Rapid Protein Extraction and Trypsin Digestion with Pressure Cycling Technology (PCT)

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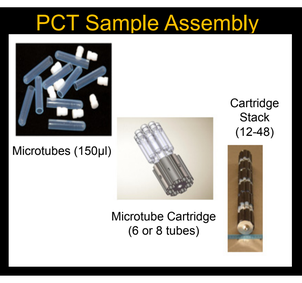
What is PCT?

PCT is the application of cycling high and low hydrostatic pressures to a solid (tissue) or liquid (trypsin digest) sample.

Suzuki C, Suzuki K. *J Biochem* 1962; **52**: 67.
Ruan K, Lange R, Bec N, Balny C. *Biochem Biophys Res Commun* 1997; **239**: 150.
Chicon R, Belloque J, Recio I, Lopez-Fandino R. *J Dairy Res* 2006; **73**: 121.
Smejkal GB, Robinson MH, Lawrence NP, Tao F, Saravis CA, Schumacher RT. *J Biomol Tech* 2006;**17**: 173.
Ringham H, Bell RL, Smejkal GB, Behnke J, Witzmann FA. *Electrophoresis* 2007; **28**: 1022.
Pevsner P, Vecchione D, Stall B, Remsen T, Anand S, Stern A. 2007. British Mass Spectrometry Society, Edinburgh, Scotland.
Pevsner P, Vecchione D, Remsen T, Kessler P, Levers N, Yang P, Stern A, Samuels H. British Mass Spectrometry Society, 2007 Robinson College,Cambridge .
Pevsner P, Vecchione D, 1 Remsen T, Kessler P, Momeni M, Duddempudi S, Francois F, Stern A, Anand S. 2007 British Mass Spectrometry LCMS Meeting, Robinson College, Cambridge, UK.

PCT Sample Assembly



PCT Barocycler

How does PCT Work?

- Pressures between 29k-145k PSI (2-10 kbar) denature protein
- Water-water and protein-water hydrogen bonds increase.
- Protein-protein hydrogen bonds decrease.

Silva JL, Weber G. *Annu Rev Phys Chem* 1993; **44**: 89.

How does PCT Work?

- Hydrophobic groups exposed to water.
- Water penetrates protein, increasing internal pressure.
- Hydrogen bonds responsible for secondary and tertiary structure of protein destabilize.

Ruan K, Lange R, Bec N, Balny C. *Biochem Biophys Res Commun* 1997; **239**: 150.

How does PCT Work?

- Non-native salt bridges, hydrophobic, and electrostatic interactions disrupted
  - –Electrostatic replusions increase.
  - –Repulsions favor unfolding.

Balny C. *Journal of Physics: Condensed Matter* 2004; **16**: S1245.

PCT and Trypsin Digest

- Pressures >35k PSI(2.4 kbar) will denature trypsin.

Pevsner P, Vecchione D, Stall B, Remsen T, Anand S, Stern A. 2007. British Mass Spectrometry Society, Edinburgh, Scotland
Pevsner P, Vecchione D, Remsen T, Kessler P, Levers N, Yang P, Stern A, Samuels H. British Mass Spectrometry Society, 2007 Robinson College, Cambridge .
Pevsner P, Vecchione D, 1 Remsen T, Kessler P, Momeni M, Duddempudi S, Francois F, Stern A, Anand S. 2007. British Mass Spectrometry LCMS Meeting, Robinson College, Cambridge, UK
Lopez-Ferrer D, Petritis K, Hixson KK, Heibeck TH, Moore RJ, Belov ME, Camp DG, Smith RD. *J Proteome Res* 2008; **7**: 3276.

Example of PCT Protein Extraction from Tissue

Extraction of Protein from Irradiated Mouse Heart

- Control (Normal Mouse Heart)
- Experimental [Mouse Heart 6 days post-total body irradiation (TBI)] -1 Gy, 2 Gy, and 4 Gy samples
- All samples run in triplicate
- Bradford Protein Assay

PCT Mouse Heart Protein Extraction

tube	sample	tissue mass mg	protein assay mg/mL	extraction efficiency (%)
HRT Na	control	28	2.25	8.0
HRT Nb		27	1.56	5.8
HRT Nc		**9**	0.68	7.6
HRT 1-6a	1 Gy	23	1.66	7.2
HRT 1-6b		**12**	0.94	7.8
HRT 1-6c		16	1.54	9.6
HRT 2-6a	2 Gy	29	3.15	10.9
HRT 2-6b		12	1.86	15.5
HRT 2-6c		**14**	1.14	8.2
HRT 4-6a	4 Gy	**10**	0.82	8.2
HRT 4-6b		18	1.41	7.8
HRT 4-6c		13	1.58	12.2
				9.1 ± 2.6

Why PCT Extraction?

- Reproducibility
- Speed
- Frozen samples can be processed
- Virtually adiabatic
- Ambient environment
- Instrument reliability
  - 30k cycles before our first repair

PCT Trypsin Digest

PCT Trypsin Digest (2007-2010)

- Remsen, T, Kessler, P, Francois, F, Stern, A, Anand, S, & Pevsner, P (**2008**). Imaging MALDI of colorectal carcinoma - field defects in satellite tissue. American Chemical Society Mid-Atlantic meeting, Queensborough College, Queens, NY, May, **2008**. *American Chemical Society Mid-Atlantic Meeting*, Queensborough College, Queens, NY.
- Remsen, T, Naftolin, F, Talebian, S, Kessler, P, Licciardi, F, Grifo, J, et al. (**2008**). LCMS identification of competent embryo biomarkers. British Mass Spectrometry Society, York, UK, September, **2008**.
- Lopez-Ferrer D, Petritis K, Hixson KK, Heibeck TH, Moore RJ, Belov ME, Camp DG,2nd, Smith RD. *J Proteome Res* **2008**; **7**: 3276.
- Pevsner PH, Melamed J, Remsen T, Kogus A, Francois F, Kessler P, Stern A, Anand S. *Biomarkers Med* **2009**; **3**: 55.

Trypsin/ Calibrant Prep

- **Trypsin**
  - –Trypsin was dissolved in 100 mM ammonium bicarbonate
- **Calibrant**
  - –Calibrants were dissolved in 0.1% TFA/Acetonitrile (ACN), 30/70.

BSA/Cyto-C Prep

- BSA*
  - –1158 µg BSA / 2 µg trypsin in 160 µL of 100 mM ammonium bicarbonate
- Cytochrome C*
  - –1158 µg Cyto-C / 2 µg trypsin in 160 µL of 100 mM ammonium bicarbonate

*No alkylation or reduction was performed on any samples.

PCT Protocol

- Experimental Protocol
  - – 35 kPSI (2.4 kbar)
  - – 40 seconds/cycle (with compressor)
  - – Cycle= 20 sec 35k PSI / 20 sec 1 atm (5 seconds with continuous N_2 gas supply)
- BSA (16) and Cyto-C (16)
  - – 1 cycle (n=4)
  - – 5 cycles (n=4)
  - – 10 cycles (n=4)
  - – 20 cycles (n=4)

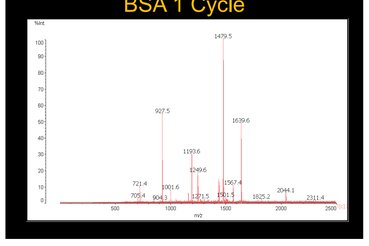
Reference Sample Protocol

–BSA (4) & Cyto-C (4)

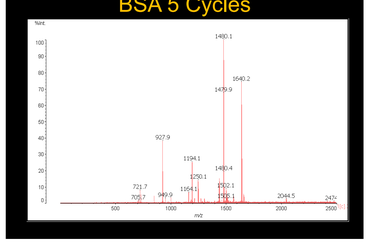
- 30 Minutes, 55˚C (n=1)
- 1 Hour, 55˚C (n=1)
- 2 Hours, 55˚C (n=1)
- 4 Hours, 55˚C (n=1)

PCT Trypsin Digest Spectra

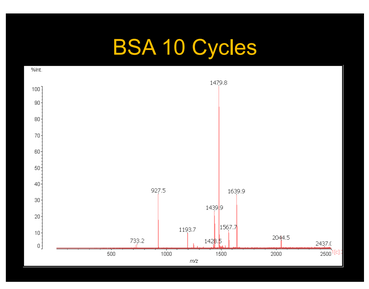
BSA 1 Cycle



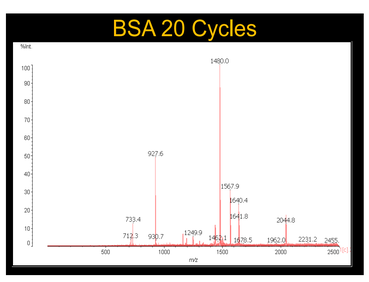
BSA 5 Cycles



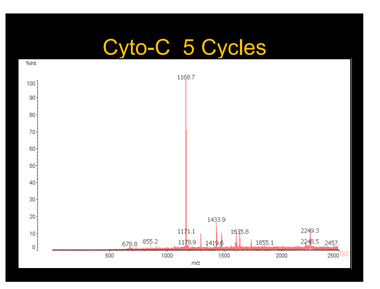
BSA 10 Cycles



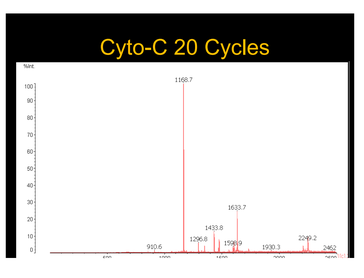
BSA 20 Cycles



Cyto-C 5 Cycles

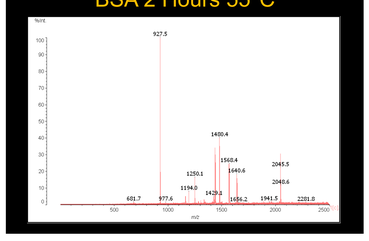


Cyto-C 20 Cycles

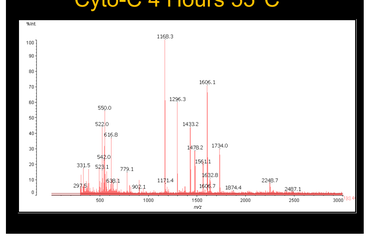


Reference Sample Spectra

BSA 2 Hours 55°C



Cyto-C 4 Hours 55°C



BSA (Bovine Serum Albumin) + Trypsin + PCT (Samples 1-8)		
158 µL BSA (1158 µg) + 2 µL trypsin (2 µg); BSA: Trypsin, 579:1		
Runs (Cycles/Seconds)	**MASCOT Score**	**Sequence Coverage (%)**
1/20	86	20
1/20	No match	N/A
1 /20	No match	N/A
1/20	No match	N/A
5/100	73	18
5/100	No match	N/A
5/100	No match	N/A
5/100	No match	N/A

BSA (Bovine Serum Albumin) + Trypsin + PCT (Samples 8-16)		
158 µL BSA (1158 µg) + 2 µL trypsin (2 µg); BSA: Trypsin, 579:1		
Runs (Cycles/Seconds)	**MASCOT Score**	**Sequence Coverage (%)**
10/200	70	17
10/200	59	15
10/200	No match	N/A
10/200	No match	N/A
20/400	54	17
20/400	69	19
20/400	65	20
20/400	No match	N/A

Cyto-C (Horse Heart Cytochrome-C) + Trypsin + PCT (Samples 1-8)		
158 µL Cyto-C (1158 µg) + 2 µL trypsin (2 µg); Cyto-C: Trypsin, 579:1		
Runs (Cycles/Seconds)	**MASCOT Score**	**Sequence Coverage (%)**
1/20	No match	N/A
1/20	No match	N/A
1/20	No match	N/A
1/20	No match	N/A
5/100	52	44
5/100	60	50
5/100	52	44
5/100	No match	N/A

Cyto-C (Horse Heart Cytochrome-C) + Trypsin + PCT (Samples 8-16)		
158 µL Cyto-C (1158 µg) + 2 µL trypsin (2 µg); Cyto-C: Trypsin, 579:1		
Runs (Cycles/Seconds)	**MASCOT Score**	**Sequence Coverage (%)**
10/200	77	50
10/200	54	44
10/200	93	62
10/200	60	50
20/400	59	50
20/400	65	50
20/400	63	50
20/400	75	50

BSA Reference Samples @ 55 °C		
Time	Mascot Score	Sequence Coverage (%)
30 Minutes	No match	N/A
1 Hour	41	16
2 Hours	54	20
4 Hours	No match	N/A

Cytochrome C Reference Samples @ 55 °C		
Time	Mascot Score	Sequence Coverage (%)
30 Minutes	No match	N/A
1 Hour	No match	N/A
2 Hours	No match	N/A
4 Hours	99	78

Data Summary

- Protein identification is cycle-dependent.
- PCT digest in minutes achieves equal coverage compared to water bath digest (55˚C) at 2 and 4 hours, respectively, for BSA and Cytochrome C.

Conclusion

PCT tissue extraction is:
1) rapid
2) reproducible
3) high-yield

PCT trypsin digest is:
1) rapid with high % coverage
2) reproducible
3)Reduced analyte / trypsin ratio (~500:1)