Enhanced Biomarker Discovery Using Pathway Markers, Density Based Fractionation and MALDI-MS/MS

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Introduction

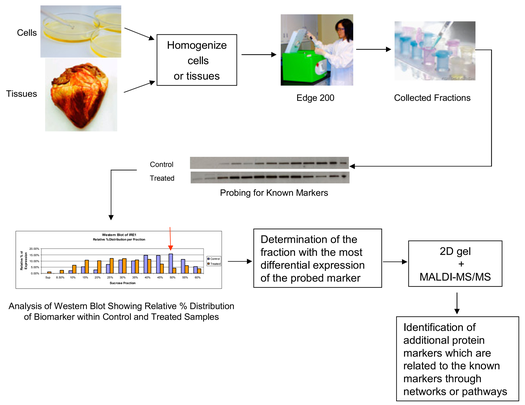
Protein biomarkers are indicators of normal biologic or pathogenic processes, or pharmacologic responses to therapeutic intervention. Information exists regarding key proteins that are part of specific biochemical pathways. To the extent that such proteins are reflective of activity within those pathways, following their movement and location can be used to identify additional proteins which may be markers of that pathway. We have developed a method to quickly and efficiently track pathway biomarkers within fractionated biological samples. Probing the distribution of known pathway biomarkers within defined fractions, we find other differentially expressed proteins related to the specific pathway. These proteins are additional markers for the pathway and can be used to track specific diseases and therapies linked to the pathway.

Experimental Design

- About 20 million INS - 1 pancreatic beta cells were grown in 5 mM glucose (control), or stimulated with 1 μM thapsigargin (treated). 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 diet for the same time. Hearts from each group were collected and snap frozen.
- Cells and hearts were homogenized, then fractionated by Centricollation™* using the Edge™ 200 Separation System. Samples from each fraction were subjected to western analyses, and were probed for IRE1 (cells) and GRP75 (heart).
- Fractions were selected for further 2DE analysis based on the relative percentage change of IRE1 or GRP75. Interesting protein spots from the cell sample were excised, digested and identified by MALDI - TOF/TOF.

***Centricollation** (cen'-tri-col-la-tion) n.– the process of using centrifugal force for the rapid, ordered extraction of substances with specific characteristics.

Work Flow

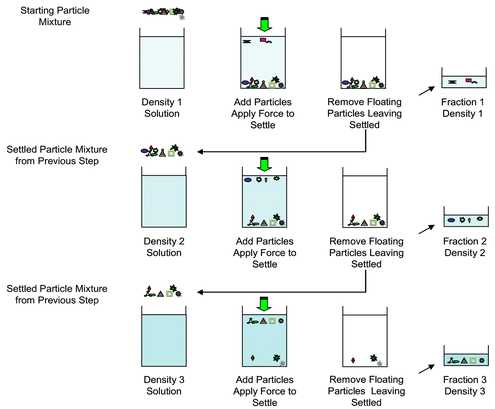


Hypothesis

Given a known pathway marker, the relative percentage distribution of the marker within fractions will differ between normal and treated (or diseased) states. A fraction can be identified which has the highest difference relative percentage distribution between normal and treated states. If 2DE analysis is performed on that fraction, in both groups, more differentially expressed proteins will be identified in this fraction than in others. These proteins are likely to be related to the known markers through networks or pathways. On the other hand, those fractions with little or no change in the relative percentage distributions of a given marker will give less differentially expressed proteins by 2DE.

Edge™ Technology

A Step-Wise Density Based Extraction



Edge 200 Separation System

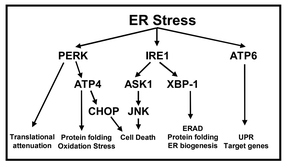
Benefits

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

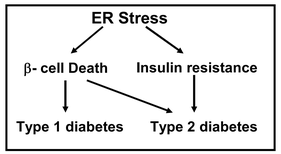
- Sample complexity is reduced significantly.
- The method DOES NOT need any density gradient medium and does not need any gradient mixer.
- The density of the extraction medium at each step is pre-defined and no other instrument is needed for density determination.
- The recovery yield is > 90%.
- Fractionation conditions are non-denaturing.
- Starting sample volume can be 50 μl to 3.5 ml.
- Extraction volume is flexible, user-defined, and can be as low as 100 μl.
- Each extraction step takes about 5 minutes.

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

Discovery of ER stress Pathway Markers

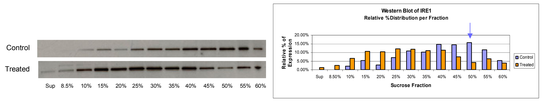


ER Stress Signaling Pathways



ER Stress and Diabetes

Western Blot of IRE1 of Pancreatic Cells



IRE1 Expression Changes versus Number of Differentially Expressed Proteins

Fr	Relative Change in IRE1 Expression*	Number of Differentially Expressed Proteins
25%	0.71	37
30%	0.10	31
35%	0.08	18
45%	-0.93	31
50%	-2.73	55

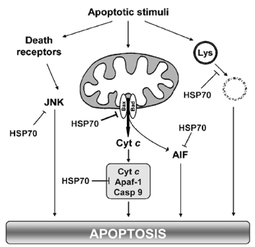
* Relative Change in IRE1 Expression = (Treated – Control)/Treated

Protein ID of Selected Differentially Expressed Spots Using MALDI-TOF/TOF

	Protein	Accession #	Relative Expression	ER Stress Pathway**	Diabetes**
Fr 25%	expressed in non-metastatic cell 1, protein (NM23A) (nucleoside diphosphate kinase)	gi|19924089	Up	✓	✓
	N-ethylmaleimide sensitive fusion protein	gi|13489067	Up	✓	✓
	similar to Eukaryotic translation initation factor 5A	gi|27672956	Up	Unkown	Unkown
	Eno 1 protein	gi|50926833	Up	✓	✓
	unnamed protein product	gi|56200	Up	Unkown	Unkown
	arginase 1	gi|8392920	Up	✓	✓
	guanine nucleotide-binding protein, beta-1 subunit	gi|13591874	Up	✓	✓
	expressed in non-metastatic cell 2	gi|55778652	Up	x	x
	signal sequence receptor 4	gi|8394364	Up	✓	✓
	unnamed protein product	gi|56082	Down	Unkown	Unkown
	aconitase 2, mitochondrial	gi|40538860	Down	✓	✓
Fr 50%	isovalery Coenzyme A dehydrogenase	gi|6981112	Up	✓	✓
	Eef1g protein	gi|51261278	Up	x	x
	similar to prohibitin (BAP 32)	gi|34873234	Up	Unkown	Unkown
	unnamed protein product	gi|56200	Up	Unkown	Unkown
	ATP synthase, H+ transporting, mitochondrial F1 complex, beta subunit	gi|54792127	Up	✓	✓
	mitochondrial aconitase	gi|10637996	Up	✓	✓
	glycerol-3-phosphate dehydrogenase 2	gi|6980978	Up	✓	
	dnaK-type molecular chaperone grp 75 precursor	gi|2119726	Down	✓	✓
	glucose regulated protein, 58 kDa	gi|38382858	Down	✓	✓
	insulin I	gi|56488	Down	✓	✓

** Relationship between Protein and ER Stress or Diabetes previously reported in literature

Discovery of Apoptotic Pathway Markers

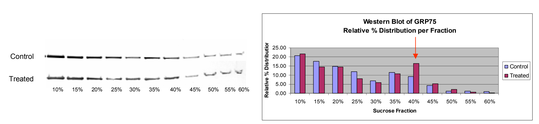


Apoptotic Pathways

(Carrido,C., Galluzzi, L., Brunet, M, et al., "Cell Death and Differentiation" 2006, *13*, 1423-1433)

Note: GRP75 is an essential protein belonging to the Hsp70 family of chaperones.

Western Blot of GRP75 of Rat Heart



Relative % Distribution of GRP75

Fr	Control	Treated
10%	20.63	21.59
15%	17.70	14.54
20%	14.69	14.41
25%	11.88	8.06
30%	6.89	6.04
35%	11.34	10.77
40%	9.18	16.47
45%	4.35	5.26
50%	1.23	2.03
55%	1.21	0.65
60%	0.90	0.18

GRP75 Expression Changes versus Number of Differentially Expressed Proteins

Fr	Relative Change in GRP75 Expression*	Number of Differentially Expressed Proteins
20%	-0.02	15
40%	0.44	59

* Relative Change in GRP75 Expression = (Treated – Control)/Treated

Conclusions

- Those fractions showing the highest difference in relative percentage distribution between control and treated for the markers IRE1 and GRP75 correlate directly with the fractions showing the highest number of differentially expressed proteins.
- Preliminary data from these two experiments validate our hypothesis for pathway biomarker discovery.
- Fractionation reveals 3.73 fold down regulation for IRE1 in treated cells in fraction 50%, and 1.79 fold up regulation for GRP75 in high fat diet rat hearts in fraction 40%.
- Density based fractionation provides rapid and valuable information connecting subcellular biology and biomarkers.
- Several unique and theoretical proteins found by MALDI demonstrate the power of Edge technology in isolating low abundance proteins and in potential biomarker discovery.
- Further investigation is needed to explore the biological roles of those unique proteins identified by MALDI.

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