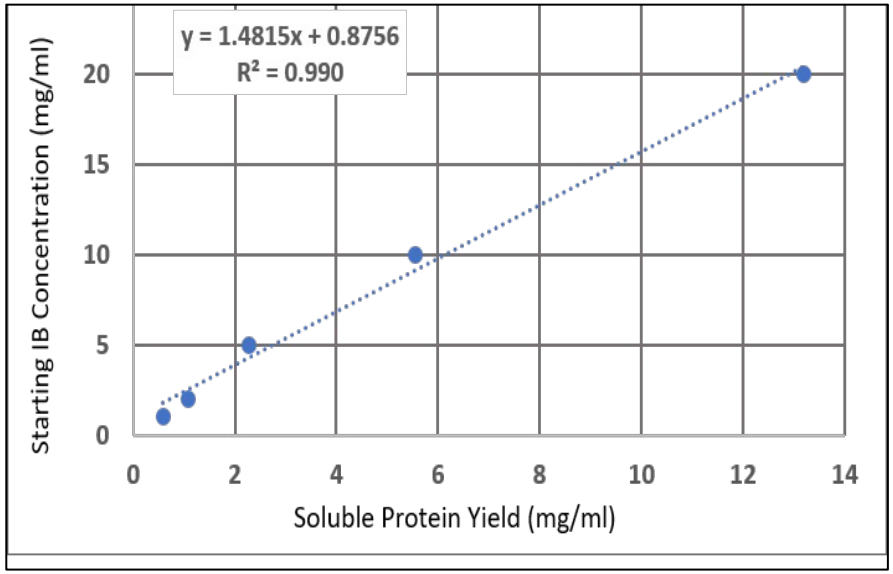
Proinsulin inclusion bodies: Protein yield is linear across a wide range of starting IB concentrations

BEFORE Pressure IB suspension is milky white



AFTER Pressure Protein solution is clear

IB solubilization for 5 minutes at 45,000 psi



Data taken from Proinsulin Application Note on PBI website

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Laser Capture Microdissection
Integration of PBI MicroTubes and Leica Sample Collection Stage

Leica MICROSYSTEMS

Fits LMT350 Stage

Collect samples directly into microtubes for subsequent processing in Barocycler

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Part 2A

Deeper Dive into Applications and Methods:

Using MicroPestles and MicroTubes

Enzymes/Digestion examples and conditions

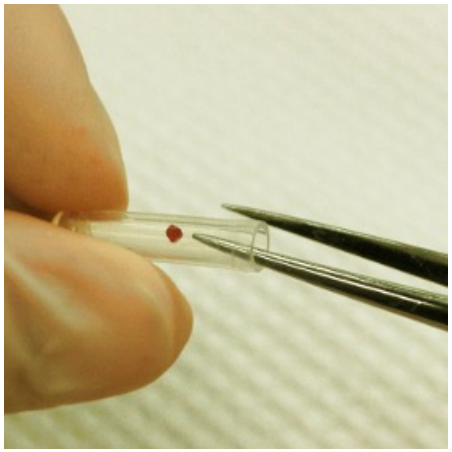
Synergy of pressure and chemistry

PCT-HD

Protein Disaggregation and Folding (Barofold)

Extraction Using Barocycler and MicroPestle Suggested Starting Conditions

Temperature: Ambient or chilled, depending on application.
Pressure: 45kpsi (310 MPa) for most applications
Time 1: 20 seconds at high pressure
Time 2: 10 seconds, no pressure
Cycles: 60 cycles. Fewer cycles may be sufficient depending on tissue type and extraction reagent. Note that excessive cycling can lead to protein aggregation and reduced yield. Optimization will be required for extraction of active enzymes under non-denaturing conditions.



Place tissue into MicroTube

Note that optimal tissue mass is in the range of <3-5mg for animal tissues.

Add **30µl** extraction reagent of choice

Insert MicroPestle using capper tool

Note: using a larger volume will result in poor tissue homogenization. Water is less compressible than air so replacing air volume with buffer will prevent the tube from flexing around the pestle leading to sub-optimal extraction yield.

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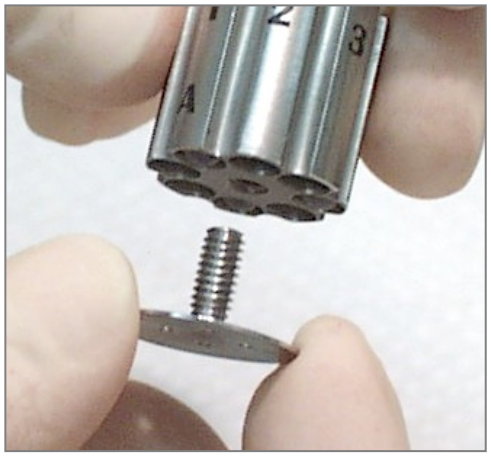
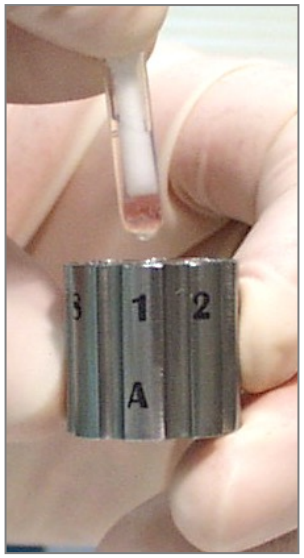
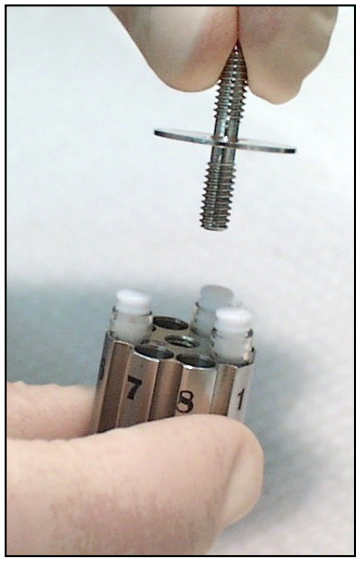
MicroTube Cartridge Assembly



Insert an end cap into the first cartridge and tighten well.



Space tubes evenly around the cartridge. If running one sample in the cartridge, use a balance tube to ensure that stack does not lean.



Use connecting screw to assemble cartridge stack. Ensure that the cartridges are tightly screwed down. A tight fit is important, but do not over tighten.

Insure that tubes in the cartridges are facing inward (“head-to-head”).

The Barocycler can hold a stack of two 8-place cartridges (16 samples total).

Note that it is not necessary to run all 16 samples at a time but is advisable to use dummy tubes if running less than 4 samples. Arrange tubes symmetrically in one cartridge. Leave second cartridge empty and assemble as shown.

Gently drop the assembled cartridge stack into the Barocycler chamber. Close the chamber and initiate pressure cycling.

After pressure cycling, use the magnetic wand (provided) to remove the cartridge stack from the Barocycler chamber.

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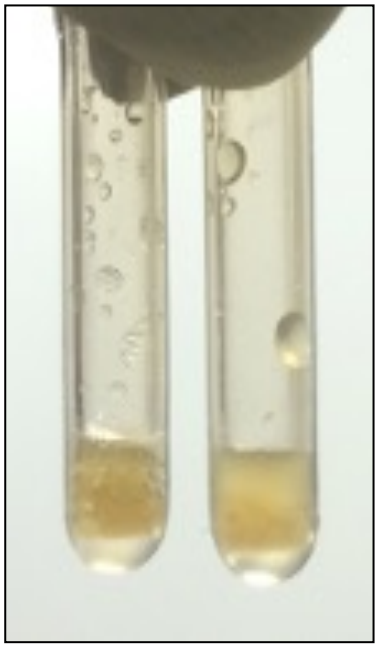
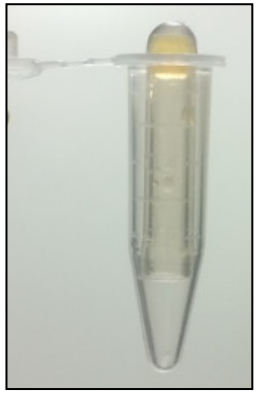
Sample Recovery After Homogenization with PCT MicroPestle

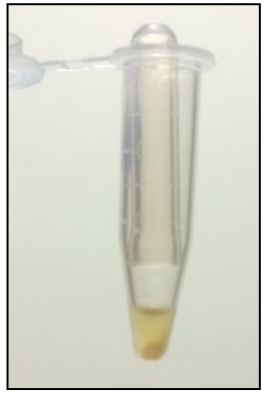
(If not proceeding to protein digestion in the same MicroTubes)



After the MicroPestle is withdrawn, droplets of sample may cling to the sides of the tube.

Transfer sample to centrifuge tube





Invert the open MicroTube into 0.65ml eppendorf-style centrifuge tube. Spin the assembly for 1min to collect sample.

or

centrifuge directly in MicroTube

To centrifuge sample directly in MicroTube, place the microTube into an 0.65 or 1.7ml eppendorf-style centrifuge tube and spin.

Note: MicroTubes can be centrifuged at up to 20,000g (20,000rcf), but lower speeds (~5000g) are recommended to prevent wedging of MicroTubes into holder tubes.

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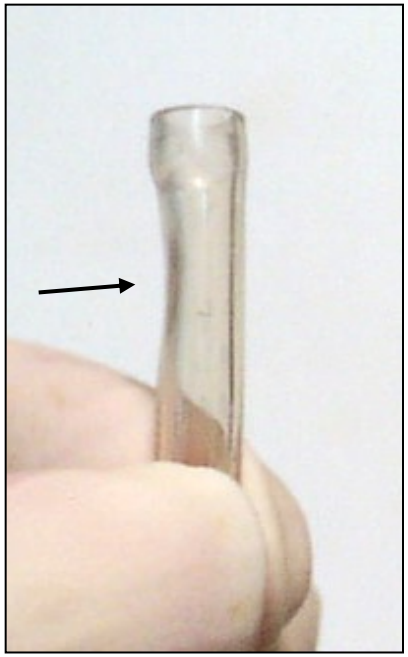
Proper Capping of MicroTubes to Avoid Tube Deformation at High Pressure

Troubleshooting

Tube denting is a result of under-loading the MicroTubes
(too little sample and too much air).



Slight under-loading results in a slight dent (left), severe under-loading results in severe denting (right). Dented tubes do not leak, but the dent will make it more difficult to insert a pipet tip into the tube. In these cases, a thin gel-loading tip may be used to recover the sample. Note that very dented tubes may be more difficult to remove from the cartridge, due to wedging of the deformed tube.

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Part 2B

Deeper Dive into Applications and Methods:

Using MicroPestles and MicroTubes

Enzymes/Digestion examples and conditions

Synergy of pressure and chemistry

PCT-HD

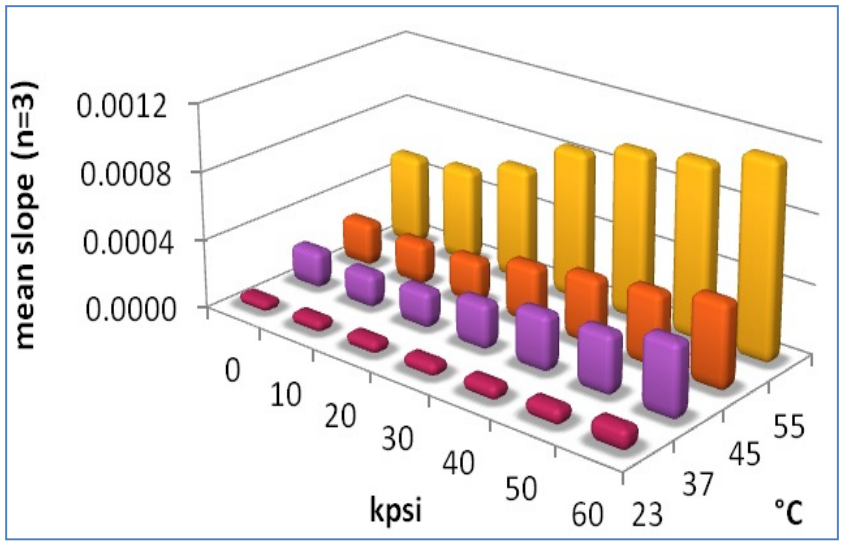
Protein Disaggregation and Folding (Barofold)

Effects of High Hydrostatic Pressure on Enzyme Activity

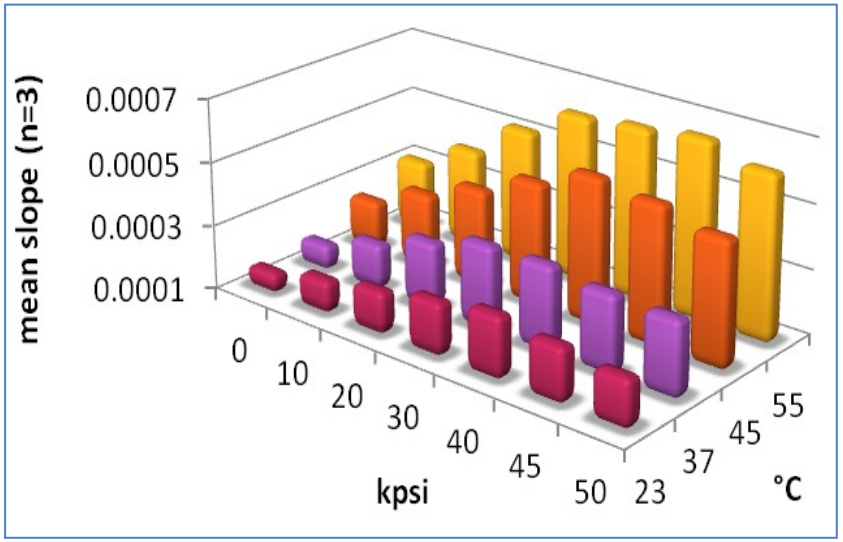
Enzymes such as Lys-C and Chymotrypsin are more active at high pressure.

We recommend 45kpsi as the starting condition for these digests.

Lys-C



Chymotrypsin



Enzyme activity measured at high pressure (Note that this is NOT the same as measuring activity at atmosphere after enzymes have been treated at high pressure).

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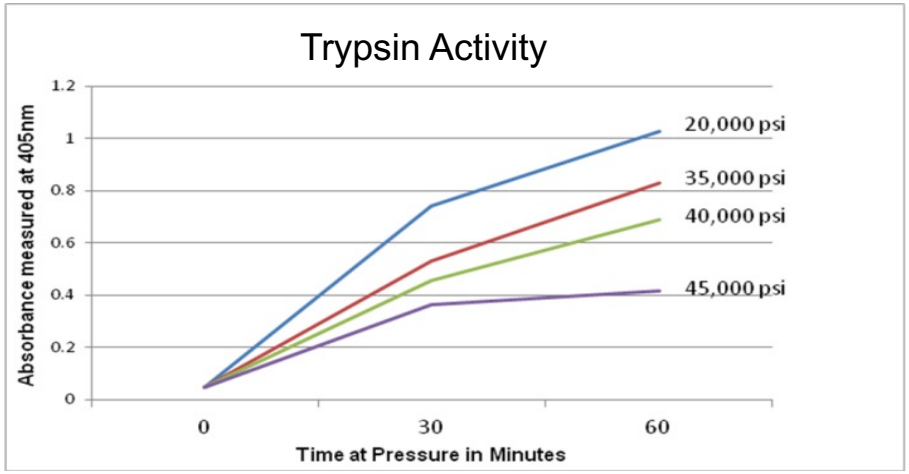
Pressure has different effects on different enzymes

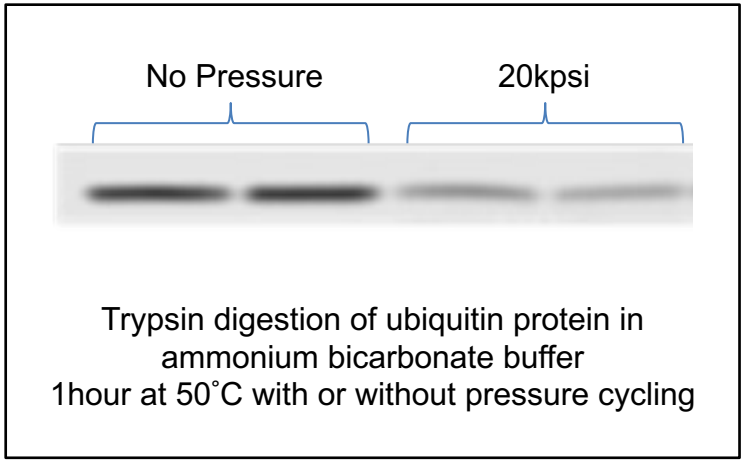
Effect of pressure on the enzyme itself is half the story

Effect of pressure on target protein is the other half of the story

Enzymes, such as Lys-C and Chymotrypsin are most active at very high pressure, so we recommend 45kpsi as the starting condition when using those enzymes. Other enzymes, such as Glu-C work best at more moderate pressure levels in the range of 20-30kpsi.

Conversely, trypsin activity actually decreases somewhat with increasing pressure. However, for real-world applications this is offset by significantly improved "digestability" of the target, resulting in better overall trypsin digestion under pressure. This is part of the reason we recommend 20kpsi for trypsin as the default pressure, increasing to 45k only if 20kpsi does not give a good result.





Trypsin digestion of ubiquitin protein in ammonium bicarbonate buffer
1hour at 50˚C with or without pressure cycling

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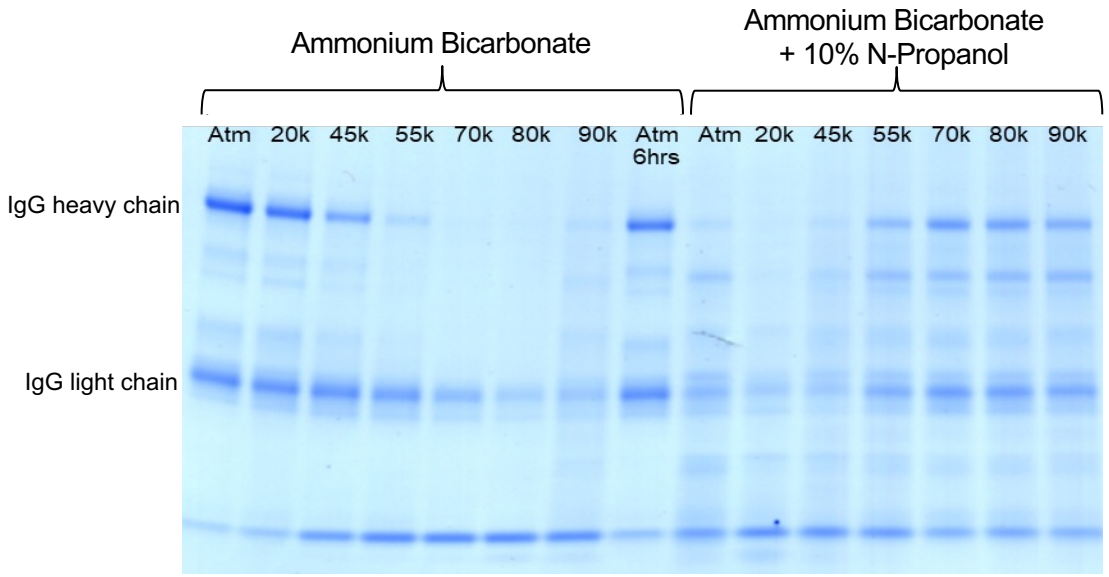
Synergy of Pressure and Chemistry

Example: Addition of 10% N-propanol lowers the optimal pressure level for rapid digestion of native IgG by Trypsin



Native IgG was incubated for 30 minute with trypsin (1:50 E/S), in 50mM AmBic buffer with or without 10% N-propanol at 50ºC at the indicated pressure.
Protein was reduced with DTT prior to loading on gel.

One Word of Caution: The synergy of pressure and chemistry can combine to denature not only substrate proteins, but enzymes as well. It is important to test enzyme activity at pressure when incorporating new reagents (solvents, detergents, etc) into any high-pressure protocol.

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Chemical compatibility data for various reagents and enzymes are available on our website

Example: High pressure **trypsin** digestion chemical compatibility

Reagent	Compatibility	Notes
Urea	Compatible at or below ~0.8M final concentration.	Higher concentrations can be tested but may inhibit reaction. See application notes for more information. Reactions containing urea must be kept at or below 37˚C to avoid urea-induced protein carbamylation.
Guanidine-HCl	Should be avoided.	If used, should be kept below 50mM.
Acetonitrile	Compatible at 10% final concentration.	Higher concentrations may inhibit reaction. Some customers have had very good results using 10% acetonitrile.
n-Propanol	Compatible at 10-15% final concentration.	Higher concentrations may inhibit reaction**. In some applications adding 10% N-propanol has shown significant benefit for trypsin digestion.** Note that this is not a commonly used reagent in proteomic labs so they may not want to use it. Do not mistake isopropanol (2-propanol) for N-propanol (1-propanol). These are NOT interchangeable.
CHAPS	Should be avoided.	Inhibits reaction even at 0.2% final concentration.
SDS	Compatible at up to 0.025% final concentration.	Up to 0.05% can be used in some applications, but higher concentrations will inhibit reaction.
RapiGest™ (This detergent is popular but can be very expensive)	Compatible at 0.05%.	Use less reagent in high-pressure digests than what the manufacturer recommends for standard digestions

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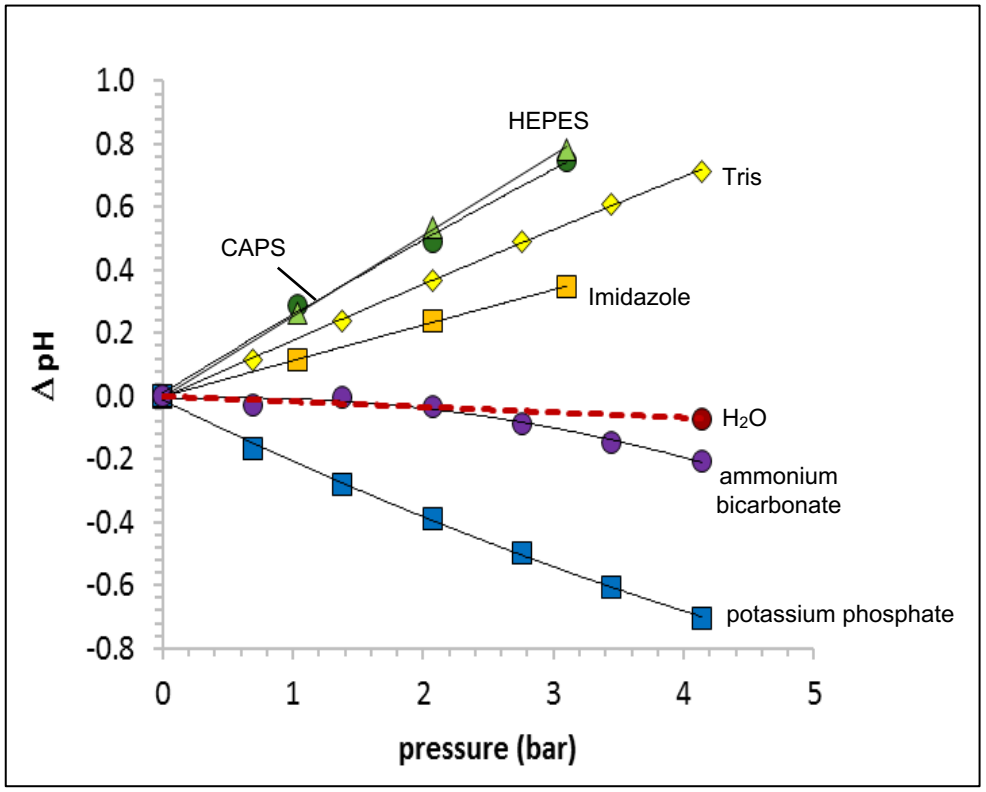
Hydrostatic Pressure and Chemistry

Effect of pressure level on pH of some common buffers



Pressure is a Thermodynamic Variable as Significant as Temperature.

High Pressure can have a Range of Effects on the Chemistry of Various Reactions.

Small but significant pH changes may occur in certain buffers under pressure.

Buffer pH will return to normal once pressure is released.

pH can go up or down, depending on the buffer used.

Changes in pH may affect enzyme digestion, protein aggregation/disaggregation, and other processes.

4 bar = ~60,000psi

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Part 2C

Deeper Dive into Applications and Methods:

Using MicroPestles and MicroTubes

Enzymes/Digestion examples and conditions

Synergy of pressure and chemistry

PCT-HD

Protein Disaggregation and Folding (Barofold)

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PCT-HD incorporates the MicroPestle and accelerated digestion methods into an efficient single tube workflow

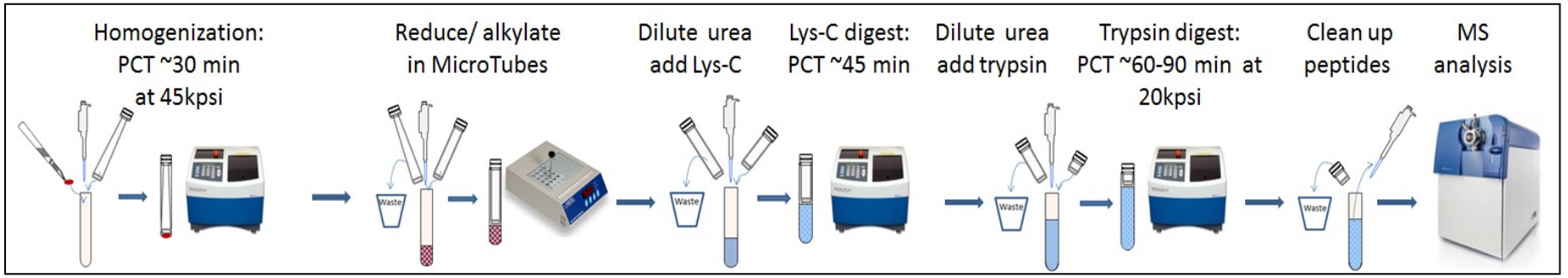
Step 1 Tissue extraction

MicroPestle and high

hydrostatic pressure

cycling.

Step 2 Protein digestion at high pressure using
Trypsin
or
Sequential digestion with Lys-C and trypsin.
or
Simultaneous digestion with trypsin + Lys-C
or
Digestion with other enzyme(s)



Conventional
reduction/alkylation
step is optional

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The Basic PCT-HD Method is Highly Modifiable for Compatibility with Customer Needs and Wants

Method (n=3)	Workflow Time (approx)	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 (0MC)	Tissue mass (mg)
Conventional method control (no PCT, o/n digest)	20 hours	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	4 hours	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	3 hours	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	2 hours	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)	2 hours	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

Example of method variations and results. Liver tissue protein extraction and digestion with Lys-C and Trypsin.

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A Few More Points to Keep in Mind Before We Move on…

- Hydrostatic pressure does not break covalent bonds (such as those in DNA or RNA, and those holding individual proteins together. This is why DNA shearing does not occur in the barocycler. Note that this is not the same as DNA breakdown/loss due to presence of DNase or other enzymes, which can occur regardless of pressure). **Pressure can disaggregate/separate from one another UNLESS the aggregates are held together by covalent bonds** (intermolecular disulfide bonds).
- **Pressure-induced protein unfolding promotes improved access of enzymes such as trypsin (and other reactants, especially water) to their target sites. It can also have a negative effect on enzyme structure and activity. Remember that enzymes are proteins too.**
- **Anything added to a digest reaction (alcohols, urea, detergents, etc) might act differently under pressure than at atm. We have tested many reagents, but we can't test everything.**
  - Pressure works in synergy with chemistry. Sometimes you need to scale back on certain reagents, or replace them with other reagents, when adding pressure to the mix.
  - Buffer pH may be affected while at pressure. This effect is usually small, but is important to keep this in mind as some enzymes and reactions are very pH sensitive
  - Most enzymes work better/faster when warm. **Pressure does not replace heating** (although may change the optimum temp for any given reaction), so almost all enzyme digestions must be carried out in a heated Barocycler. Customer demos at room temp almost never work well.
  - Pressure will not compensate for the presence of chemical inhibitors in a reaction. If trypsin inhibitors are added during extraction, they will still be active and will negatively affect subsequent digestion with trypsin (this seems obvious but was an issue in some early PCT-SWATH publications).

Part 2D

Deeper Dive into Applications and Methods:

Using MicroPestles and MicroTubes

Enzymes/Digestion examples and conditions

Synergy of pressure and chemistry

PCT-HD

Protein Disaggregation and Folding (Barofold)

"Barofold" applications use high-pressure hold times ranging from a few minutes to several hours. These applications generally call for static, rather than cycled, pressure conditions

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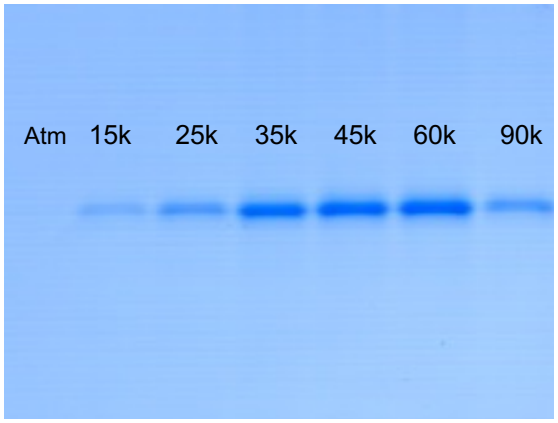
Inclusion Bodies: Solubilization at High Pressure

Inclusion bodies (IB) are VERY DIFFERENT from other protein aggregates in the way they respond to disaggregation by pressure. It is important to know whether the target of disaggregation is an inclusion body or a different type of aggregate.

Moderate pressure levels in the 30-60kpsi range are sufficient for solubilization of most inclusion bodies, making the 2320EXT Barocycler a convenient bench-top instrument for screening large numbers of IB-incorporated proteins and solubilization reagent formulations



Example of a Protein extracted from IB at indicated pressure levels for 30 minutes in ammonium bicarbonate pH8 with 30mM DTT

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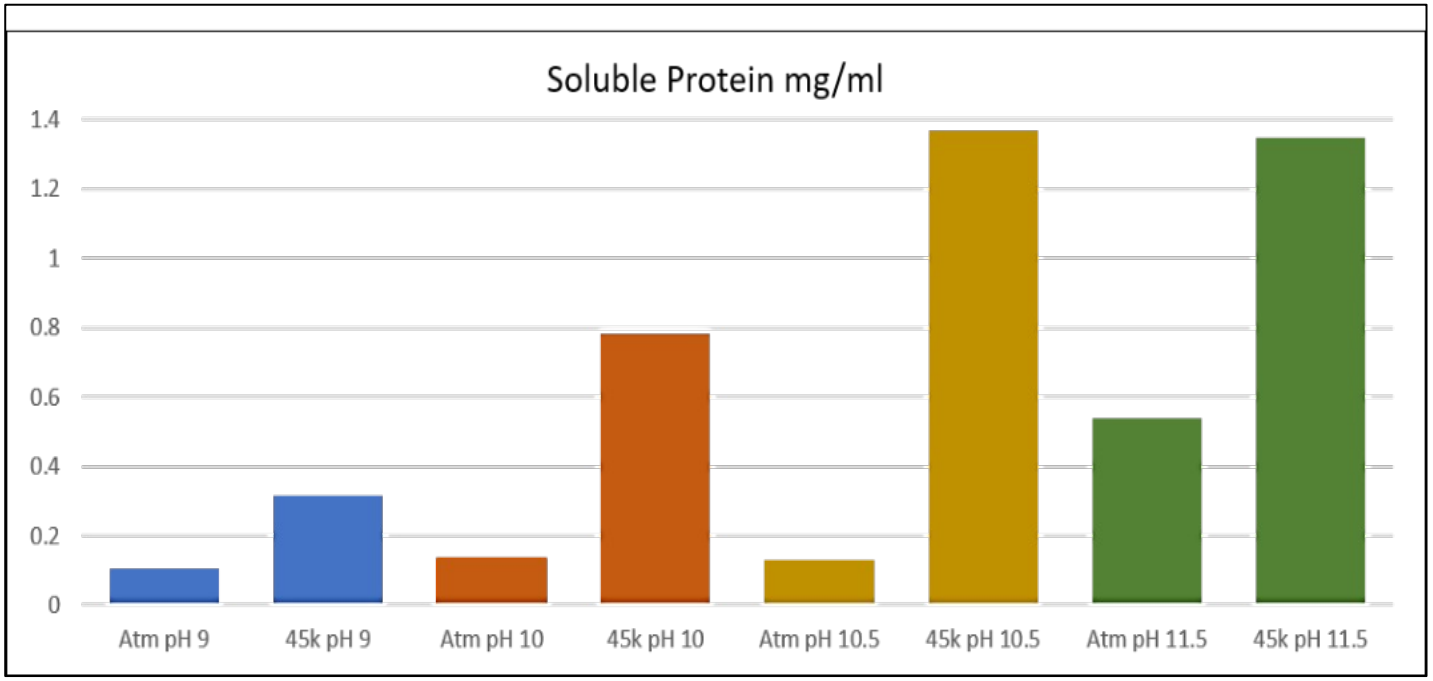
Inclusion Bodies solubilization and Extraction Reagent pH

For most of the inclusion bodies we have studied, high-pressure extraction is more effective in alkaline buffers than near neutral pH.



Disaggregation of Proinsulin IB and release of soluble protein. IB were suspended in buffer adjusted to the indicated pH. Samples were incubated at ambient pressure for 10 mins or at **45kpsi for 5 mins.** Insoluble residue was removed by centrifugation.

Proinsulin inclusion bodies dissolve rapidly at high pressure. Other inclusion bodies may require longer time at pressure, or higher/lower pressure level, or a different extraction buffer formulation.

Data taken from Proinsulin Application Note on PBI website

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Inclusion bodies are not like other protein aggregates

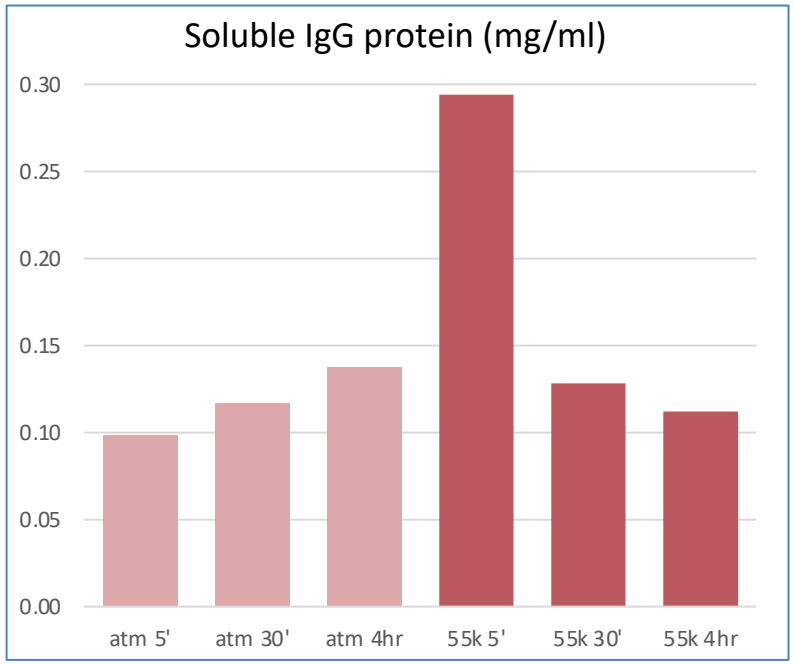
IB are formed in bacteria by a specific process that results in large, tight, homogenous aggregates with very specific characteristics. IB can be solubilized very effectively by high pressure. Conditions for high-pressure extraction of protein from IB are more predictable and consistent than for other types of aggregates. Therefore, relatively little optimization is usually required to get high yields of soluble protein from a suspension of inclusion bodies incubated briefly at high pressure.

However, it is VERY important to not conflate IB solubilization with protein folding into a stable and active final conformation. Folding requires formation of specific 3D structures, often with disulfide bonds, chelated metal ions, or other complex structures. In some cases, extraction and refolding may be carried out in a single step, but for most proteins extraction and refolding will likely be sequential processes.

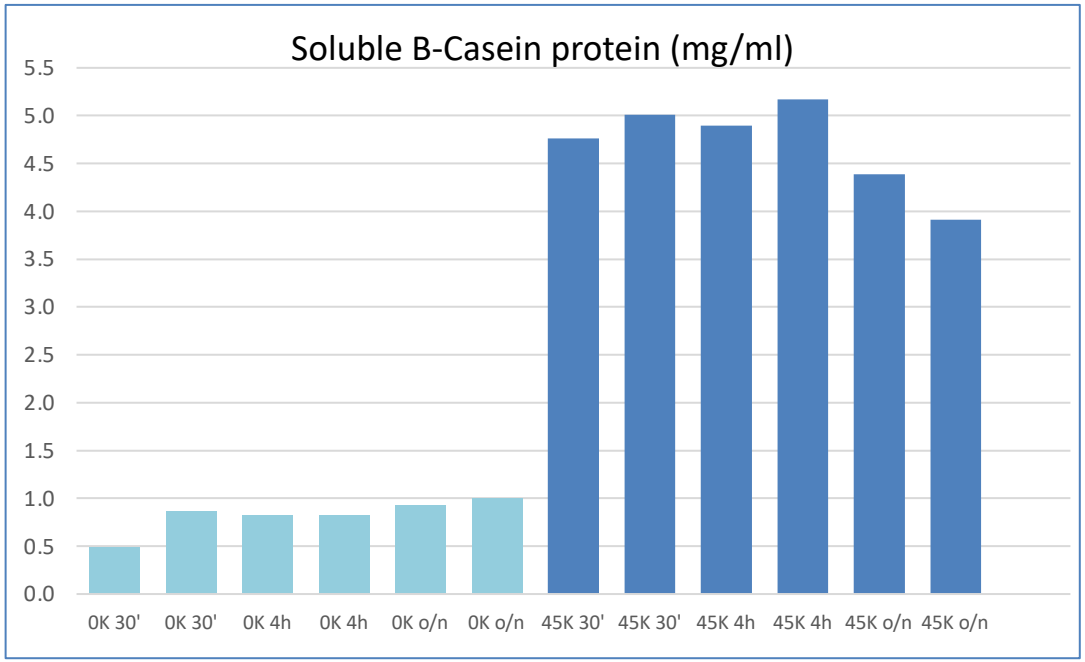
High Pressure can also be used to disaggregate and solubilize various proteins that have aggregated during extended or improper storage.
This type of protein aggregate is different from inclusion bodies and may require protein-specific pressure and chemical conditions to generate the desired result

Effect of time at pressure on yield of soluble protein from different types of protein aggregates is protein-specific

Note that temperature has a significant effect on protein solubilization at pressure. Optimal temperature, as well as time and pressure level may vary for different proteins



In this example, rapid solubilization occurs within 5min at high pressure. Note that continuing to hold this protein at high pressure results in re-aggregation and loss of soluble material.



In this example, the protein dissolves in less than 30min at 45kpsi, but remains soluble even after 4hrs at high pressure.

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Summary: Barofold Disaggregation and Solubilization of Inclusion Bodies and Other Protein Aggregates

- High Pressure can be used to help solubilize insoluble aggregates and/or disaggregate soluble protein aggregates.
  - Most applications will require static hold times, not pressure cycling
- Optimal pressure levels, time at pressure, pH, and temperature may need to be determined empirically for different sample types
  - Inclusion bodies are (usually) relatively easy to dissolve with pressure, but may require alkaline pH
  - Note that one protein may require very different conditions depending on the type of aggregates that have formed.
- So far, data suggest that long (e.g., >4hrs) pressure holds are not required to solubilize most aggregates.
  - longer pressure holds may turn out to be required for some targets, however, it should not be assumed that longer is always better.
- Current data suggest that pressure ramping is not required for solubilization (at least for the proteins we have tried to-date). It is possible that ramping pressure down could be beneficial for refolding of some proteins.

Thank You!