

Comparative Proteomics of Swine Tissue

2DLC/MS of Tissue Digestion
Preliminary Protein Analysis

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Briefing

- Treating and monitoring diseased tissue
- Sharing swine tissue for continued protein characterization

- They provide tissue
- We provide preparation, analysis

Win-Win

The Goal

Find proteins to monitor disease treatment

- Find many proteins
 - Efficient protein extraction
 - Good sensitivity (low protein concentration)
- Specific Subsets of proteins
 - Growth Factors
 - Mitochondrial
 - Anti Apoptosis
 - Glycolosis
- Consistency

Constraints

- Tissue amount
- Time and temperature sensitive samples
- Ease of analysis

Homogenization Tools

Suited for larger amounts of tissue (grams)
Consistent volume loss for tissue
Single sample at a time

- Rotary homogenizer



- Hand-Pestle



Smaller tissue amounts
Low volume loss
Inconsistent sample processing
Single sample at a time

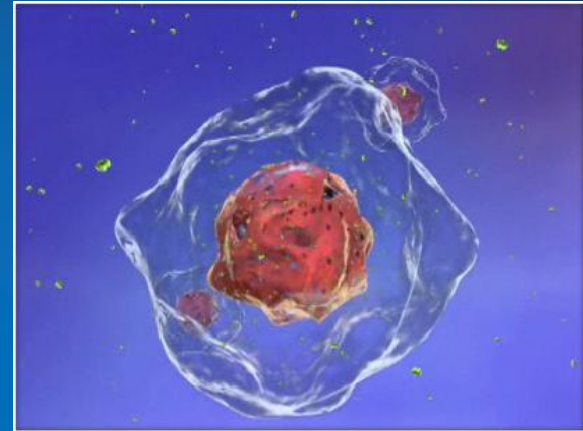
- Pressure Cycling Technology (PCT)



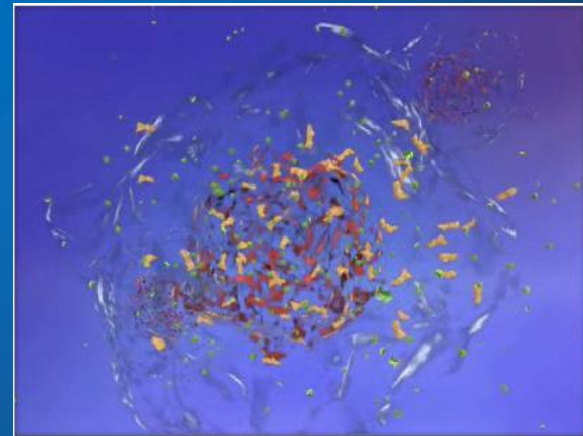
Smaller tissue amounts
Low volume loss
Consistent sample processing
Multiple samples (up to 48)

PCT Technology Overview

- In closed container, PCT applies and releases pressure on the sample from ambient to 35,000 psi in user customizable intervals
- PCT uses the hydrostatic compression differential of various cellular components to disrupt the cell with gentle yet highly powerful forces



Intact Cell



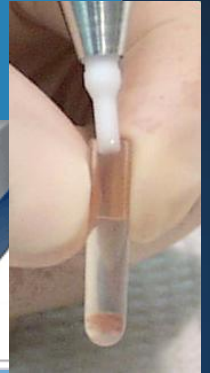
Cell Lysis with PCT

Sample Preparation Procedure

HEPES Buffer extraction

- Group A: Normal tissue, separate study
 - Group B: Diseased tissue
 - Group C: Diseased tissue with vehicle control
 - Group D: Treated diseased tissue
 - Group E: Normal tissue
- N=3 each

es at 35,000 psi)



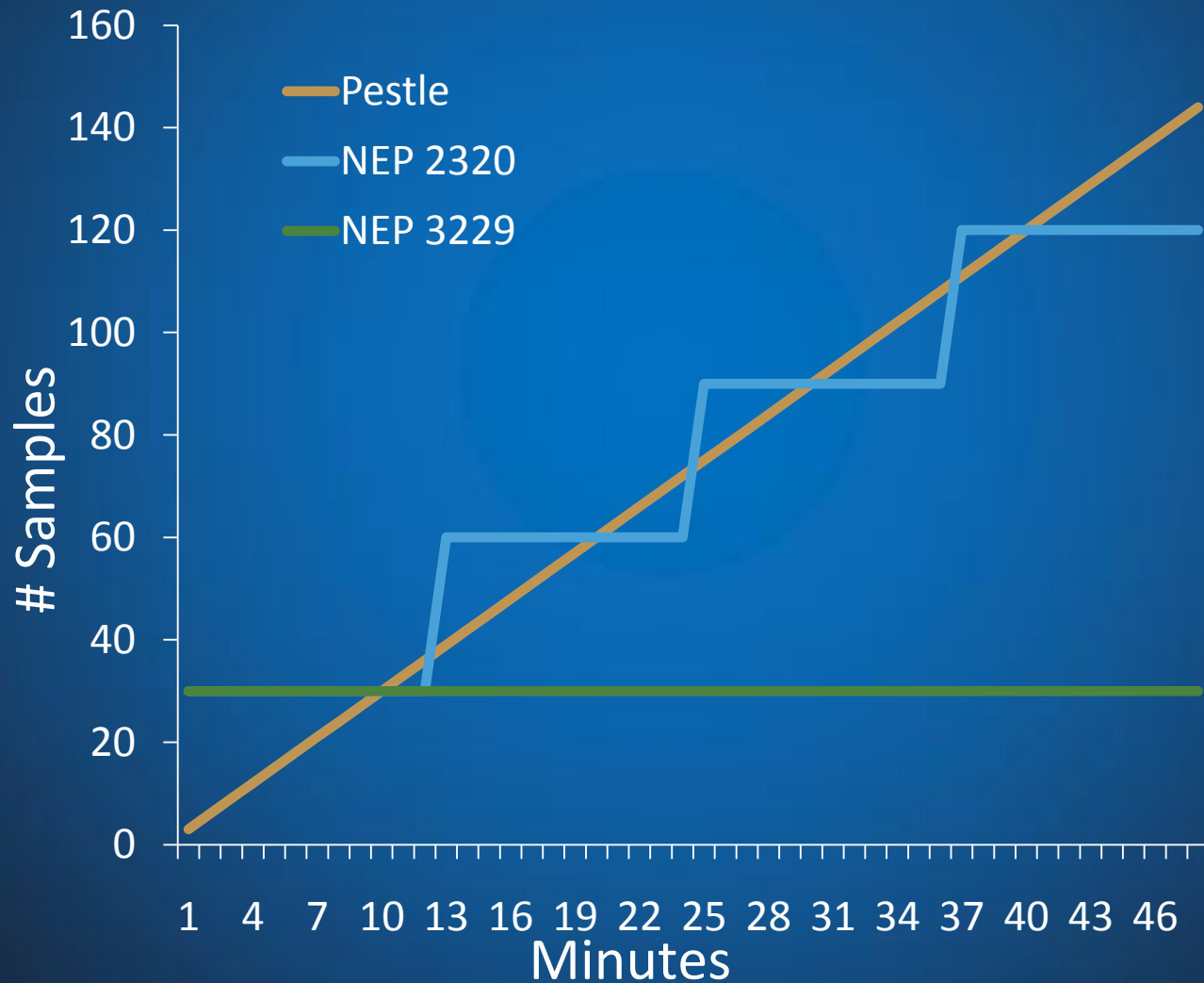
Inject onto
LC/MS



Digest with
Trypsin

Homogenization Time

Barocycler use is more efficient at more than 10 samples



Sample Preparation Procedure

Homogenize Tissue

2 Dimensional LC/MS

4 Step strong cation exchange (SCX)

40 minute reverse phase gradient

Thermo LTQ:

Data Dependant Top 5 analysis

Dynamic Exclusion



Extract Proteins

Reduced and Alkylated

Digested with Trypsin, ProteaseMax

Overnight at 37°C or

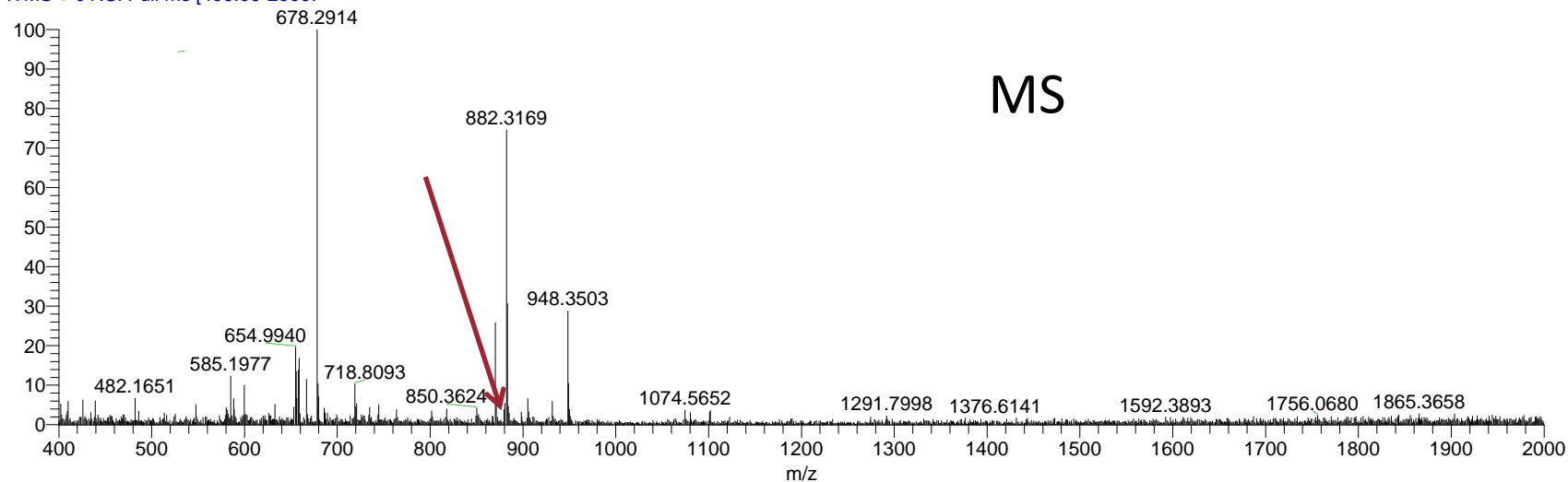
Barocycler PCT (30 cycles at 20,000 psi)



Example Spectrum

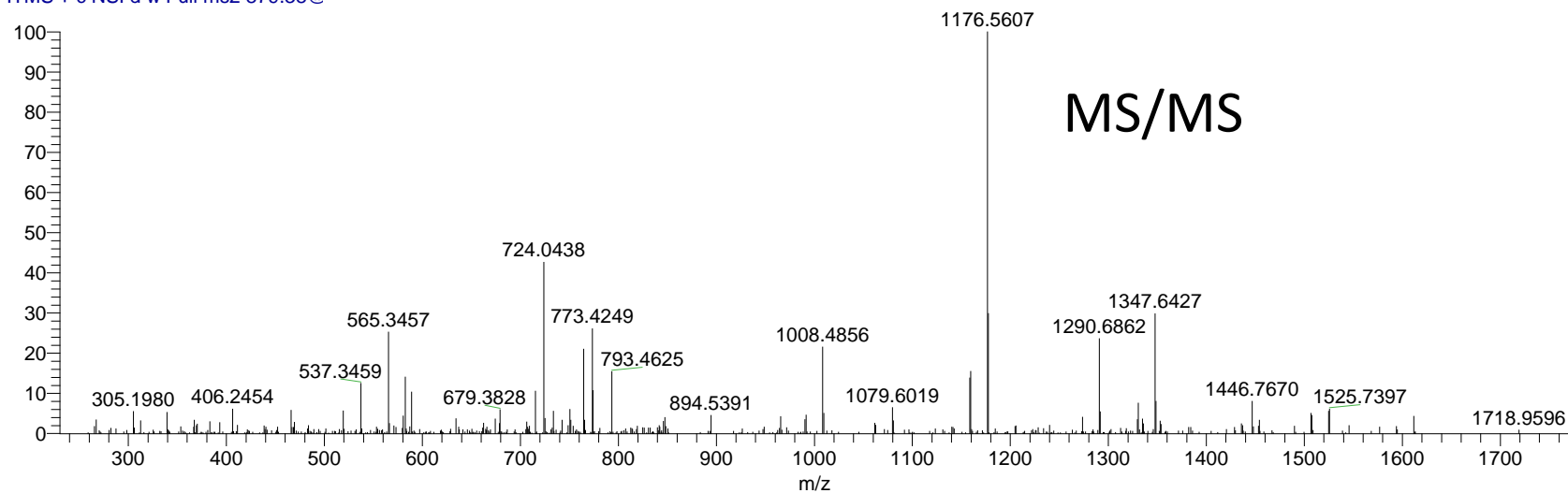
20110512_C3_Patch_Baro-Baro_Inject #2679 RT: 16.14 AV: 1 NL: 5.17E5

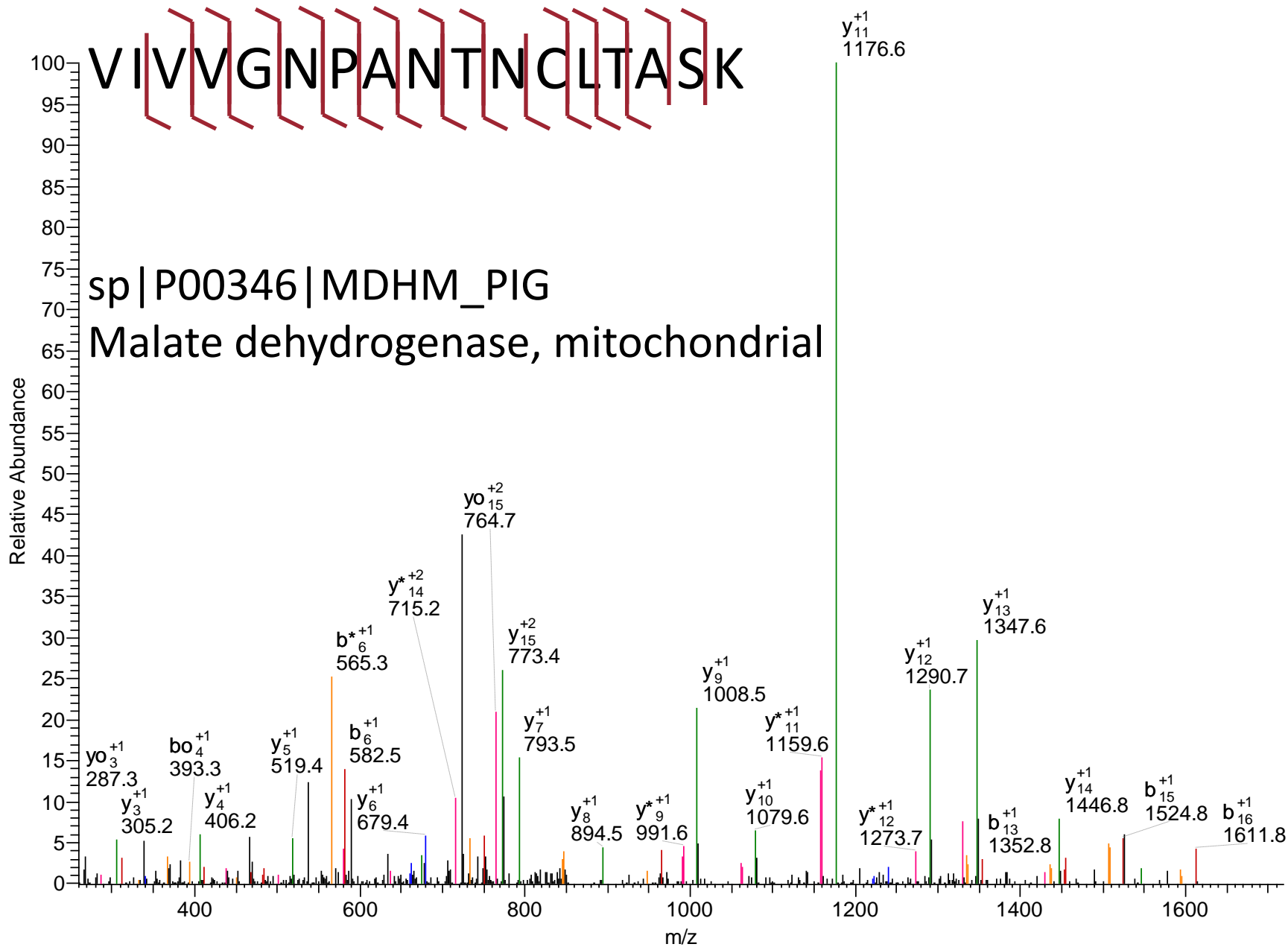
T: ITMS + c NSI Full ms [400.00-2000.]



20110512_C3_Patch_Baro-Baro_Inject #2683 RT: 16.16 AV: 1 NL: 8.68E2

T: ITMS + c NSI d w Full ms2 879.38@





Sequest Database Search

147,157 spectra identified as peptides

	Scan(s)	Reference	MH+	ΔM	z	Peptide	P (pep)	XC	ΔCn	
	7	20110519_C4_Patch_sp Q71FK5 ACTB_CAVPO Actin, cytoplasmic 1 OS=Cavia porcellus	1198.52	0.09	2	K.DSYVVGDEAQSK.R	2.2e-007	3.087	0.461	
	21	20110519_C4_Patch_sp P00346 MDHM_PIG Malate dehydrogenase, mitochondrial OS=Sus	1073.58	0.11	2	R.IQEAGTEVVK.A	8.6e-004	2.326	0.370	
	28	20110519_C4_Patch_sp Q767M3 SYVM_PIG Valyl-tRNA synthetase, mitochondrial OS=Su	1625.79	0.15	2	R.YVVEAAWYPWWVR.E	4.0e-004	1.404	0.051	
	33	20110519_C4_Patch_sp P08835 ALBU_PIG Serum albumin OS=Sus scrofa GN=ALB PE=1	1471.67	0.12	2	K.YIC@ENQDTISTK.L	5.1e-007	3.590	0.630	
	34	20110519_C4_Patch_sp P00761 TRYP_PIG Trypsin OS=Sus scrofa PE=1 SV=1	1045.56	0.05	2	K.LSSPATLNSR.V	1.2e-005	1.947	0.528	
	37	20110519_C4_Patch_sp P19620 ANXA2_PIG Annexin A2 OS=Sus scrofa GN=ANXA2 PE=	1222.59	0.00	2	K.TPAQYDASELK.A	2.7e-006	3.719	0.629	
	43	20110519_C4_Patch_sp P02543 REVERSED - VIME_PIG Vimentin (Fragments) OS=Sus scr	1146.56	0.01	2	K.MNQIEDQLR.G	3.4e-004	1.823	0.136	
	45	20110519_C4_Patch_sp P08835 ALBU_PIG Serum albumin OS=Sus scrofa GN=ALB PE=1	1414.65	0.02	2	K.YICENQDTISTK.L	2.8e-009	3.210	0.685	
	47	20110519_C4_Patch_sp P08835 ALBU_PIG Serum albumin OS=Sus scrofa GN=ALB PE=1	910.45	0.10	2	K.ADFTEISK.I	5.3e-006	2.705	0.497	
	56	20110519_C4_Patch_sp P67937 TRPM4_PIG Transmembrane protein, alpha-4 chain OS=Sus scrofa GN	1614.77	0.06	2	K.IDAIQQQADFAENR.A	1.8e-009	3.759	0.589	
or		sp Q5XLD3 KCRM_PIG Creatine kinase M-type OS=Sus scrofa GN=CKM	1083.54		0.03	2	K.TDLNHENLK.G			8.1
or		sp P01965 HBA_PIG Hemoglobin subunit alpha OS=Sus scrofa GN=HBA	1422.71		0.03	2	K.VGGQAGAHGAEALER.M			1.1
or		sp P08835 ALBU_PIG Serum albumin OS=Sus scrofa GN=ALB PE=1 SV	1471.67		0.16	2	K.YIC@ENQDTISTK.L			3.6
or		sp P02189 MYG_PIG Myoglobin OS=Sus scrofa GN=MB PE=1 SV=2	1488.65		0.06	2	K.HPGDFGADAQGAMSK.A			3.1
or		sp Q5XLD3 KCRM_PIG Creatine kinase M-type OS=Sus scrofa GN=CKM	1180.54		0.14	2	K.AEEEYPDLISK.H			1.9
or		sp P08835 ALBU_PIG Serum albumin OS=Sus scrofa GN=ALB PE=1 SV	910.45		0.06	2	K.ADFTEISK.I			5.4
	110	20110519_C4_Patch_sp P02067 HBB_PIG Hemoglobin subunit beta OS=Sus scrofa GN=H	1314.66	0.11	2	K.VNVDEVGGEALGR.L	6.9e-008	3.320	0.503	
	121	20110519_C4_Patch_sp Q767L7 TBB5_PIG Tubulin beta chain OS=Sus scrofa GN=TUBB P	1446.69	0.10	2	K.EVDEQMLNVQNK.N	1.2e-004	3.458	0.425	
	127	20110519_C4_Patch_sp P19619 ANXA1_PIG Annexin A1 OS=Sus scrofa GN=ANXA1 PE=	1064.49	0.18	2	R.SEIDMNDIK.A	2.6e-007	2.429	0.499	
	130	20110519_C4_Patch_sp P08835 ALBU_PIG Serum albumin OS=Sus scrofa GN=ALB PE=1	910.45	-0.02	2	K.ADFTEISK.I	2.0e-005	2.445	0.446	
	137	20110519_C4_Patch_sp P08835 ALBU_PIG Serum albumin OS=Sus scrofa GN=ALB PE=1	910.45	-0.02	2	K.ADFTEISK.I	1.0e-004	1.994	0.497	
	139	20110519_C4_Patch_sp P02189 MYG_PIG Myoglobin OS=Sus scrofa GN=MB PE=1 SV=2	1592.84	0.11	3	K.VEADVAGHGQEVLR.L	2.4e-011	3.529	0.538	
	141	20110519_C4_Patch_sp P02189 MYG_PIG Myoglobin OS=Sus scrofa GN=MB PE=1 SV=2	1592.84	0.09	2	K.VEADVAGHGQEVLR.L	1.6e-009	3.837	0.536	
	142	20110519_C4_Patch_sp Q19AZ8 THRB_PIG Prothrombin OS=Sus scrofa GN=F2 PE=2 SV	898.54	-0.86	2	R.VTAEIIPR.S	7.4e-005	2.579	0.336	
	144	20110519_C4_Patch_sp P08835 ALBU_PIG Serum albumin OS=Sus scrofa GN=ALB PE=1	1072.57	0.09	2	K.FQPLVDEPK.N	4.4e-004	2.289	0.319	
	148	20110519_C4_Patch_sp P16293 FA9_PIG Coagulation factor IX (Fragment) OS=Sus scrof	2166.02	0.11	2	K.ITVVAGEYNTETEPTEQ	3.1e-013	4.837	0.716	
	150	20110519_C4_Patch_sp P02067 HBB_PIG Hemoglobin subunit beta OS=Sus scrofa GN=H	1314.66	-0.93	2	K.VNVDEVGGEALGR.L	1.3e-004	2.741	0.138	
	151	20110519_C4_Patch_sp P16293 FA9_PIG Coagulation factor IX (Fragment) OS=Sus scrof	2166.02	0.03	2	K.ITVVAGEYNTETEPTEQ	9.6e-004	2.369	0.563	
	155	20110519_C4_Patch_sp P26234 VINC_PIG Vinculin OS=Sus scrofa GN=VCL PE=1 SV=4	1314.74	0.18	2	K.QVATALQNLQTK.T	1.1e-006	2.998	0.269	
	158	20110519_C4_Patch_sp P08835 ALBU_PIG Serum albumin OS=Sus scrofa GN=ALB PE=1	1072.57	0.06	2	K.FQPLVDEPK.N	2.0e-007	1.664	0.433	

Data Analysis Workflow

1. Find proteins of interest

Protein Search

2. Compare different sample tissue

Ontology Search

Spectral Count Quantification

3. Find up/down-regulated proteins

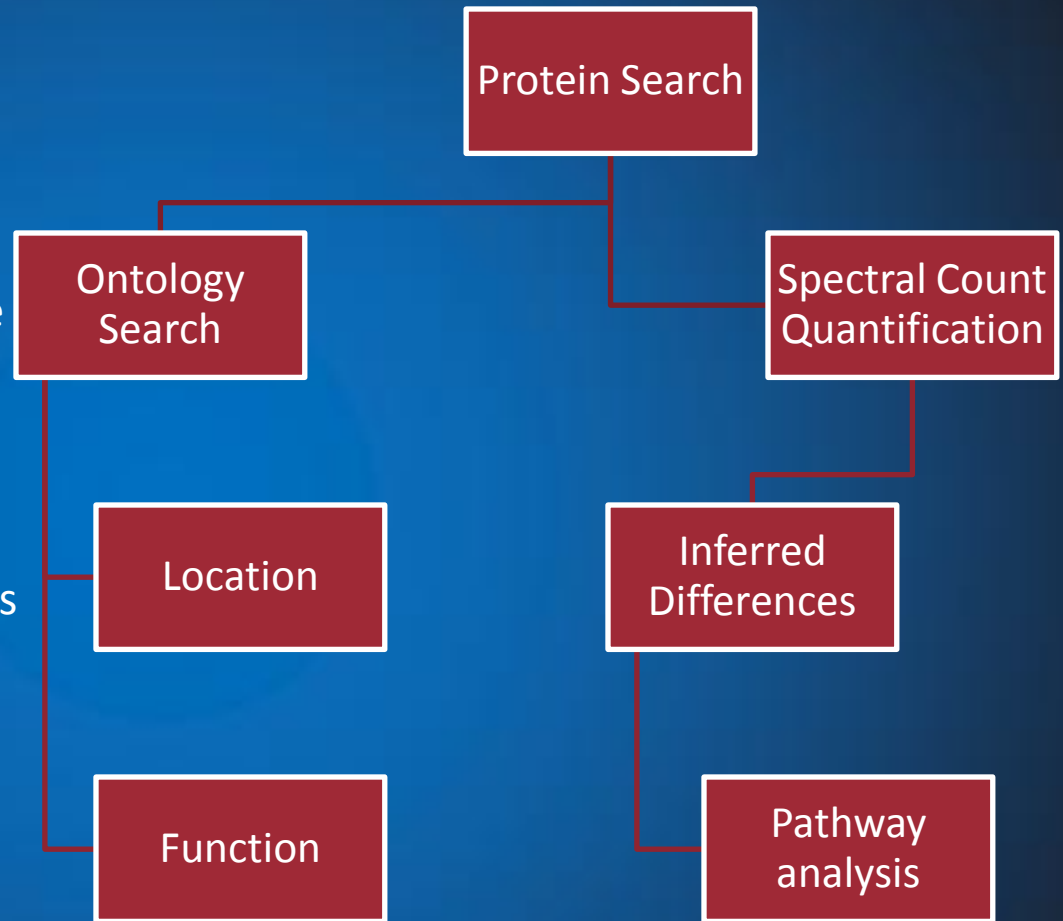
Location

Inferred Differences

4. Determine important pathways

Function

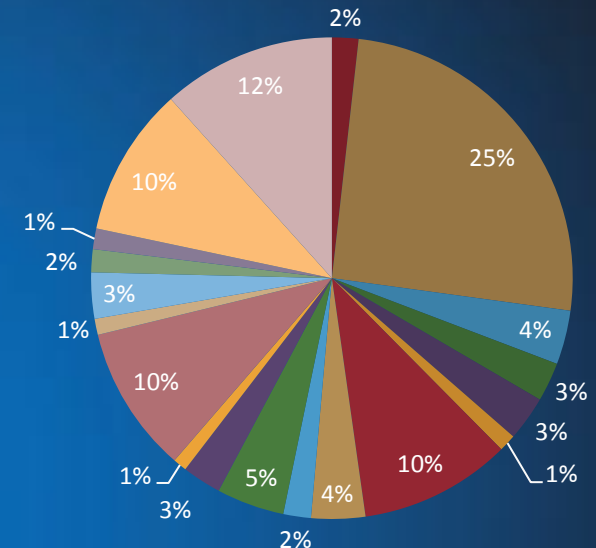
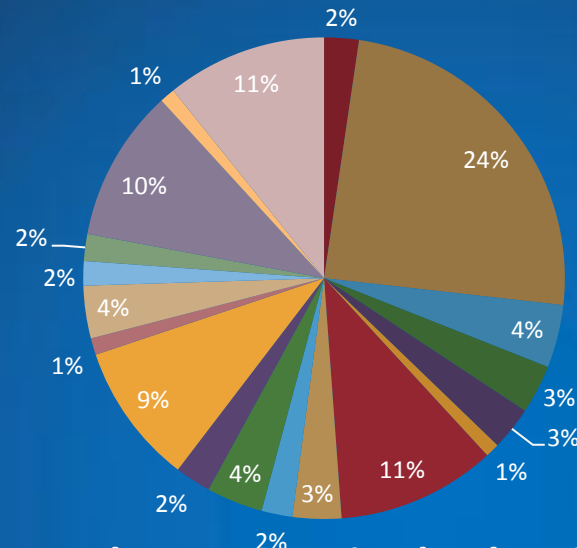
Pathway analysis



Gene Ontology: Protein Location

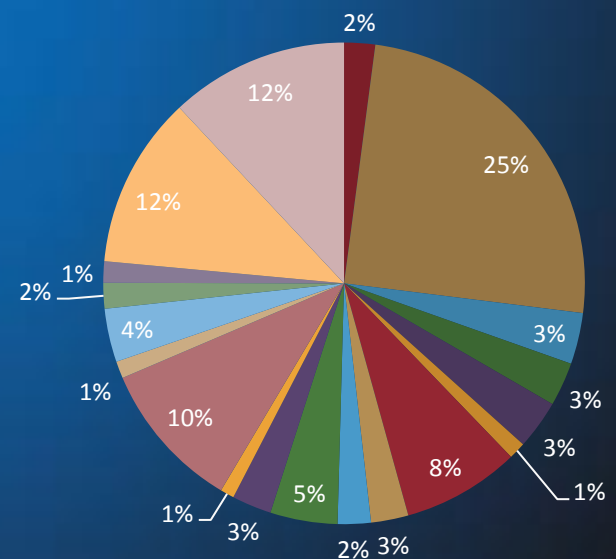
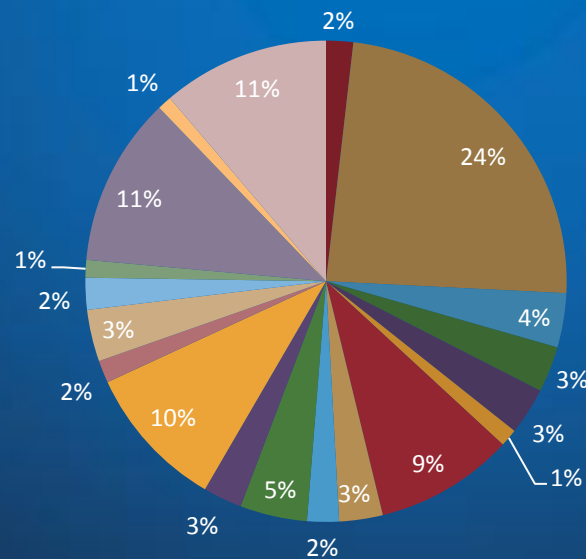
PCT Homogenized 18hr Digest

PCT Homogenized PCT Digest



Pestle Homogenized 18hr Digest

Pestle Homogenized PCT Digest



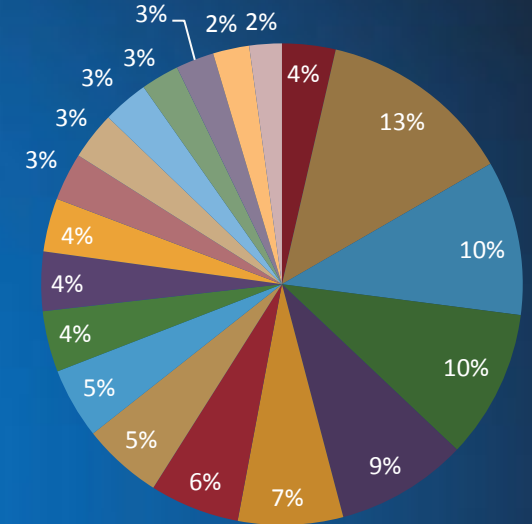
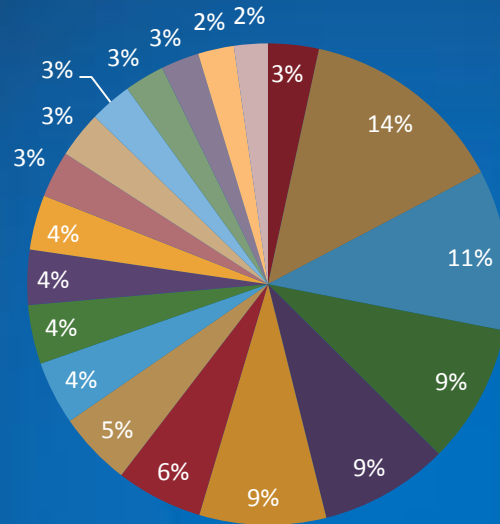
- unannotated
- cytoplasm
- cytoskeleton
- cytosol
- endoplasmic reticulum
- endoplasmic reticulum membrane
- extracellular region
- extracellular space
- Golgi apparatus
- integral to membrane
- intracellular
- membrane
- microsome
- mitochondrial inner membrane
- mitochondrial matrix
- mitochondrial outer membrane
- mitochondrion
- myosin complex
- nucleus

Gene Ontology: Function

PCT Homogenized 18hr Digest

PCT Homogenized PCT Digest

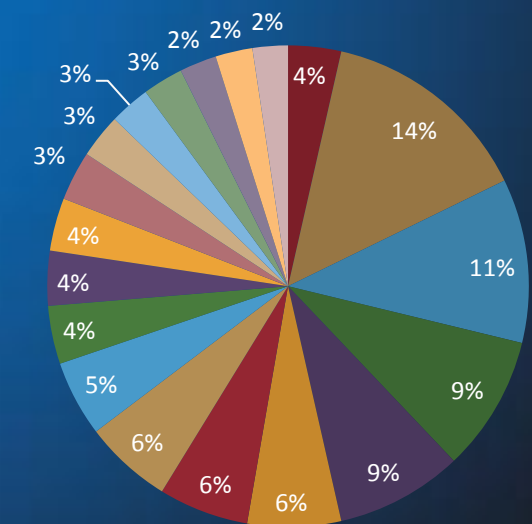
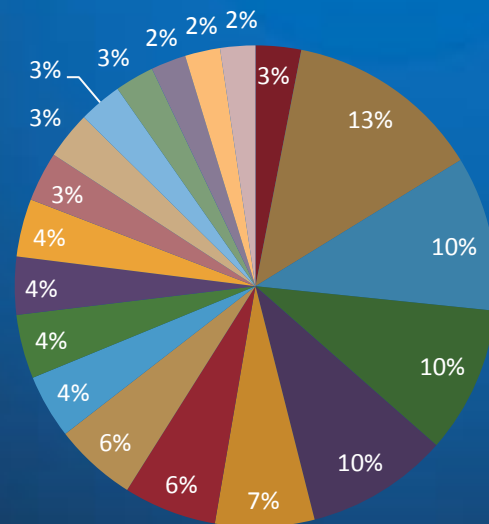
- unannotated
- ATP binding
- nucleotide binding
- oxidoreductase activity
- metal ion binding
- hydrolase activity
- catalytic activity
- transferase activity



Pestle Homogenized 18hr Digest

Pestle Homogenized PCT Digest

- actin binding
- GTP binding
- binding
- calcium ion binding
- kinase activity
- peptidase activity
- protein serine/threonine kinase activity
- heme binding

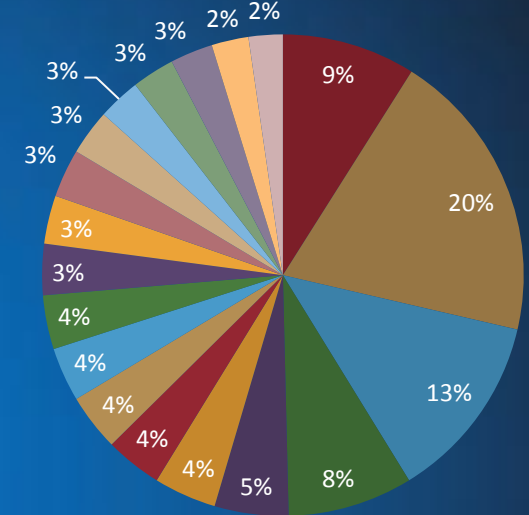
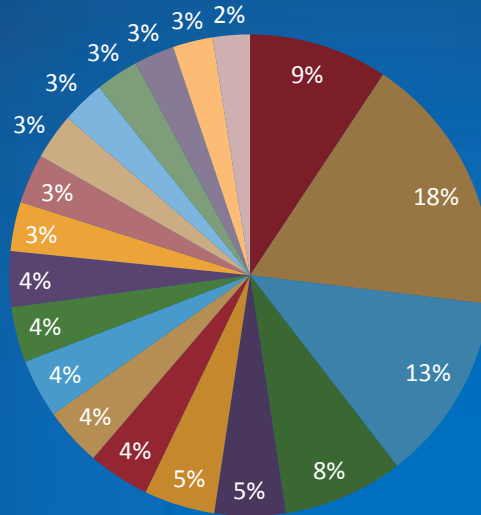


Gene Ontology: Process

PCT Homogenized 18hr Digest

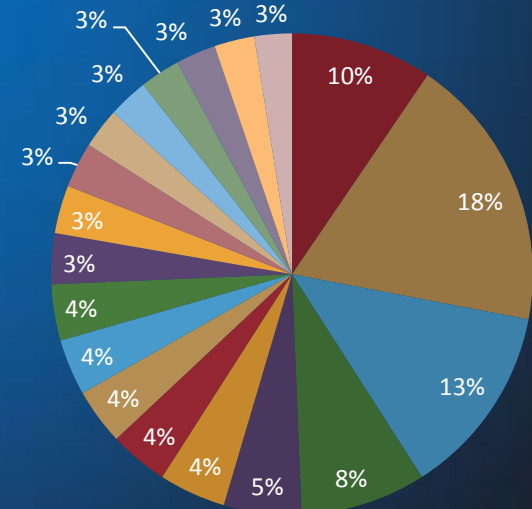
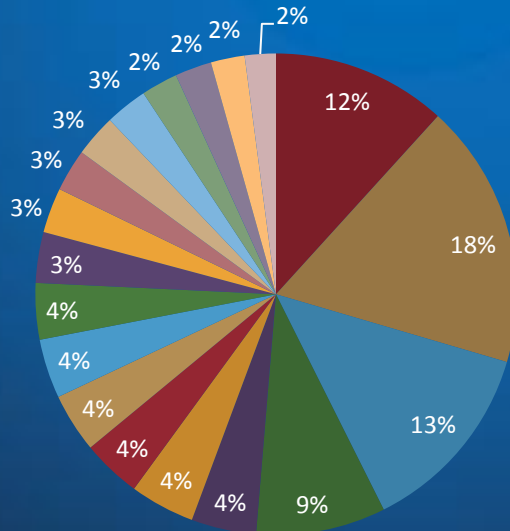
PCT Homogenized PCT Digest

- Unannotated
- oxidation-reduction process
- transport
- metabolic process
- phosphorylation
- proteolysis
- cell adhesion
- tricarboxylic acid cycle
- glycolysis
- translation
- signal transduction
- electron transport chain
- response to oxidative stress
- protein phosphorylation
- negative regulation of apoptosis
- ion transport
- carbohydrate metabolic process
- lipid metabolic process
- oxygen transport



Pestle Homogenized 18hr Digest

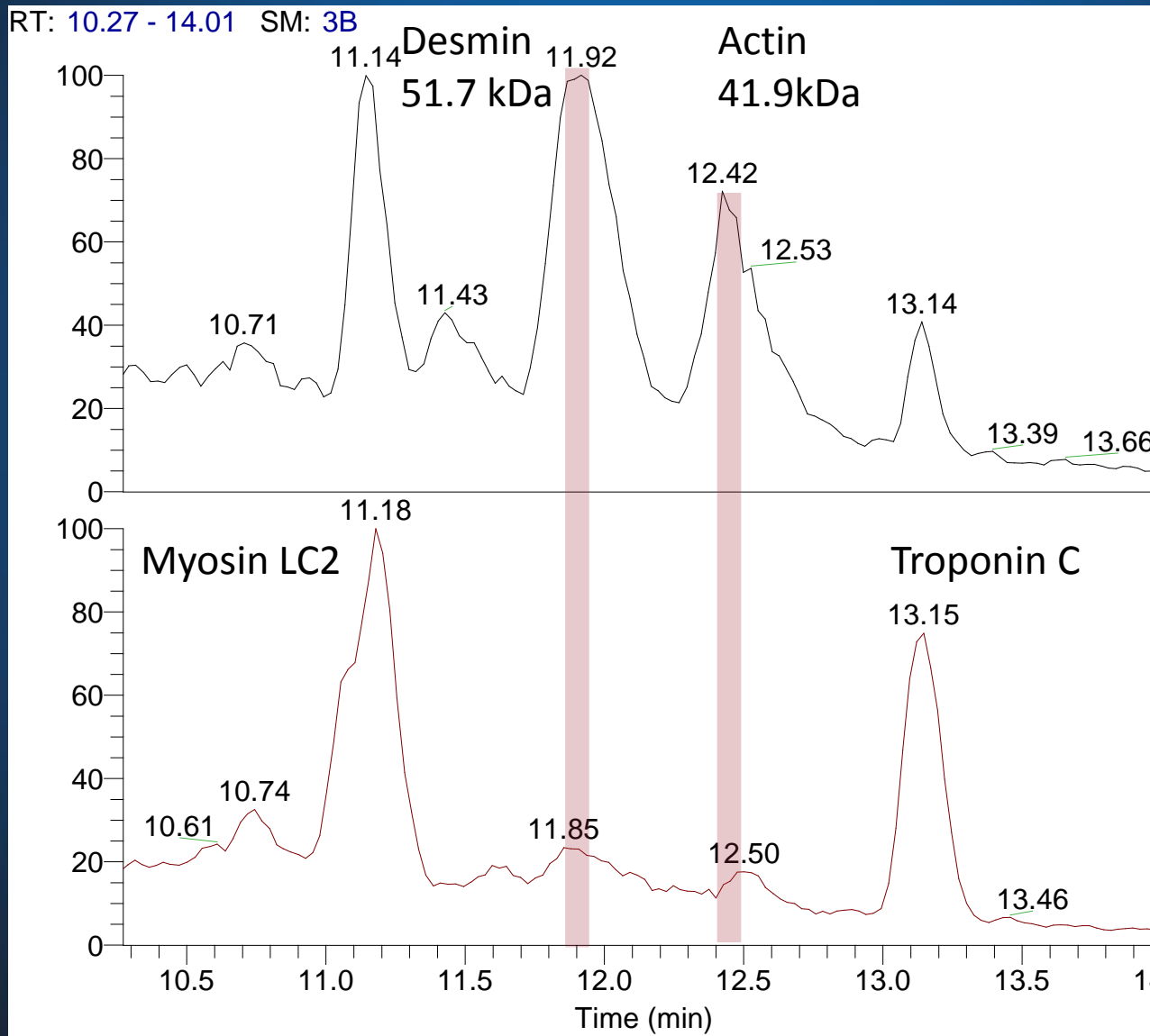
Pestle Homogenized PCT Digest



1mg Tissue: Secondary Extraction

Acid extracted myofilament proteins

Desmin and Actin abundance is reduced in PCT Homogenized samples



Pestle
Homogenate

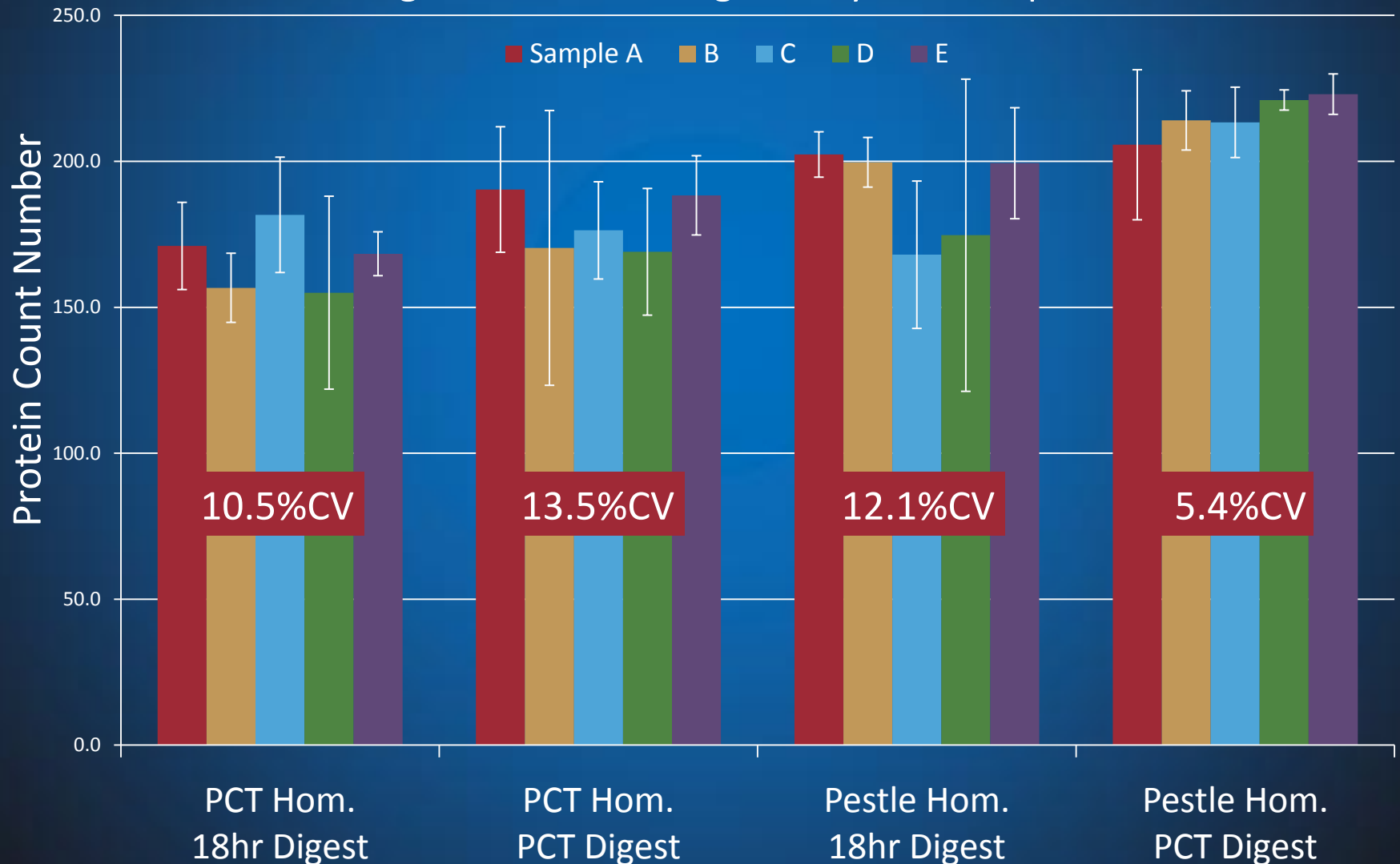
PCT
Homogenate

Quantifying Proteins

Spectral Counting is used to quantify proteins

1 spectral count = 1 MS/MS spectrum related to a specific protein

Pestle Homogenate and PCT Digest may be an improvement

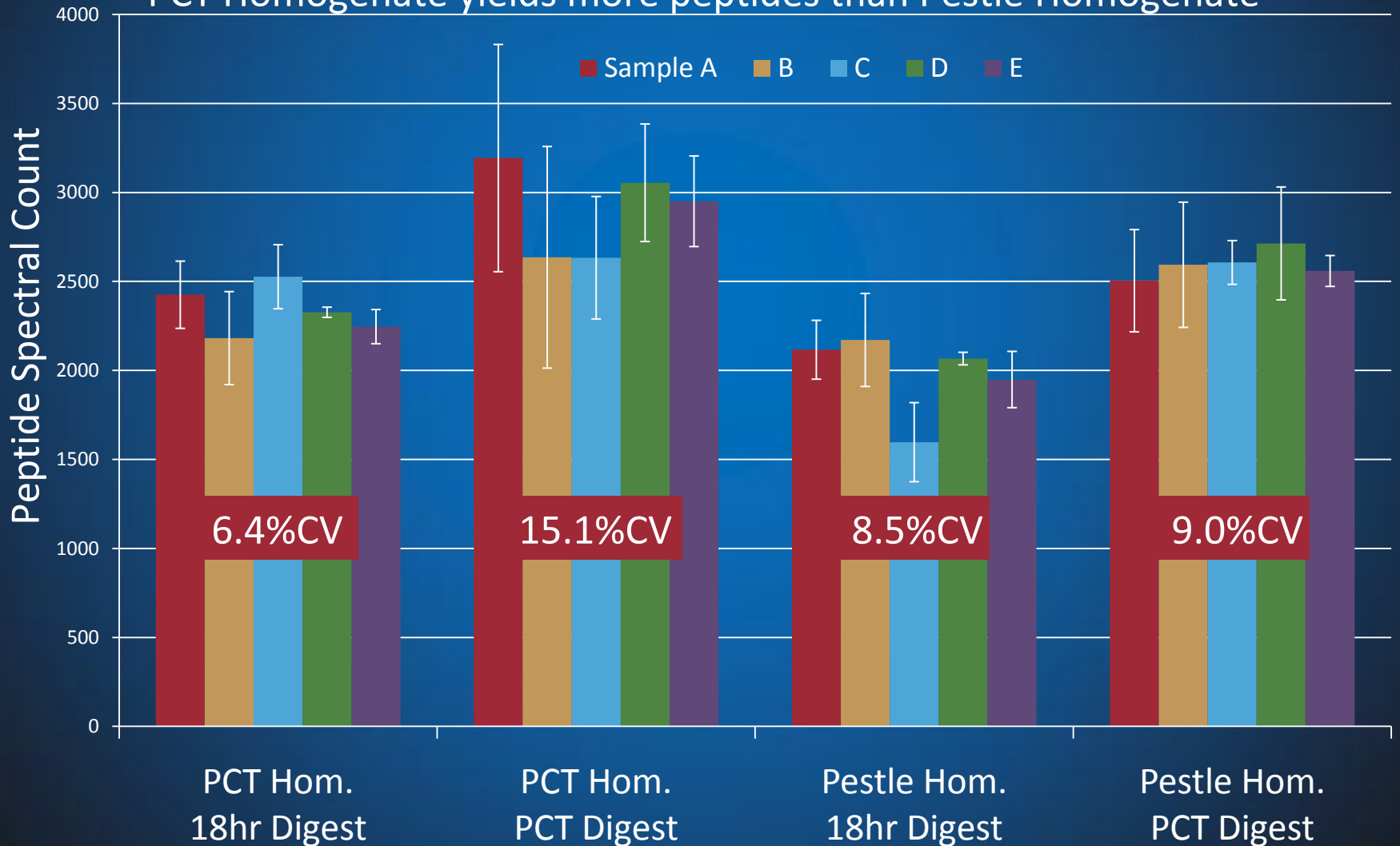


Quantified Peptides

Spectral Counting is used to quantify proteins

1 spectral count = 1 MS/MS spectrum related to a specific protein

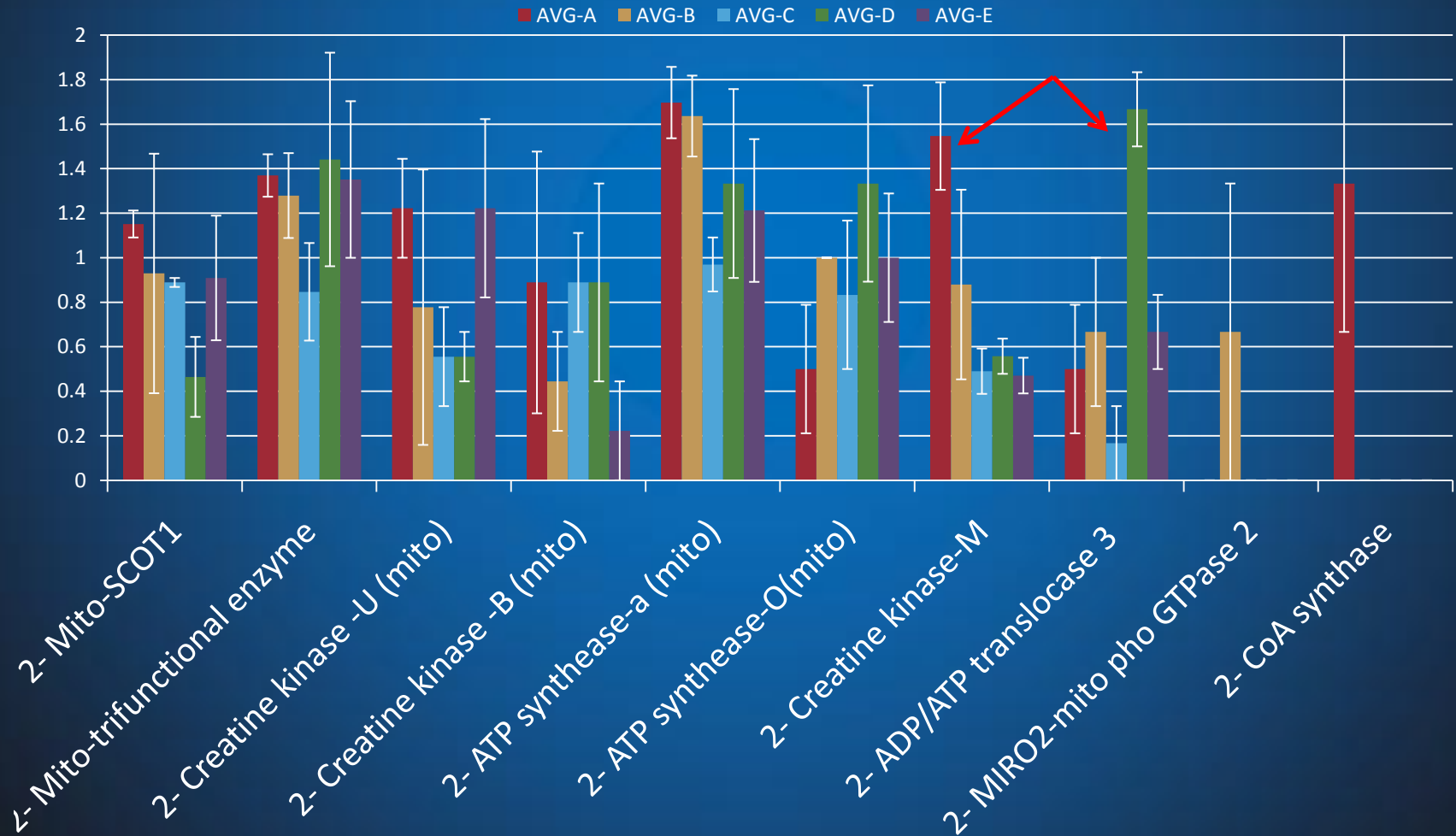
PCT Homogenate yields more peptides than Pestle Homogenate



Normalized Protein Comparison

Sample to sample comparison of proteins of interest

Proteins may be up or down-regulated if results are consistent and error is low



Results

- Faster sample preparation is achieved with the Barocycler
 - Efficient sample homogenizations above 10 samples
 - 30 minute digestion is fast enough for same-day LCMS injection
- Protein and peptide yield results are equivalent to traditional methods
- Pathways can be monitored for up/down regulated proteins based on disease state or therapy effect

Future Work

- Continue sample preparation development to reduce variables like analyzed tissue amount, extraction efficiency
- Complete sample analysis (60 tissues) will yield consistent data for protein comparison
- Develop software tools that automate data analysis workflow

Acknowledgements

Pressure Biosciences: Tom Strader and the use of their Barocycler NEP 2320

Human Proteomics Program:

Hard work and long hours put in by Han Zhang, Huseyin Guner and Ying Ge

Thanks for listening!