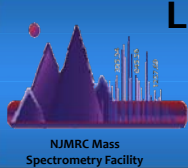


# Label-Independent Quantitative Analysis of Rat Mitochondrial Proteomes Using Quadrupole Time-of-Flight Mass Spectrometry

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## Abstract

Global analysis of mitochondrial proteomes from skeletal muscle has presented unique challenges, particularly due to high amounts of myosin, a muscle protein contaminant that occurs during mitochondrial enrichment. We applied various protein fractionation techniques to skeletal mitochondrial proteomes from an obesity resistant rat model. Previous attempts to fractionate mitochondrial proteins using reverse phase chromatography were complicated by indiscriminate binding of myosin to mitochondrial proteins, resulting in poor protein separation. Molecular weight cut off, strong cation exchange, and antibody-based depletion of myosin were similarly ineffective. We therefore used 1-dimensional SDS-PAGE to separate myosin and resolve muscle mitochondrial proteins. Intact proteins from obesity resistant (OR) and obesity prone (OP) rats were separated on a 10.5% to 14% gradient gel. Gel lanes were cut into 40 slices and subjected to in-gel trypsin digests. Peptides were first analyzed in MSMS mode on a quadrupole time-of-flight (QTOF) mass spectrometer for global protein identification. While our reverse phase method resulted in the identification of 330 mitochondrial proteins, the SDS-PAGE fractionation technique yielded over 400 mitochondrial proteins. To determine quantitative differences between mitochondrial proteins from OR and OP rats, we employed a label-independent method using accurate mass and retention time for molecular feature assignments. Peptides were analyzed on a QTOF in MS mode (n=3). Features were aligned for mass and retention time in GeneSpring MS (Agilent Technologies). After filtering and statistical analysis, features that passed a two-fold change filter were exported for targeted MSMS analysis to assign protein identifications. Quantitative differences were evident between OR and OP rats. In three consecutive gel slice sets we found 669 molecular features with changes ranging from 2 to >40 fold. Targeted MSMS analysis results allowed matching of a subset of these features to 60 proteins identified with  $\geq 2$  peptides. Three proteins identified with higher abundance levels in OR rats function in lipid metabolism pathways. This study underscores challenges inherent in tissue-specific mitochondrial proteomics, and illustrates an effective quantitative method for the mitochondrial proteome.

## Methods

**Sample Preparation:** Mitochondria from the hindlimb skeletal muscle of female OP and OR rats were isolated. Mitochondria were lysed with 0.1% PPS with Complete Protease Inhibitor cocktail (Santa Cruz), and protein concentrations were determined by Bradford method. 40 $\mu$ g of mitochondrial proteins from both OP and OR rats were separated on a 10.5% to 14% gradient SDS-PAGE gel (BioRad). Proteins were stained by Bio-Safe Coomassie (BioRad), and 40 - 2 mm x 7mm gel slices were removed from each lane using a GridCutter (The Gel Company).

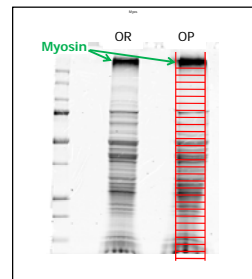
**Digestion:** Proteins were digested using a standard in-gel digest protocol with DTT, IAA and Trypsin. Digests were subsequently cleaned using C18 spin columns (Pierce), dried to completion and reconstituted 20  $\mu$ l in 3% ACN + 0.1% formic acid.

**Instrumentation:** Peptides were analyzed via LC/MS/MS on an Agilent QTOF (model 6510) mass spectrometer with an HPLC-chip interface. The chip contains a 40 nI enrichment column and a 75  $\mu$ m x 150 nm analytical column packed with C18 SB-ZORBAX 300A particles. Instrument parameters and gradient details are listed in the tables below. For MS acquisition, three replicate injections (0.5  $\mu$ l) were analyzed for each sample. For targeted MS/MS analysis, the MS m/z range was narrowed to include only targeted ions and reference ions.

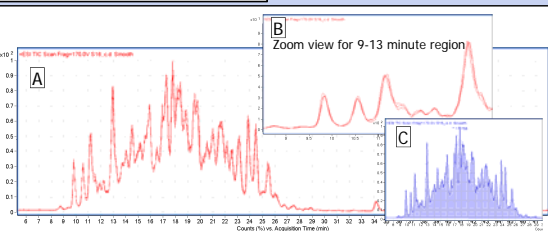
Parameter	MS only	MS/MS	Targeted MS/MS	Time	% B
Voltage	1800	1800	1800	0	3
Drying gas	4.0 L/min	4.0 L/min	4.0 L/min	1	3
Temperature	300	300	300	45	45
m/z range	295-3000	MS: 295-3000 MSMS: 50-1800	MS: 295-1225 MSMS: 50-1800	48	45
Precursor ions	N/A	5	N/A	48.1	80
Active exclusion	N/A	Yes	N/A	52	80
Scan rate	1 spec/second	MS: 8 spec/sec MSMS: 3 spec/sec	MSMS: 3 spec/sec	52.1	3

**Data Analysis:** Raw data was extracted using Mass Hunter software (Agilent Technologies) with the following parameters: absolute threshold = 5,000, isotope abundance profile = peptide-like, ion count threshold = 2 or more. Data was then imported as MHD files into GenespringMS (Agilent). Mass and retention time alignments were performed, and features filtered by the following criteria: present in at least 50% of replicates from each gel slice set, analysis of variance (ANOVA)  $p \leq 0.05$ , and fold change  $\geq 2$ -fold. Masses passing these filter criteria were exported as an inclusion list for targeted analysis. Abundance values were normalized against median values per mass, and against integrated TIC values to correct for gel loading. Final peak lists were used to generate an inclusion list, which was imported into the QTOF acquisition software and used for targeted MS/MS acquisition. Spectra were submitted to IPI and SwissProt databases using the Spectrum Mill search engine (Agilent Technologies). Data were validated by cross-referencing the exported mass and retention time from the targeted list and search engine results.

## Results



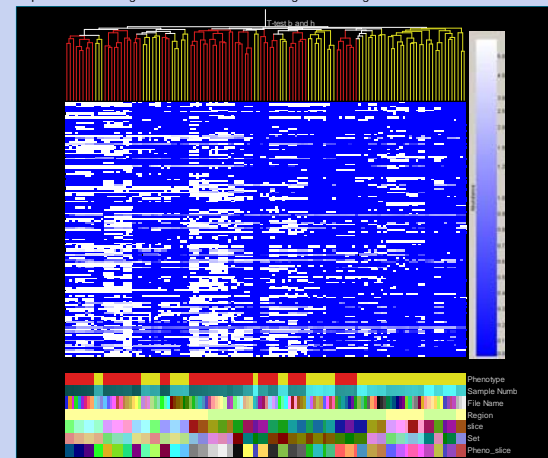
**Figure 1:** SDS-PAGE separation of mitochondrial proteins. 40  $\mu$ g protein was loaded in each lane. A GridCutter was used to extract 40 gel bands from each lane, represented by red grid. OP, obesity prone; OR, obesity resistant.



**Figure 2: Reproducibility of chromatography.** Tryptic peptides were resolved on a 150 mm C18 column and analyzed on a 6510 QTOF. Panel A above shows overlaid TICs for three replicate injections of a sample derived from a single gel slice. There was good retention time and abundance reproducibility between injections as shown in the zoom view (B). To correct for protein loading in the gel, the TIC of one technical replicate per sample was integrated between 8 and 30 minutes (C) and the average total areas were used as a normalization factor in GeneSpring MS.

**GeneSpring MS Analysis:** MHD files from 20 gel slice sets (40 gel slices, 120 files) were imported into GeneSpring MS. Molecular features were aligned using appropriate retention time and mass tolerance values. Feature abundance values were corrected by per mass and per run normalization. Initially 8,520 features resulted after alignments. Features were then filtered on relative frequency (present in 100% of technical replicates in at least one gel slice) which reduced the feature number to 4,468. A t-test was performed ( $p < 0.05$ , ) and a 2 fold change filter was applied. Of the 669 features that passed the ANOVA, 520 had higher abundance values in OP mitochondria, 146 had higher values in OR mitochondria.

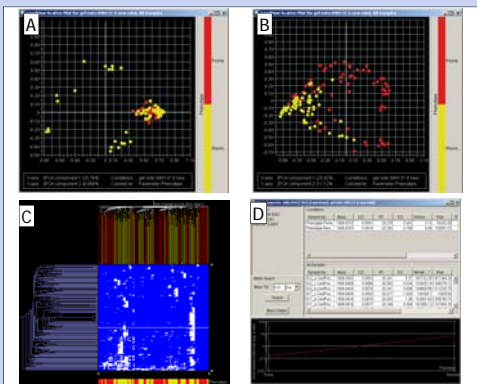
Unsupervised hierarchical cluster analysis was performed to assess the variance and reproducibility of the data. Figure 3 shows clustering of all 120 samples based on the masses that passed the T-test filter. Masses cluster closely within gel slices, and phenotype-dependent clustering is evident within distinct regions of the gel.



**Figure 3:** Unsupervised hierarchical clustering of molecular features. Features are colored by abundance with higher abundance features in white. Bottom color legend: Samples from OP rats are colored red, samples from OR rats in yellow. Additional parameters such as sample number and gel slice are indicated by other colors.

**1D SDS-PAGE Separation of Proteins:** Previous efforts to fractionate mitochondrial proteins using column chromatography methods were unsuccessful due to contaminating myosin from muscle tissue. 1D-SDS-PAGE was therefore used to resolve mitochondrial proteins and segregate proteins of interest from myosin. A total of 40 - 2mm x 7mm gel slices were excised from each lane, and proteins were cleaved by in-gel trypsin digests.

**LC/MS:** The LC/MS portions of analysis were extremely reproducible as demonstrated in Figure 2 which shows overlaid total ion chromatograms (TIC) for three replicate analyses. To correct for gel loading, in one replicate for each sample, the total ion current was integrated between 8 and 30 minutes. Integration areas were used as correction factors in GeneSpring MS.



**Figure 4:** Covariance and Fold Change Analysis. Molecular features passing the  $p < 0.05$  T-test filter were filtered for 2-fold or greater change. Principal components analyses were performed on filtered features with higher abundance values in OR (A) and OP (B) samples. Panel C, Condition and mass tree clustering for features with higher abundance values in OR samples 4, D, Raw and normalized abundance values for mass 1806.9318 showing a 40-fold difference. This feature was identified as a peptide from 3-ketoacyl-CoA.

Protein	Peptide #	Avg Fold Change
3-ketoacyl-CoA thiolase	3	18
Medium-chain specific acyl-CoA dehydrogenase	6	6.3
Trifunctional enzyme alpha subunit	6	2.75
Trifunctional enzyme beta subunit	1	20

**Figure 4:** Protein Identification and Relative Quantitation. In the fractions analyzed by targeted MS/MS 73 proteins were identified. The table above lists 4 proteins up-regulated in obesity resistant rats. All four map to lipid metabolism pathways.

**Principal Components and Fold-Change Analysis:** PCA was performed on mass features passing a 2-fold difference filter. Samples segregated based on phenotype-specific abundance differences, as well as by gel slice.

**Targeted MS/MS Analysis:** Inclusion lists were generated from masses with 2-fold changes and imported into QTOF acquisition software. Using the same gradient used for MS acquisition, features of interest were targeted based on mass and RT values. The resulting spectra were subjected to a SwissProt Rat database search using the Spectrum Mill search engine. In 6 gel slices targeted to date, 128 peptides and 73 proteins were identified. Peptides were validated by cross-referencing the exported mass and retention time list with actual peptide fragmentation (MS/MS) data. Of these, three proteins identified with higher abundance levels in OR rats function in lipid metabolism pathways.

**Automated MS/MS Analysis:** While our reverse phase method resulted in the identification of 330 mitochondrial proteins, the SDS-PAGE fractionation technique yielded over 400 mitochondrial proteins.

## Discussion

- We have demonstrated the feasibility of performing label-free proteomics quantitation in a complex mixture following fractionation by 1D SDS-PAGE. Although variability in gel slice location prevented direct lane-to-lane comparison of proteins at specific slice locations, GeneSpring MS allowed overall comparison between OP and OR phenotypes.
- In 3 gel slice sets analyzed thus far we identified 73 proteins with abundance values 2-fold different between phenotypes. Importantly three proteins upregulated in obesity resistant rat mitochondria are involved in lipid metabolism.
- As two pooled animals were used initially from each phenotype, we are now repeating the experiments with larger sample numbers. Proteins identified as differently expressed between OR and OP rats will be validated using western blotting.

## References

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