APPLICATION NOTE

High-Throughput Genomic DNA and Protein Extraction from Multiple Tissue Types using the Bead Ruptor 96 Bead Mill Homogenizer

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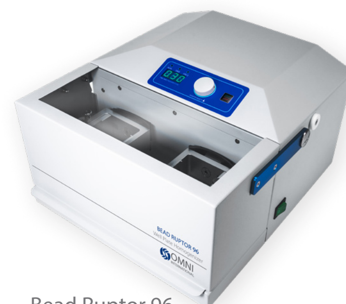
Introduction

There have been many studies of correlating gene and protein expression from single tissue samples[1,2,3,4]. Clinical samples can be especially challenging as sample sourcing and quantity are often limited. This is certainly the case for tissue samples of human origin that represent rare disorders. To make the most of rare tissues an ideal extraction method would be suitable for extracting a multitude of analytes, including nucleic acids, proteins and small molecules. Furthermore, the method would be capable of processing multiple samples simultaneously with high reproducibility.

The Bead Ruptor 96, Bead Mill Homogenizer is capable of rapid tissue dissruption via bead milling in 96 well plates, tubes or cryomilling in stainless steel chambers. Herein, we evaluate the Bead Ruptor 96 for high-throughput tissue dissruption for the purification of genomic DNA and proteins. Genomic DNA integrity is evaluated, and the protein repertoire is analyzed via electrophoresis and bottom up semi-quantitative proteomics.

Materials and Methods

- **Bead Ruptor 96 (Cat# 27-000)**
- **2 mL deep well plates pre-filled with 1 X 5 mm stainless steel bead (Cat# 27-6005)**
- **96 well plate holder (Cat# 27-101)**
- **Sealing mats for 2 mL deep well plates (Cat# 27-510)**
- **Omni Tissue DNA Extraction Kit (Cat# 26-007)**



Bead Ruptor 96
Cat#27-000

DNA Extraction

Rat brain, heart and small intestine was obtained from Bioreclamation and immediately stored at -80 °C. 30 mg of each tissue type was placed in separate wells of a pre-filled 96 well plate containing one 5 mm stainless steel bead that had been previously certified as nuclease free. 220 µL of DLB buffer (Omni Tissue DNA Kit) was added to each sample well. The well plate was sealed with a silicone mat and the well plate was loaded into the Bead Ruptor 96. Samples were dissociated per the settings in Table 1. All samples were fully homogenized in under two minutes.

Tissue Type	Processing Speed (Hz)	Time (sec)	Number of Cycles
Brain	20	30	1
Heart	20	30	3
Small Intestine	20	30	2

Table 1: Bead Ruptor 96 processing parameters requires to achieve complete sample disruption.

After processing, the plate was removed, and homogenates were transferred to 1.5 mL microcentrifuge tubes. Genomic DNA extraction was carried out per the Omni Tissue DNA Extraction Kit protocol. Nucleic acid concentrations were analyzed on the NanoDrop Spectrophotometer (Thermo Fisher Scientific) and are summarized in Table 2.

Tissue Type	DNA Concentration (ng/µl)	Protein Concertration (µg/µl)
Brain	226.3	4.2
Heart	170	5.1
Small Intestine	527.4	10.8

Table 2: Average DNA and protein concentrations measured via spectrophotometry.

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Protein Extraction

30 mg of each tissue type was placed in separate wells of a pre-filled 96 well plate containing one 5 mm stainless steel bead. 1 mL 100 mM Tris-HCL pH 7.6 was added to each well. The well plate was sealed with a silicone mat and processed on the Bead Ruptor 96 per the settings in Table 1. After processing, the homogenates were transferred to 1.5 mL microcentrifuge tubes and centrifuged at 8,000 x g for 10 minutes. The supernatant, containing soluble proteins, was removed, and protein concentration was determined on the NanoDrop Spectrophotometer and summarized in Table 2.

DNA and Protein Separation

Both harvested genomic DNA and proteins were separated by electrophoresis and visualized. For genomic DNA, 300 ng of each DNA sample was mixed with 5 µL of TBE/Urea loading buffer. The samples were loaded onto a 1% agarose gel and separated at a constant 140 V for 45 minutes. The gel was stained with ethidium bromide, washed with dd H_2O, and visualized on a GelDoc EZ System (Bio-Rad) as shown in Figure 1.

20 µg of each protein sample was mixed with 5 µL Laemmeli sample buffer and incubated at 95°C for 5 minutes. The proteins were then loaded on a 4-20% Mini Protean TGX gel (Bio rad) and separated at a constant 200 V for 30 minutes. The gel was stained with Coomassie and visualized on a GelDoc EZ System as shown in Figure 2.

Protein Digestion

Each gel lane was cut into three equal sections and washed twice with dd H_2O. Proteins were reduced with 5 mM dithiothreitol and 100 mM ammonium bicarbonate at 57°C for 1 hour and carboxyamidomethylated with 50 mM iodoacetamide and 100 mM ammonium bicarbonate for 1 hr at room temperature. Enzymatic digestion was performed with porcine trypsin (1:50 w/w) at 37°C in the dark. Samples were then acidified with 5% formic acid and desalted on a C_{18} ZipTip (SigmaAldrich).

LC-MS/MS

Peptides were separated by reverse-phase liquid chromatography on a 21 cm nano-HPLC column (100 µm ID) packed with Reprosil-Pur 120 C18-AQ 1.9 µm beads. Buffer A was 0.1% formic acid, 1% acetonitrile and buffer B was acetonitrile with 0.1% formic acid. Peptides were eluted over a 2 hr linear gradient from 4-80% buffer B and electrosprayed into a hybrid LTQ-XL Orbitrap mass spectrometer (ThermoFisher Scientific). Data dependent acquisition parameters were set as follows: parent scan centroid at 30,000 resolution, MS/MS collision energy 35%, activation Q 0.25 and activation time 30 ms. Dynamic exclusion was enabled for 20 sec.

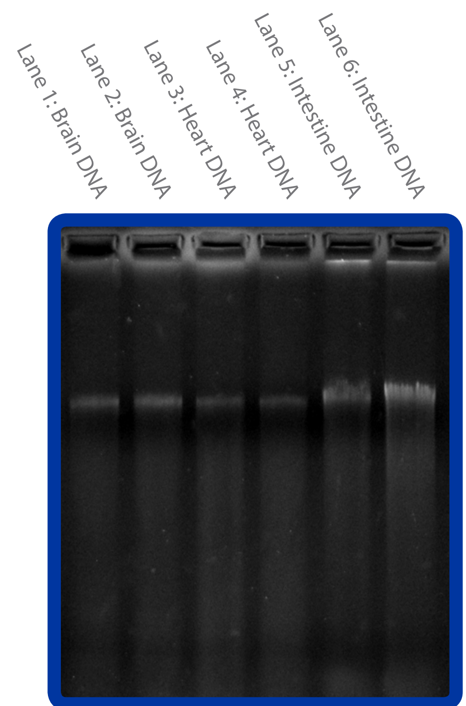

Figure 1: Genomic DNA extracted from rat brain, heart and small intestine. Electrophoresis and staining indicated the extracted genomic DNA was intact with no observable shearing. Processing parameters requires to achieve complete sample disruption.

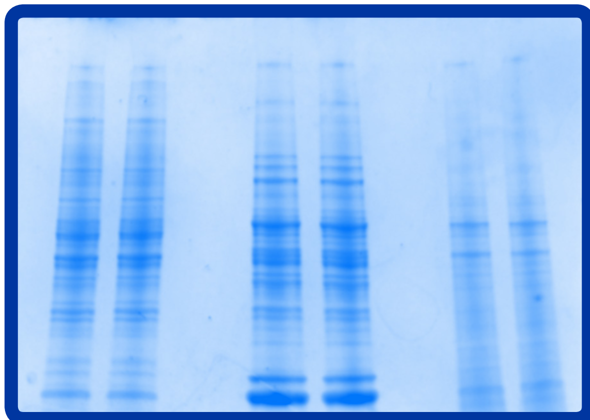

Figure 2: Total protein extracted from rat brain, heart and small intestine. Proteins were analyzed by 1D-PAGE and stained with coomassie blue. While 20 µg of each sample was loaded, the heart and brain samples appear to have the broadest and most abundant protein repertoire compared with the small intestine sample. MS/MS analysis confirmed this observation.

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Peptide and Protein Identification

Raw MS/MS spectra were extracted using ExtractMSn (ThermoFisher Scientific) and searched against a concatenated target/decoy Rattus norvegicus reference protein database (UniProt) using the SEQUEST algorithm deployed in the Sorcerer (SAGE-N), Parameters were: precursor tolerance 50 ppm, fragment tolerance 0.5 Da, fully tryptic enzyme specificity, two missed cleavages, variable modifications on methionine (+16). Peptide identifications were filtered at a 1% FDR taken as FDR = 2Xnd/nt. A second pass filter was performed by removing peptide identifications in excess of 10 ppm precursor mass error. Peptides were clustered to proteins and proteins were assembled into protein groups based on parsimony rules where the protein group is represented by the maximum number of assigned peptides.

Results

High-throughput extraction of nucleic acids and proteins requires instrumentation capable of processing multiple samples per cycle coupled with the ability to apply the force required to dissociate challenging samples while maintaining analyte integrity. Herein, we evaluated the ability of the Bead Ruptor 96, bead mill homogenizer to extract high quality genomic DNA and proteins from a variety of animal tissues. For all tissue types, a complete homogenate was observed after processing for less than 90 seconds (Table 1). Both genomic DNA and protein concentrations varied by tissue type.

DNA concentration was determined by spectrophotometry. Average DNA concentration for all tissues ranged from 170 ng/µL to 527.4 ng/µL. The DNA was then separated and visualized by gel electrophoresis. Analysis showed high quality DNA bands with no shearing. Nucleic acid yields for the small intestine sample were double that of heart and brain. Yields were corroborated by the gel analysis as shown in Figure 1. In Figure 1, it was clear that intact genomic DNA was isolated with very little shearing and no observable RNA contamination. The average protein concentration ranged from 4.2 µg/µL to 10.8 µg/µL for the three tissue types with the small intestine sample exhibiting the highest yields. The proteins were then separated by 1D-PAGE and imaged through Coomassie staining. While the yields for the small intestine sample were the highest, the gel analyses revealed what appeared to be a smaller diversity of proteins when compared to the brain and heart samples. Produce abundance, as visualized by 1D-PAGE was further confirmed by the MS/MS analysis as shown in Table 3.

Bottom up proteomic analysis resulted in the identification of 1,288 proteins from 5,954 peptides at a 1% protein false discovery rate. While >850 proteins were identified from the heart and brain samples, the small intestine sample resulted in the identification of 335 proteins.

Sample	Proteins	Peptides	Unique Peptides	Spectral Counts (PSM)
Brain	867		3413	14441
Heart	857		3645	11984
Small Intestine	335		1037	2860
Total	1288	5954	5359	29285

Table 3: Proteomic sampling across multiple tissue types.

A key feature in any extraction method is that the analyte integrity is maintained. To evaluate if the milling method was altering protein expression profiles, a semi-quantitative proteomic analysis was performed in which spectral counts were compared between each tissue type. The goal of this analysis was to compare the expression of well characterized protein markers for the tissues processed. Tables 4-6 display the proteins identified uniquely in each tissue type. Heart and brain unique proteins were filtered at > 20 spectral counts (PSMs) while the small intestine unique protein list was filtered at > 8 PSMs.

Unique brain proteins included well characterized tissue specific markers such as calbindin, syntaxin and dynamin; as well as proteins known to be associated with neuronal differentiation[5], neurotransmitter secretion[6] or dopamine receptor function[7] (Table 4).

Accession	Name	MW[kDA]	Peptides	#PSMs
P07171	Calbindin OS	30	14	76
P61765-2	Isoform 2 of Syntaxin-binding protein 1	68.7	24	61
F1MA36	Spectrin beta 3	270.8	28	57
F1LNT0	Dihydropyrimidinase-related protein 4	61.9	16	51
P21575	Dynamin-1	97.2	21	48
D4A133	Protein Atp6v1a	68.2	20	47
P50554	4-aminobutyrate aminotransferase, mitochondrial	56.4	12	42
P63012	Ras-related protein Rab-3A	25	5	33
G3V6S0	Protein Sptbn1	273.3	16	32
Q9QUL6	Vesicle-fusing ATPase	82.6	22	31
P30009	Myristoylated alanine-rich C-kinase substrate	29.8	11	29
Q64559	Cytosolic acyl coenzyme A thioester hydrolase	42.7	7	27
B4F772	Heat shock 70 kDa protein 4L	94.2	10	26
P37805	Transgelin-3	22.5	7	26
P15146	Microtubule-associated protein 2	202.3	14	23
O35179	Endophilin-A1	39.9	8	23
P61265	Syntaxin-1B	33.2	5	20

Table 4: Unique Proteins in Brain Sample

While the overall sampling of the small intestine proteome was limited, a subset of unique proteins was observed. They included proteins associated with nutritional uptake[8], carbohydrate and protein digestion[9,10,] including fatty acid-binding protein, sucrase-isomaltase and alanyl aminopeptidase (Table 5).

Accession	Name	MW[kDA]	Peptides	#PSMs
P02692	Fatty acid-binding protein, liver	14.3	4	14
F1M792	Sucrase-isomaltase, intestinal	210.1	8	13
P00884	Fructose-bisphosphate aldolase B	39.6	4	13
Q9Z144	Galectin-2	14.7	2	9
G3V7W7	Alanyl (Membrane) aminopeptidase	109.4	5	8

Table 5: Unique Proteins in Intestine Sample.

In this study, the heart proteome was the most diverse with seventeen unique proteins detected with over 20 PSMs (Table 6). While heart\muscle tissue markers such as myomesin and creatine kinase were detected the largest number of unique proteins detected in heart were associated with the fatty acid oxidation pathway[11,12] as shown in Figure 3.

Table 7 shows as summery of proteins with no change in expression between the heart and brain samples. These included "housekeeping" proteins such as the heat shock family and proteins associated with carbohydrate metabolism[13,14,15].

Accession	Name	MW[kDA]	Peptides	#PSMs
P15650	Long-chain specific acyl-CoA dehydrogenase, mitochondrial	47.8	14	137
P13437	3-ketoacyl-CoA thiolase, mitochondrial	41.8	12	68
Q64428	Trifunctional enzyme subunit alpha, mitochondrial	82.6	17	53
D3ZZN3	Protein Acss1	74.8	11	49
D4A882	Propionyl-CoA carboxylase alpha chain, mitochondrial	70.9	17	47
Q5M9H2	Acyl-Coenzyme A dehydrogenase, very long chain	70.8	19	46
P45953	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial	70.7	18	45
P14882	Propionyl-CoA carboxylase alpha chain, mitochondrial	81.6	16	43
G3V796	Acetyl-Coenzyme A dehydrogenase, medium chain	46.5	11	34
P14604	Enoyl-CoA hydratase, mitochondrial	31.5	8	32
G3V7K1	Myomesin 2	164.6	16	30
G3V7K3	Ceruloplasmin	120.6	15	29
B0BMW2	3-hydroxyacyl-CoA dehydrogenase type-2	27.2	12	28
Q499N5	Acyl-CoA synthetase family member 2, mitochondrial	67.8	15	27
F1LPV8	Succinyl-CoA ligase subunit beta	46.6	12	24
Q6IMX3	Acetyl-Coenzyme A dehydrogenase, short chain, isoform CRA_a	44.9	8	24
M0R6J6	Creatine kinase S-type, mitochondrial	46.4	4	22
Q5BJZ3	Nicotinamide nucleotide transhydrogenase	113.8	10	21

Table 6: Unique Proteins in Heart Sample

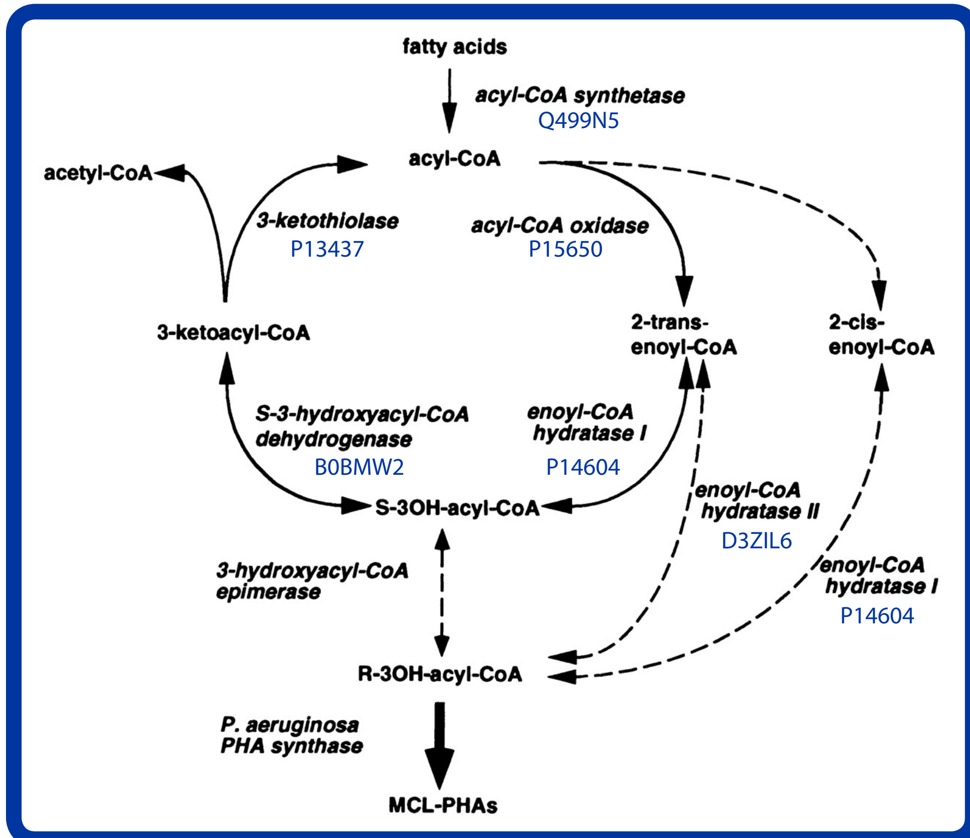

Figure 3: Key Regulation Sites of Fatty Acid β-oxidation.
(http://lipidlibrary.aocs.org/Biochemistry/content.cfm?ItemNumber=39187

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			Brain		Heart	
Accession	Name	MW[kDA]	#PSMs	#Unique Peptides	#PSMs	#Unique Peptides
P63018	Heat shock cognate 71 kDa protein	70.8	200	35	134	24
M0R8M9	Heat shock cognate 71 kDa protein	70.8	177	31	121	20
F1LZI1	Protein LOC680121	70.8	160	30	106	20
M0R5J4	Uncharacterized protein	47	147	19	125	16
M0R590	Glyceraldehyde-3-phosphate dehydrogenase	35.8	130	13	153	17
P42123	L-lactate dehydrogenase B chain	36.6	104	14	139	16
P00507	Aspartate aminotransferase, mitochondrial	47.3	97	14	101	18
Q6P6V0	Glucose-6-phosphate isomerase	62.8	93	19	45	15
P04636	Malate dehydrogenase, mitochondrial	35.7	85	13	106	15
P63039	60 kDa heat shock protein, mitochondrial	60.9	69	24	72	30

Table 7: High abundance housekeeping proteins in brain and heart

Conclusion

Genomic DNA and proteins were extracted from multiple rat brain, heart and small intestine using the Bead Ruptor 96. All tissues were fully dissociated in less than 90 seconds. High quality, unsheared genomic DNA was purified and the protein extraction method was shown to conserve well known protein abundance levels.

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