Rapid Method for Biomarker Evaluation and Validation Using Step-Wise Density Gradient Extraction and Statistical Analysis

Wenkui Lan, Neena Bitritto-Garg and Marc J. Horn, Prospect Biosystems, Inc., Newark, NJ

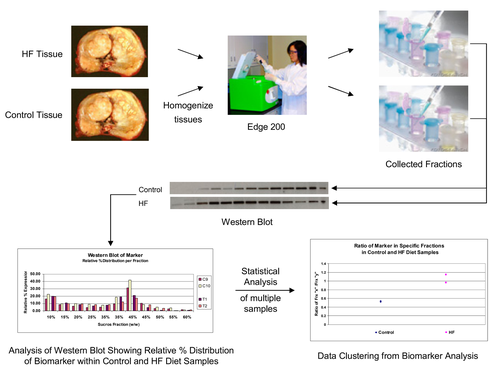
Introduction

In recent years, the role of biomarkers in drug development and diagnostics has expanded across a broad range of applications from research tools during early discovery to surrogate endpoints in the clinic. Due to the development of high-throughput proteomics, including high sensitivity mass spectrometry and automation of protein identification, the number of publications on experimental biomarkers has extended into the several thousands each year. However, only a small number of these markers will achieve clinical success. Biomarker evaluation and validation constitute critical steps in biomarker development. In this study, we demonstrate a rapid method for biomarker evaluation and validation using, A: Edge™ (Enhanced density gradient extraction) technology, a step-wise density based extraction method for simple and reproducible fractionation of tissue homogenates/cell lysates, and B: a unique statistical method for evaluation of potential biomarkers based on the relative percentage distribution of the marker across several fractions. The utility of the method in biomarker evaluation was shown using rat models of high fat diet, since high fat diet has a profound impact on brain function and apoptosis risk.

Method

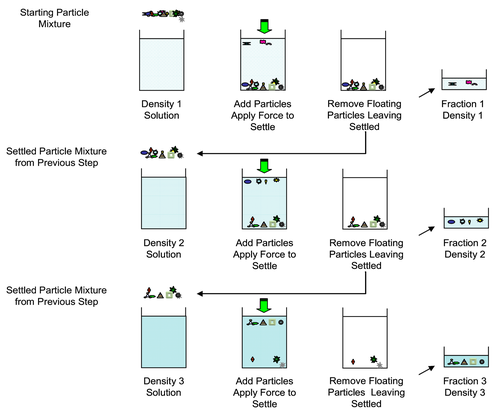
- Twenty rats were separated into two groups of 10, one group fed with special high fat diet for 6 weeks and one group fed with regular low fat diet for the same time.
- Brains and hearts from each group were collected and snap frozen.
- Tissues were homogenized, then fractionated using Edge 200 Separation System.
- All fractions from each sample were subjected to western analyses using synaptic dysfunction marker p-synapsin I, oxidative stress marker GRP75, and apoptosis marker HSP70.
- Results from each group were analyzed statistically.

Work Flow



Edge™ Technology

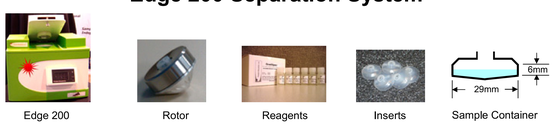
A Step-Wise Density Based Extraction



The technology can be accelerated and enhanced by various external forces, such as centrifugal, magnetic, electrical force, etc. When the process was driven by centrifugal force for the rapid, ordered extraction of substances, the process is called Centricollation™

Centricollation = Edge + Centrifugal force

Edge 200 Separation System



Benefits

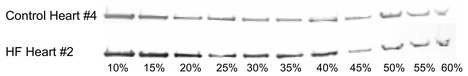
Edge Technology and Centricollation provide powerful, density based sample fractionation methods for proteomics analysis, including low abundance protein isolation and enrichment, biomarker discovery, as well as information on subcellular location.

- Sample complexity is reduced significantly.
- The method DOES NOT need any density gradient medium and does not need any gradient mixer.
- The density of the extraction medium at each step is pre-defined and no other instrument is needed for density determination.
- The recovery yield is > 90%.
- Fractionation conditions are non-denaturing.
- Starting sample volume can be 50 µl to 3.0 ml.
- Extraction volume is flexible, user-defined, and can be as low as 100 µl.
- Each extraction step takes about 5 minutes.
- The process is reproducible and straightforward.

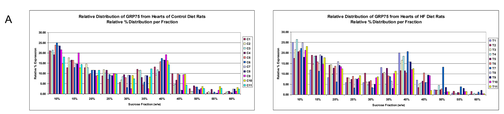
Extraction may start at any density step of interest without the need for going through the whole process.

Evaluation of GRP75 in Heart

Western Blot



Western blots of GRP75 from Edge 200 fractionated rat hearts. One sample each of a control and a HF diet rat heart is shown.

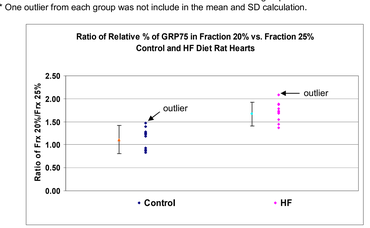


Western blot analyses of GRP75 from fractionated rat heart samples. A: Relative distributions of GRP75 within fractions of control hearts. B: Relative distributions of GRP75 within fractions of HF diet hearts. Note: Two groups of 11 rats each were used in this study.

Statistical Analysis of GRP75 Distribution Data

	Ratio of Relative % of GRP75 in Frx 20% vs Frx 25%											Mean	SD
Control	1.40	0.89	1.22	1.26	0.87	1.28	1.24	1.47*	0.83	1.18	0.93	1.11	0.207
HF	1.72	1.89	1.55	1.79	2.09*	1.87	1.73	1.44	1.69	1.37	1.69	1.67	0.172

Tabulated data from western blot analysis above showing a specific ratio (Frx 20%/ Frx 25%) of the relative % of GRP75 in control and HF diet rat hearts. The mean and SD are also shown for each group.
* One outlier from each group was not include in the mean and SD calculation.



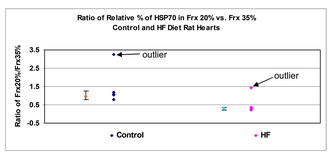
Graphical representation of data above. Error bars indicate mean +/- 1.50 SD.

Evaluation of HSP70 in Heart

Statistical Analysis of HSP70 Distribution Data

	Ratio of Relative % of HSP 70 in Frx 20% vs. Frx 35%					Mean	SD
Control	0.78	1.02	1.06	1.17	3.25*	1.01	0.16
HF	0.27	0.3	0.36	1.43*	0.23	0.29	0.05

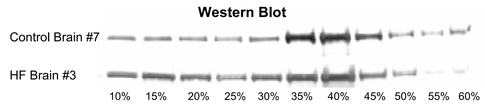
Tabulated data from western blot analysis (data not shown) showing a specific ratio (Frx 20%/ Frx 25%) of the relative % of HSP70 in control and HF diet rat hearts. The mean and SD for each group.
* One outlier from each group was not include in the mean and SD calculation.



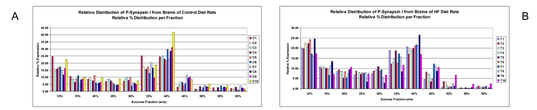
Graphical representation of data above. Error bars indicate mean +/- 1.5 SD.

Evaluation of p-Synapsin I in Brain

Western Blot



Western blots of p-Synapsin I from Edge 200 fractionated rat brains. One sample each of a control and a HF diet rat brain is shown.

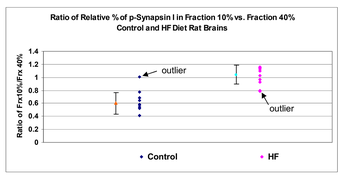


Western blot analysis of p-Synapsin I from fractionated rat brain samples. A: Relative distributions of p-Synapsin I within fractions of control brains. B: Relative distributions of p-Synapsin I within fractions of HF diet brains. Note: Two groups of 10 rats each were used in this study.

Statistical Analysis of p-Synapsin I Distribution Data

	Ratio of Relative % of p-Synapsin I in Frx 10% vs Frx 40%										Mean	SD
Control	1.01*	0.68	0.58	0.69	0.78	0.58	0.64	0.41	0.52	0.54	0.60	0.103
HF	0.96	1.15	1.16	1.10	1.13	1.15	0.80	0.79*	1.03	0.93	1.04	0.119

Tabulated data from western blot analysis above showing a specific ratio (Frx 10%/ Frx 40%) of the relative % of p-synapsin I in control and HF diet rat brains. The mean and SD for each group.
* One outlier from each group was not include in the mean and SD calculation.



Graphical representation of data above. Error bars indicate mean +/- 1.5 SD.

Conclusions

- Three potential biomarkers, p-synapsin I, HSP70 and GRP75, were evaluated as indicators of synaptic dysfunction, oxidative stress, and apoptosis respectively, using Edge technology and a propriety statistical analysis method.
- Relative distributions of these markers within fractions of samples are different between control and HF diet samples.
- Ratios of the relative % of a marker between two specific fractions within a sample are different for control and HF diet samples, with relatively tight clustering of data for each of the three markers.
- The ratio of the relative % of a marker between two specific fractions can be used to determine if the sample is control or HF diet.
- The method DOES NOT require internal or external standards.
- Because the method relies on the ratio between internal fractions of the sample, the method is independent of sample amount.
- Non-genetically identical animals were used in this experiment, and it is expected that results using this method with genetically identical animals will generate tighter clustering of data.

Prospect Biosystems, Inc.
www.prospectbiosys.com