TargetDiscovery
FROM OMICS TO KNOWMICS

Recovery and Immunoaffinity Mass Spectrometric Analysis of Integral Membrane Proteins from Metastatic Ovarian Cancer Tissue Using PCT™ and ProteoSolve™-TD Buffers

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Membrane Protein Extraction

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYIQKESTLHLVLRLRG

- **Membrane proteins remain a significant unmet proteomics challenge**
  - Surfactant Extraction incompatible with
    - ELISA or Ab arrays
    - Chromatography (SEC, RPLC, or Electrophoresis)
    - Mass Spectrometry
  - In Sample Proteolysis
    - Disconnects protein domains (intra-, extra-, and trans-membrane)
    - Can't study trans-membrane domains
- **ProteoSolve™-TD Buffers with PCT allows**
  - recovery of membrane proteins intact
  - proteins are stable in the extract (can be frozen and thawed)
  - compatible with common proteomic methods:
    - immunoaffinity capture (ELISA, Ab arrays, immunoaffinity chromatography)
    - SDS-PAGE and 2-D gels.
    - mass spectrometric analysis (post-affinity capture)
  - simple one step extraction procedure
    - ProteoSolve™-TD series buffers and PULSE tubes
    - Pressure Cycling Technology (Pressure Biosciences)

How does it work?

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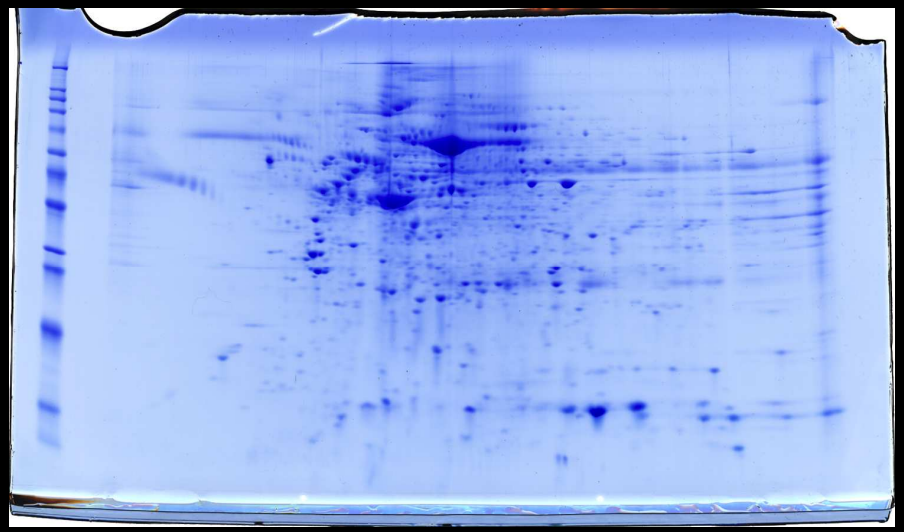
- **Pressure induces lipid membrane phase change (crystalization)**
  - Clausius-Clapyron Equation
  - $\Delta T_m = 56\ ^\circ C$
- **Work done on fluid causes adiabatic heating**
  - Energy Balance
  - $\Delta T = 6\ ^\circ C$
- **Multiple crystalization cycles**
  - force proteins from membrane
  - buffer solubilizes proteins

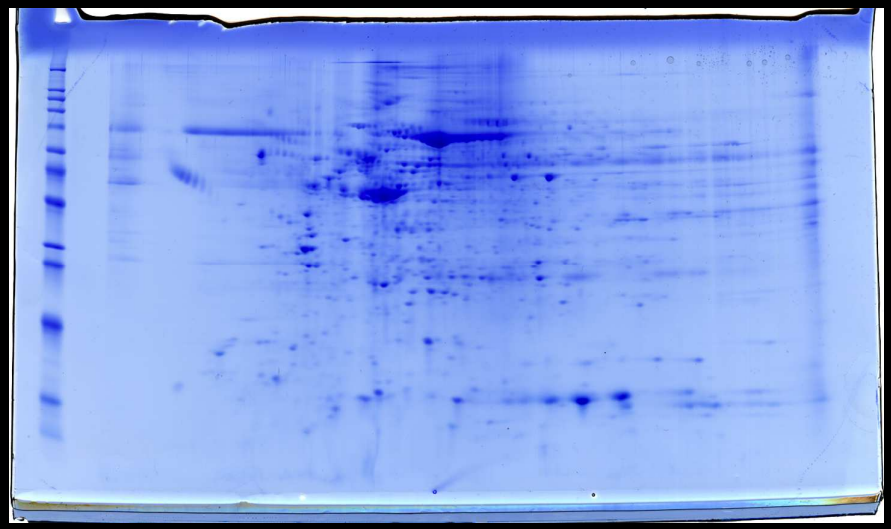
Total Protein Recovery from OvCA Tumors

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYIQKESTLHLVLRLRG

20 mM HEPES (pH 7.8)



ProteoSolve-TD2 (pH 7.8)



- **Tumor ground cryogenically with mortar and pestle under liquid N_2**
- **Proteins extracted using PCT**
  - 35,000 psi
  - 20 cycles
  - 20 sec at top and bottom of each cycle
- **IEF dimension**
  - 1:8 sample dilution into denaturing ProteoSolve™-IEF gel buffer
  - pH 3-10 IPG Ready Strip (Bio-Rad)
  - **IsoelectrIQ² IEF (Proteome Systems) 8 h at 10,000V (90kV/h total)**
- **SDS-PAGE dimension**
  - precast 8-16% TRIS-Cl (Criterion)
- ProteomIQ Blue Colloidal Coomassie stained (Smejkal, 2004).

Gel Comparison

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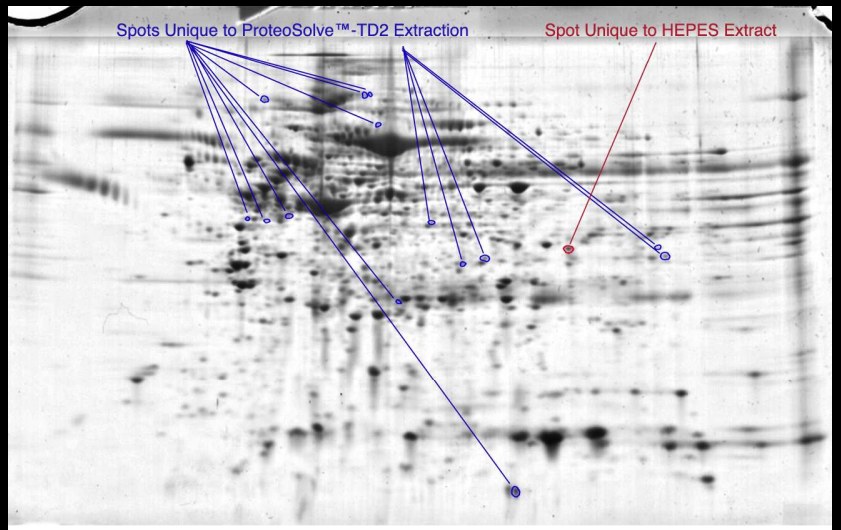

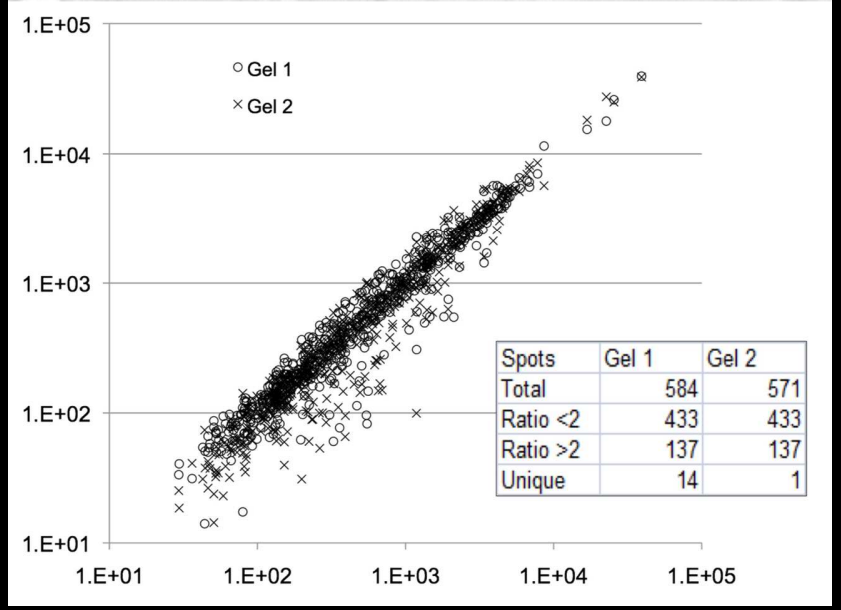

Spots	Gel 1	Gel 2
Total	584	571
Ratio <2	433	433
Ratio >2	137	137
Unique	14	1

- **Automated spot analysis(Redfin)**
  - 97% of proteins appear in both tissue preparations with similar abundance
  - 14 proteins unique to ProteoSolve™ TD2 Extract
  - 1 protein unique in the Control
- **Only abundant proteins are seen by Coomassie staining**
  - structural proteins
  - metabolic proteins
  - few membrane proteins

ProteoSolve-TD buffers are gel-compatible:
Isoelectric Focusing
SDS-PAGE

Nearly Identical patterns of protein recovery

Membrane Protein Extraction from OvCA Tumors

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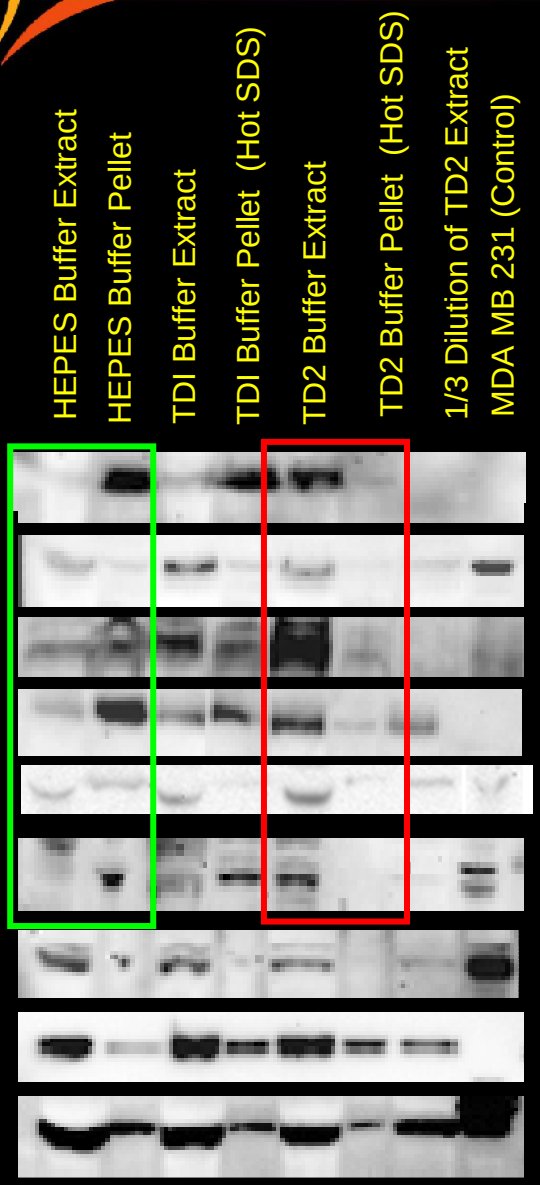

Western blots of various metastatic ovarian tumor proteins after Barocycler™ (Pressure Biosciences) extraction with various buffers.
All samples run at 35,000 psi 20 cycles 20 sec/cycle.
Samples were cryogenically-ground under liquid N_2 prior to pressure cycling.

FAS, GPC3, KDR, NRP1, EDG2, EDG4	Membrane Proteins
CCNE2	Nuclear DNA-binding Protein
Bcl2	Miitocondrial Membrane Protein
AKT	Cytosolic Protein

FAS, NRP1, and KDR are single transmembrane sequences

GPC3 is lipid anchored

EDG2 & EDG4 are 7 transmembrane GPCRs

Use of ProteoSolve-TD buffers in ELISAs

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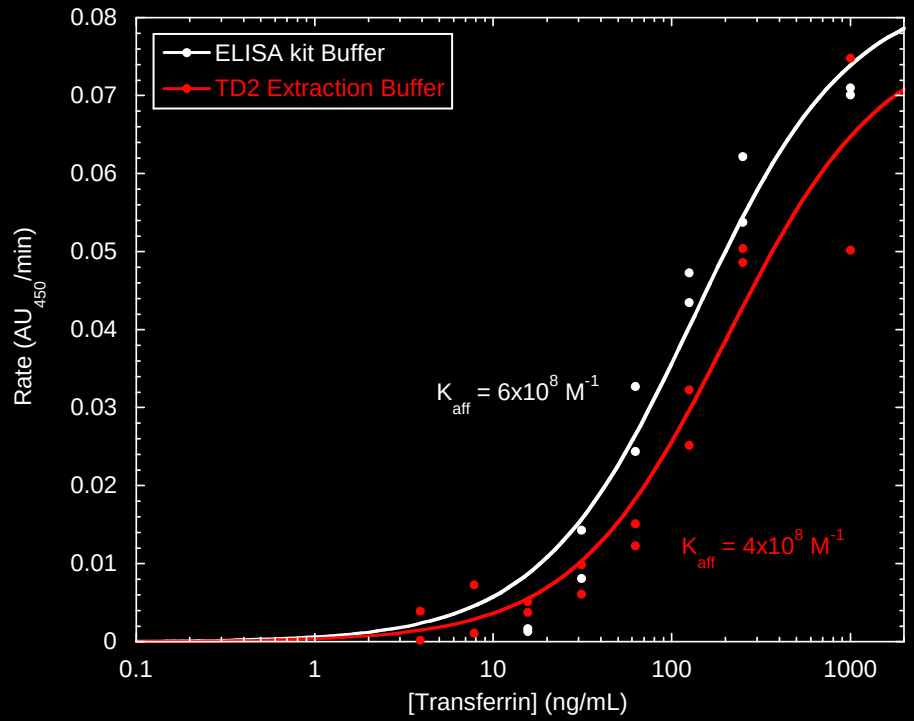

- **TD1 fully compatible with most commercial ELISA assays**
- **TD2 compatible when diluted 1:10 in TDiluent**
- **Negligible effect on assay affinity and avidity**

Biomarker Protein	Extract Concentration (ng/mL)	Tumor Concentration (ng/g)
MMP2	BDL (<10)	BDL (< 70)
MMP3	Å1	Å7
AKT1	40	20 x 10^3
VEGF R2 / sVEGF R2	0.2	1 ± 0.1

ProteoSolve™-TD buffers are compatible with immunoaffinity techniques

Adapt commercial ELISAs for soluble fractions to detect integral membrane proteins

Immunoaffinity MS Compatibility

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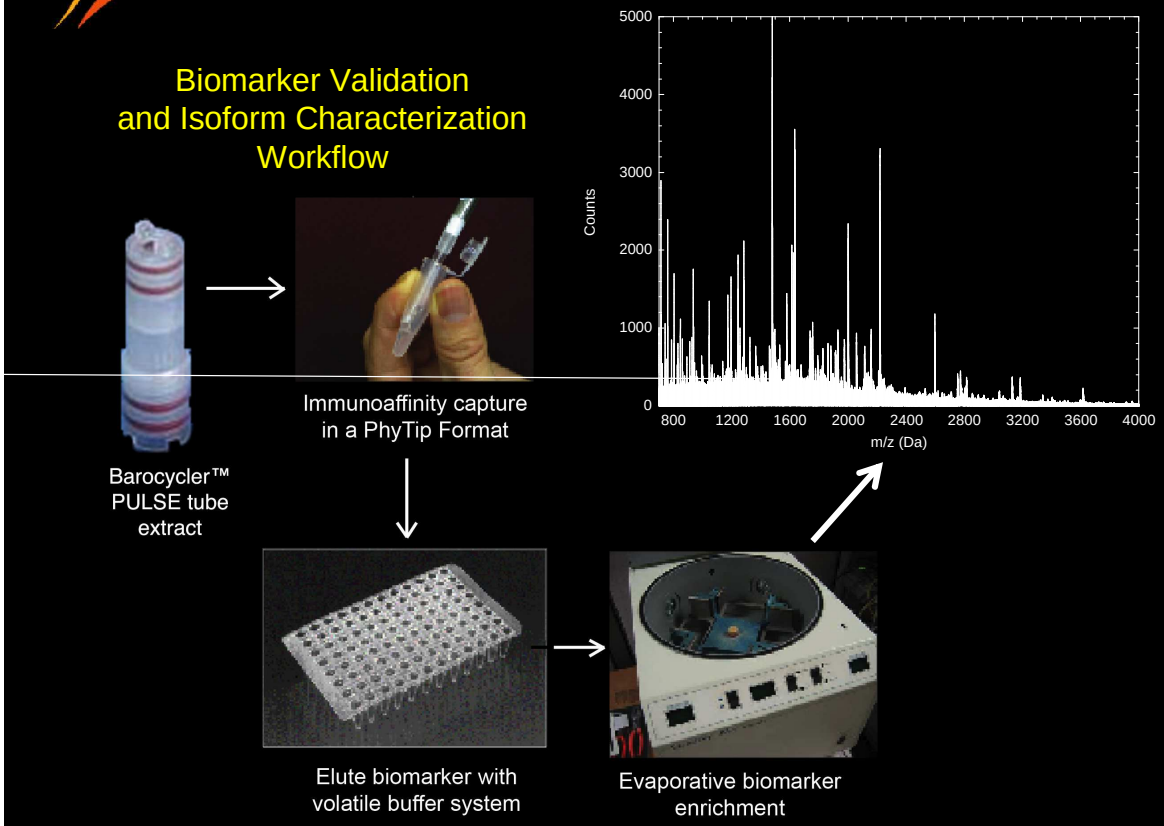
Biomarker Validation
and Isoform Characterization
Workflow



- **Transferrin (Tf) 10 µg captured on anti-Tf (Bethyl Labs)—conjugated PhyTips™ (Phynexus)**
- **Eluted with 50:50 acetic acid:water**
- **Concentrated by evaporation (Savant SpeedVac)**
- **Tryptic Digest**
  - TEP/4-VP (Hale et al., 2004)
  - 20mM NH_4HCO_3 buffer
- **Evaporate to remove volatile buffers**
- **Resuspend in MALDI matrix and spot.**
- **24 Tf peptides out of 34 total (36 % sequence coverage by MASCOT)**

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  1 MRLAVGALLV CAVLGLCLAV PDKTVRWCAV SEHEATKCQS FRDHMKSVIP SDGPSVACVK KASYLDCIRA IAANEADAVT LDAGLVYDAY LAPNNLKPVV
101 AEFYGSKEDP QTFYYAVAVV KKDSGFQMNQ LRGKKSCHTG LGRSAGWNIP IGLLYCDLPE PRKPLEKAVA NFFSGSCAPC ADGTDFPQLC QLCPGCGCST
201 LNQYFGYSGA FKCLKDGAGD VAFVKHSTIF ENLANKADRD QYELLCLDNT RKPVDEYKDC HLAQVPSHTV VARSMGGKED LIWELLNQAQ EHFGKDKSKE
301 FQLFSSPHGK DLLFKDSAHG FLKVPPRMDA KMYLGYEYVT AIRNLREGTC PEAPTDECKP VKWCALSHHE RLKCDEWSVN SVGKIECVSA ETTEDCIAKI
401 MNGEADAMSL DGGFVYIAGK CGLVPVLAEN YNKSDNCEDT PEAGYFAVAV VKKSASDLTW DNLKGKKSCH TAVGRTAGWN IPMGLLYNKI NHCRFDEFFS
501 EGCAPGSKKD SSLCKLCMGS GLNLCEPNNK EGYYGYTGAF RCLVEKGDVA FVKHQTVPQN TGGKNPDPWA KNLNEKDYEL LCLDGTRKPV EEYANCHLAR
601 APNHAVVTRK DKEACVHKIL RQQQHLFGSN VTDCSGNFCL FRSETKDLLF RDDTVCLAKL HDRNTYEKYL GEEYVKAVGN LRKCSTSSLL EACTFRRP
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Acknowledgements

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Gary Smejkal

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Webinar available: Membrane Protein Extraction & Biomarker Analysis for Solid Metastatic Ovarian Tumorshttp://www.pressurebiosciences.com/pub/media.htm

Financial Interest Disclosure: TDI has gets royalties on ProteoSolve™-TD buffers sold by PBI