Expression Pathology

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Biomarker Discovery and Quantitation for Tissue Proteomics

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Chief Scientific Officer

Tissue Proteomics

Focus on Formalin Fixed Tissue

1. Large Tissue Collections Exist
   - discover and validate protein biomarkers
   - associated with clinical outcomes

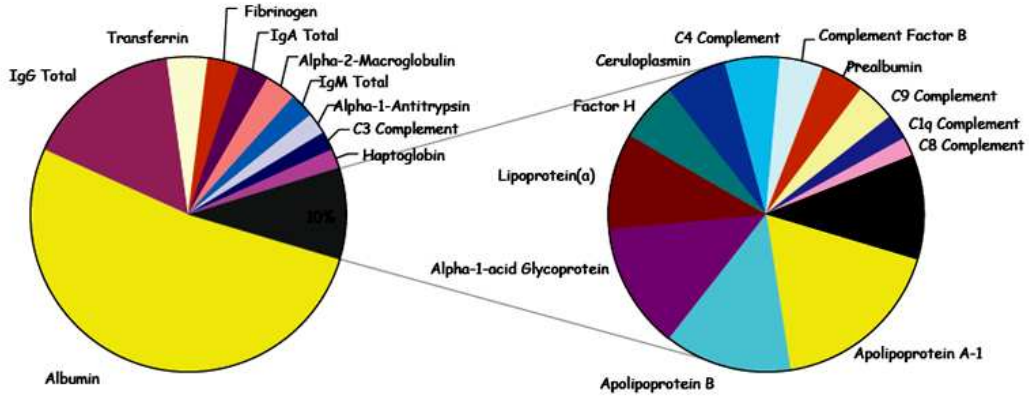
Archived Tissue Collections ⟷

- Disease Progression
- Drug Response
- Toxicity

2. Standard Form of Tissue Preservation
   - Clinical Anatomic Pathology Applications
3. Cannot be Duplicated by Freezing
   - Collection, storage, ease of use issues

The Human Proteome

Serum



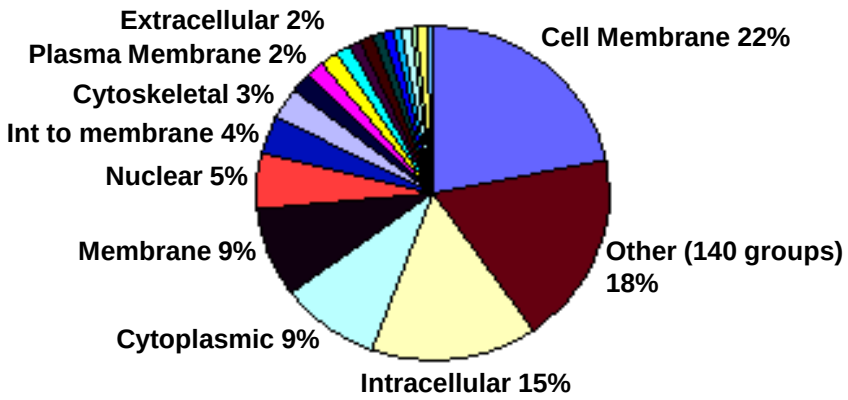
0 - 90%

(10 proteins)

90 - 99%

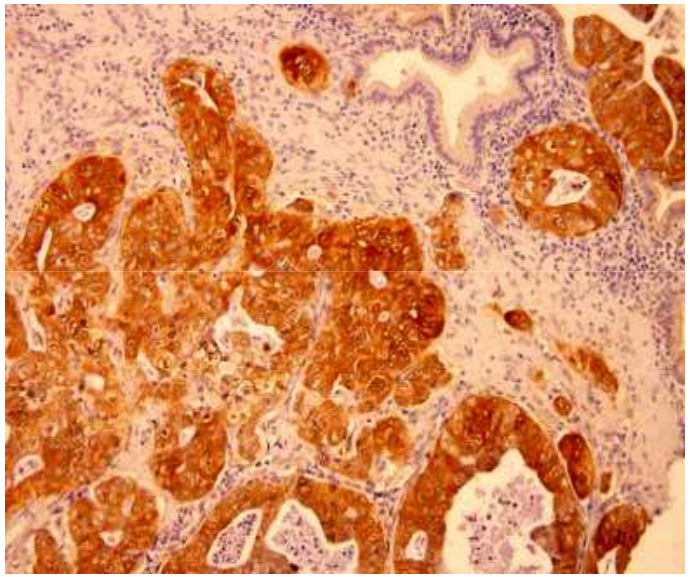
(12 proteins)

Cell



Protein Analysis in FFPE Tissue

Immunohistochemistry



- **Tissue Context**
- **Correlate to Histology**

- **Labor Intensive**
- **Limited Multiplexing**
- **Lack of Specificity**

EPI Technology

Proprietary Platform for Antibody-Free Protein Analysis of Tissue

- **Liquid Tissue®**
  - Method enables extraction of proteins from FFPE tissue
  - Patent pending – EPI's own invention

- **Director™**
  - Novel microdissection technology - fast, automatable
  - Exclusive rights of joint invention with US Naval Research Lab

- **Additional Patent Applications**
  - Diagnostic applications of Liquid Tissue®
  - Biomarkers of endometrial cancer
  - Biomarkers of cancer metastasis
  - Discovery in tissue of proteins likely to secrete into body fluids
  - NCI Her2 quantitation

Consumable Products

Liquid Tissue® MS Prep Kits

Sold for research use only

DIRECTOR™ Microdissection Slides

Can be used with Leica and Zeiss instruments

Advantages in automation, no contamination, speed over plastic film and adhesive systems

Research Opportunities

Tissue Proteomics CRO Services
Perform Completely Integrated Projects

ORIDIS Biomed
MDR
ProteoGenex

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nextgensciences
Monarch LifeSciences™
Advancing proteomics • Transforming biomarker research
University of Pittsburgh Cancer Institute

Ontario Cancer Biomarker Network

- Commercial tissue sources

- Director™ microdissection
- Liquid Tissue® processing
- Bioinformatics
- Final Report

- High quality mass spec services validated for Liquid Tissue®

Liquid Tissue® MS Protocol

Tissue Preparation:

30,000 cells from standard tissue section in 20µl Liquid Tissue® Buffer

- Needle dissection
- Laser microdissection (Leica, Zeiss, Arcturus…)

Protocol:

1. Heat tissue in Liquid Tissue® Buffer (95°C/90 min.)
2. Add trypsin, incubate (37°C/overnight)
3. Assay total protein by micro BCA

- Complete solublization of entire tissue sample; no separation
- 1 µg injection for LC MS/MS
- Use directly – detergent free – mass spec ready
- Remaining Liquid Tissue® prep can be stored at -20°C

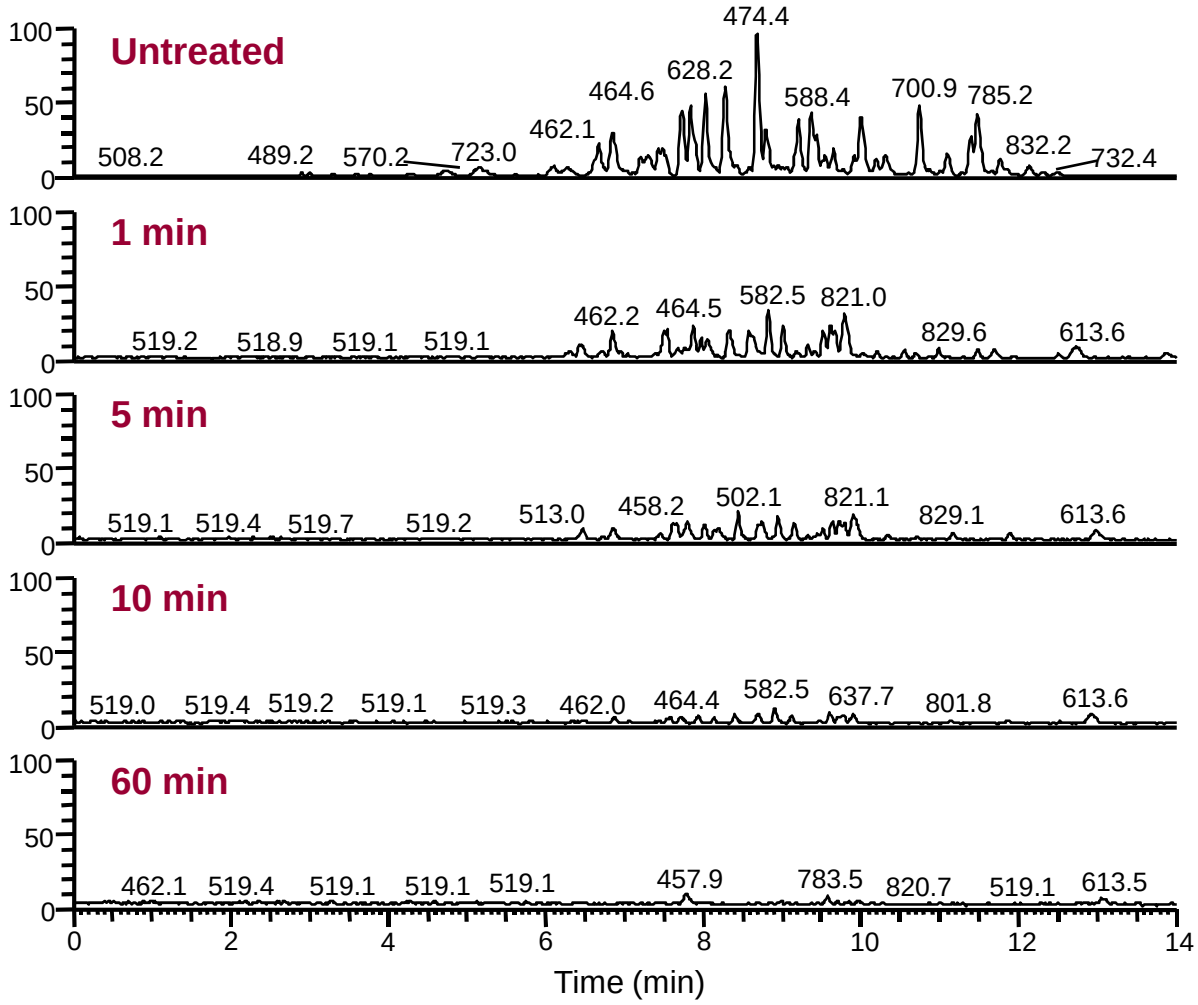
Formalin Fixation of BSA

Impact on LC MS/MS Analysis

Purified BSA

1, 5, 10, 60 minutes treatment with formalin

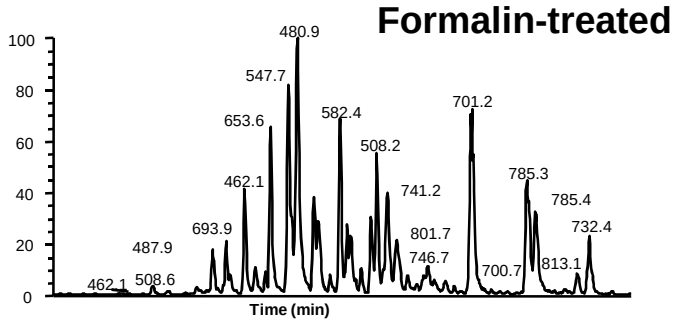
Conventional Processing



Liquid Tissue® Preparation of BSA

Impact on LC MS/MS Analysis

Liquid Tissue® Prep



Coverage 51.1%

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FPKAEFVEVTKLVTDLTKVHKECCHGDLLECADDRADLAKYICDNQDTISSKLKEC
CDKPLLEKSHCIAEVEKDAIPENLPPLTADFAEDKDVCKNYQEAKDAFLGSFLYEYS
RRHPEYAVSVLLRLAKEYEATLEECCAKDDPHACYSTVFDKLKHLVDEPQNLIKQN
CDQFEKLGEYGFQNALIVRYTRKVPQVSTPTLVEVSRSLGKVGTRCCTKPESERM
PCTEDYLSLILNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSALTPDETYVPKAFD
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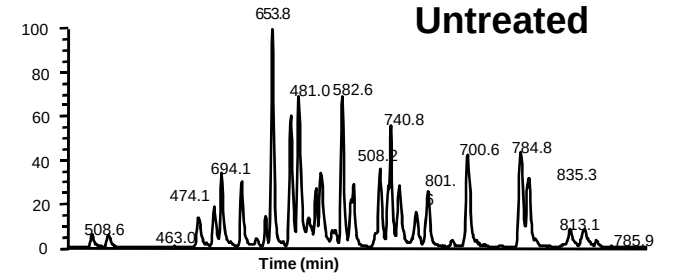
ADDKEACFAVEGPKLVVSTQTALA

Liquid Tissue® Prep



Coverage 57.8%

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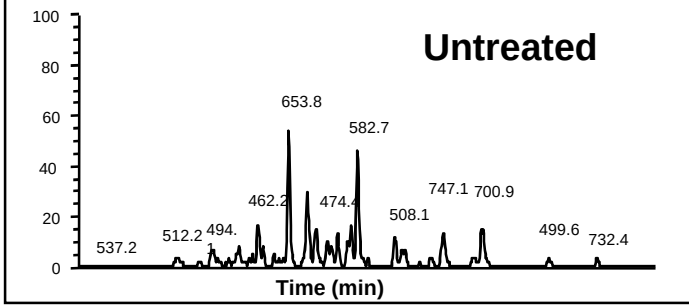
Conventional Prep



Coverage 52.5%

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AEFVEVTKLVTDLTKVHKECCHGDLLECADDRADLAKYICDNQDTISSKLKECCDKP
LLEKSHCIAEVEKDAIPENLPPLTADFAEDKDVCKNYQEAKDAFLGSFLYEYSRRHP
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KLGEYGFQNALIVRYTRKVPQVSTPTLVEVSRSLGKVGTRCCTKPESERMPCTEDY
LSLILNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSALTPDETYVPKAFDEKLFTFH
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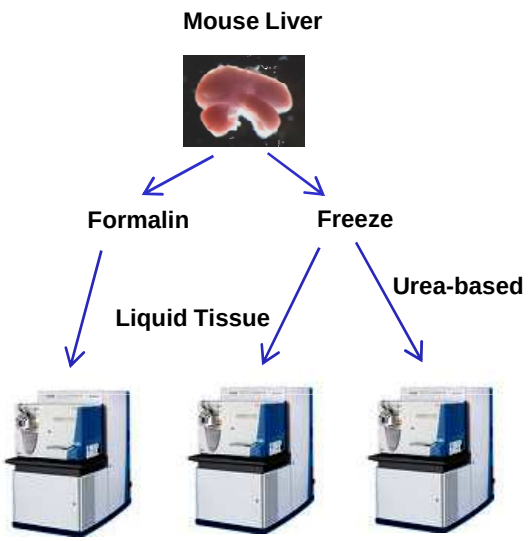
FAVEGPKLVVSTQTALA

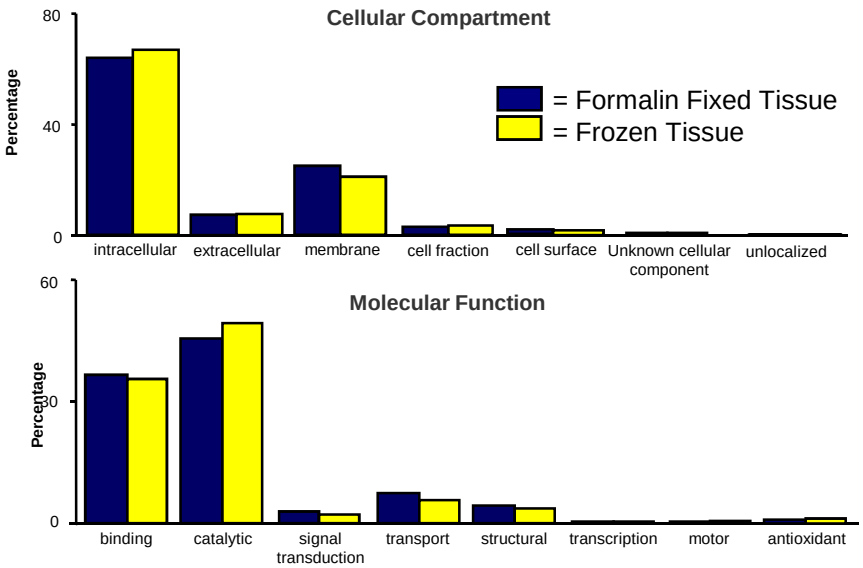
Liquid Tissue® Preparation of BSA

Impact on LC MS/MS Analysis



Sample	Unique Peptides	Unique Proteins
FFPE - LT	1710	684
Frozen - LT	2001	776
Frozen - Urea	180	122

Top 10 Proteins-Spectral Count	Accession Number	Unique Peptides FFPE	Unique Peptides Frozen-LT
Carbamoyl-phosphate synthase	Q8C196	72	91
78 kDa glucose-regulated protein	P20029	33	21
ATP synthase beta chain	P56480	32	34
60 kDa heat shock protein	P63038	32	33
10-formyltetrahydrofolate dehydrogenase	Q8R0Y6	31	41
Catalase	P24270	30	12
Hydroxymethylglutaryl-CoA synthase	P54896	29	23
Acetyl-CoA acyl transferase	Q8BWT1	27	27
Glutathione S-transferase	P10649	25	21
Pyruvate carboxylase	Q05920	24	21



Hood BL et al. *Mol Cell Proteomics* 4(11):1741-1753, 2005.

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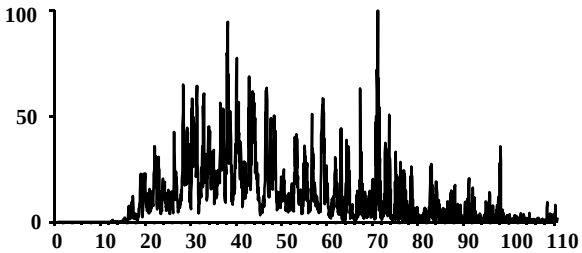
Global Profiling of Pancreatic Lesion

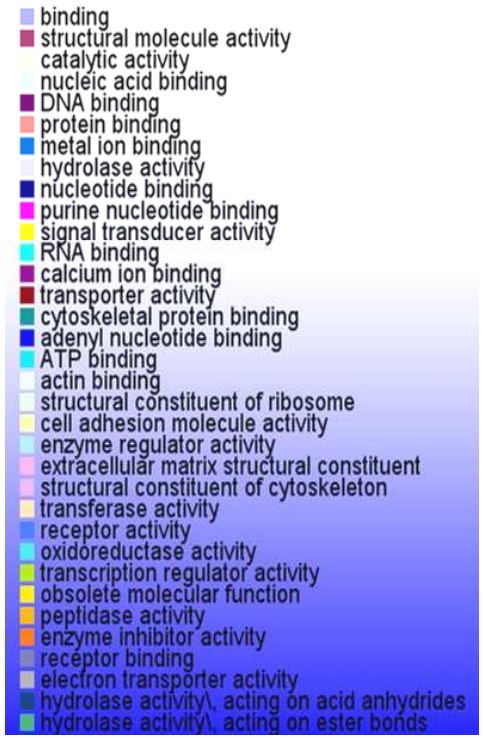
LC MS/MS Analysis

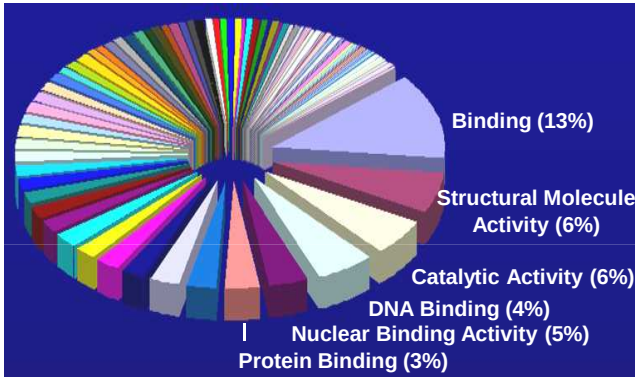
Liquid Tissue®



425 Proteins

Molecular Function





- **DMBT1**
- **Tissue Transglutaminase**

Cheung et al. *Pancreas* in press, 2008

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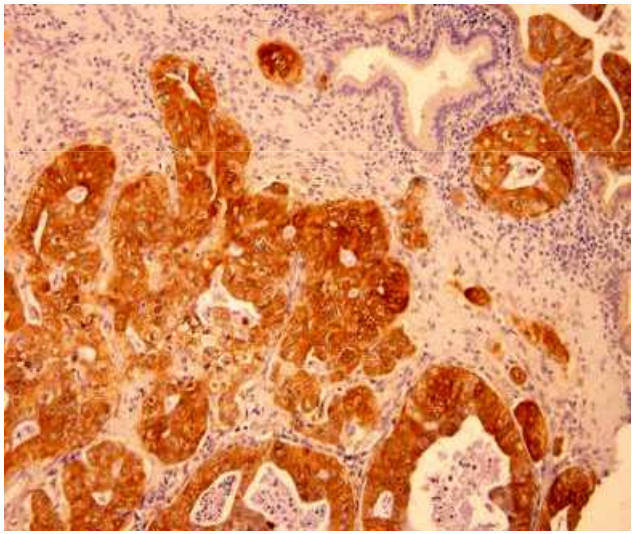
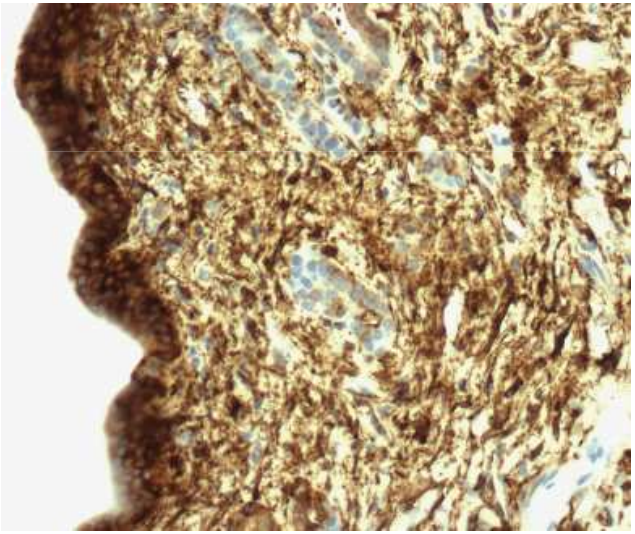
Global Profiling of Pancreatic Lesion

IHC Validation

DMBT1



Tissue Transglutaminase



Cheung et al. *Pancreas* in press, 2008

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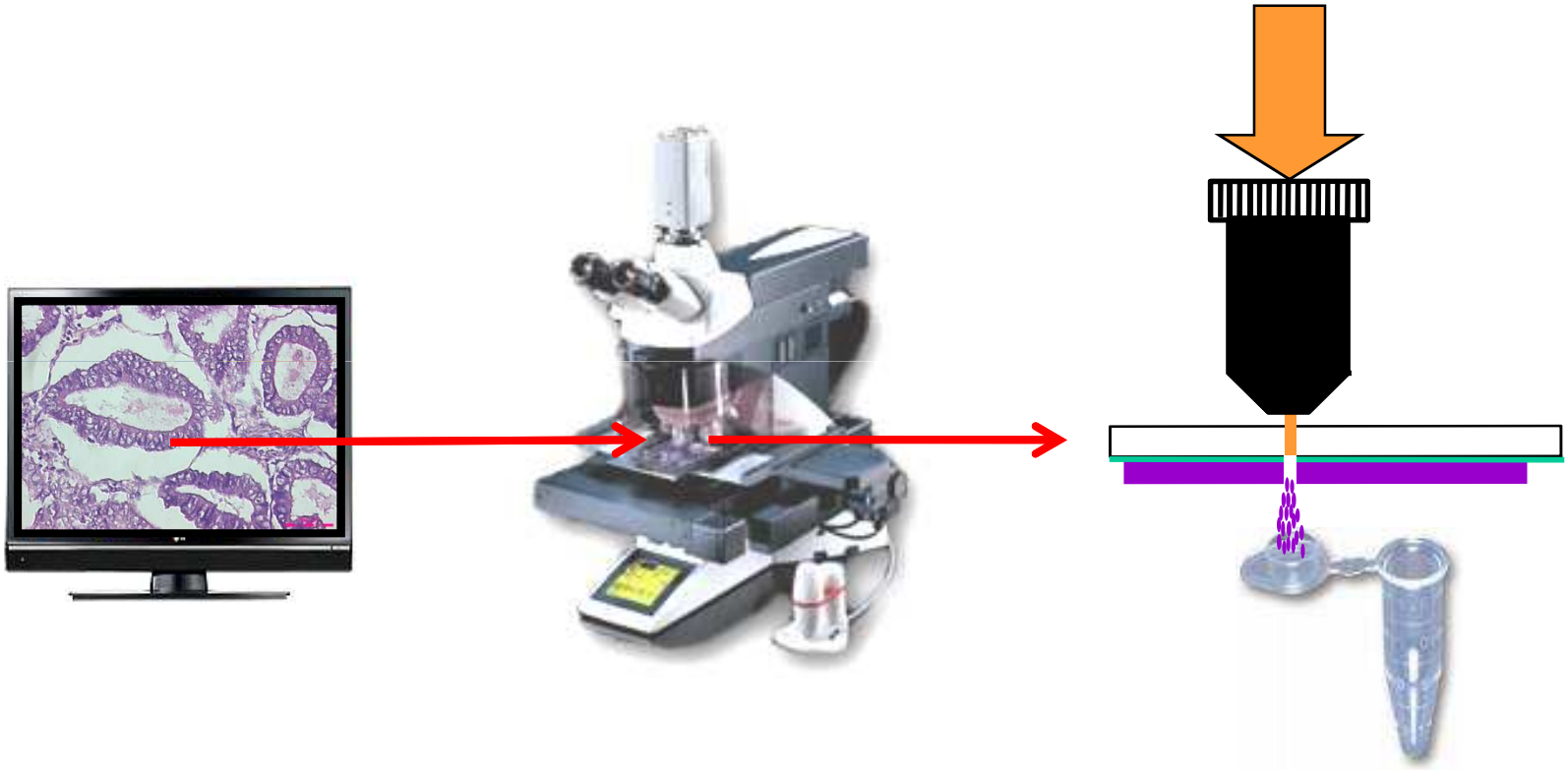
EPI Technology

DIRECTOR™ Tissue Microdissection



Hood BL et al. *Mol Cell Proteomics* 4(11):1741-1753, 2005.

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EPI Technology

DIRECTOR™ Tissue Microdissection

Laser-based, non-contact, automatable for collection of specific cell populations from complex tissue

Hood BL et al. *Mol Cell Proteomics* 4(11):1741-1753, 2005.

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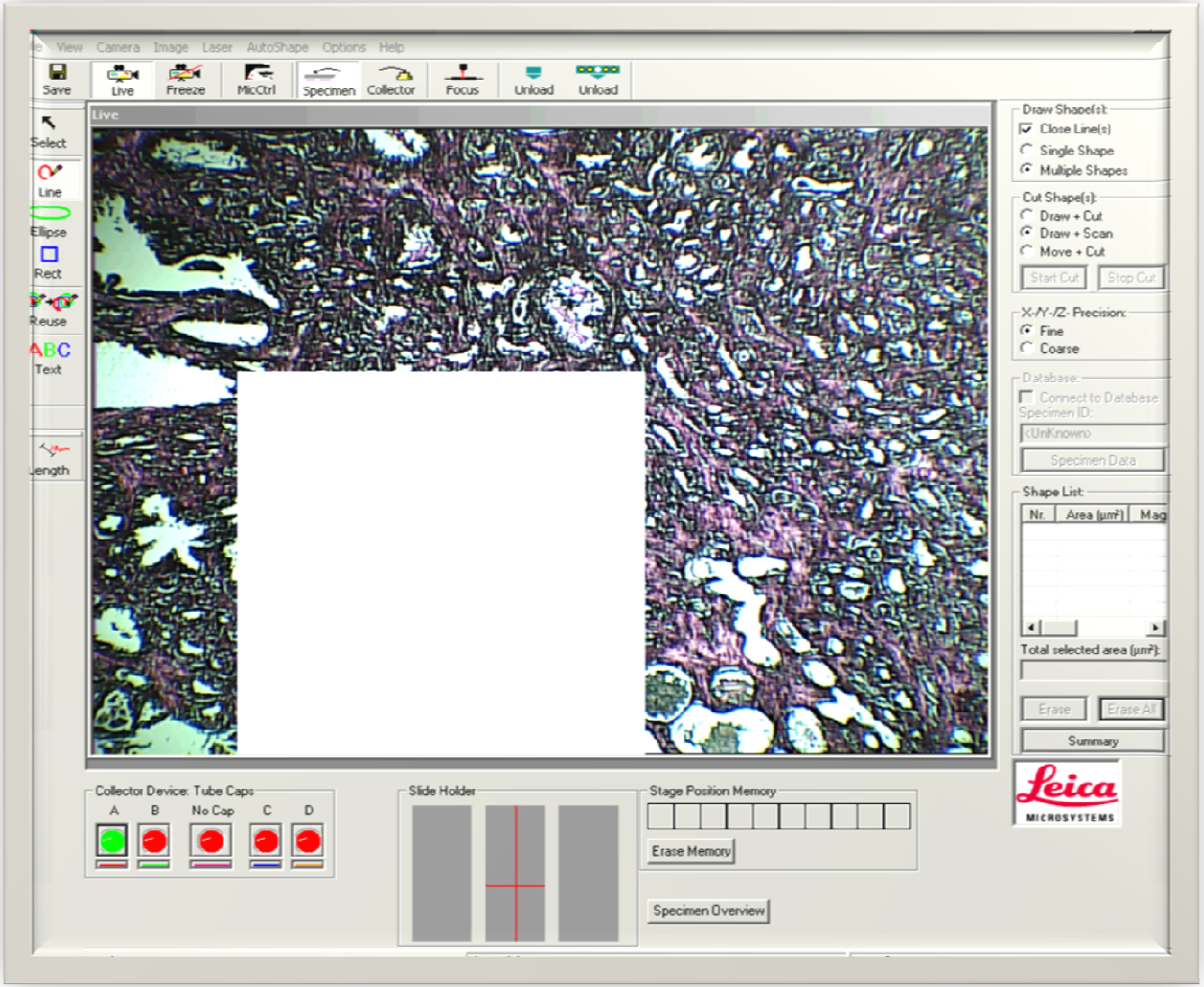
EPI Technology

DIRECTOR™ Tissue Microdissection



Hood BL et al. *Mol Cell Proteomics* 4(11):1741-1753, 2005.

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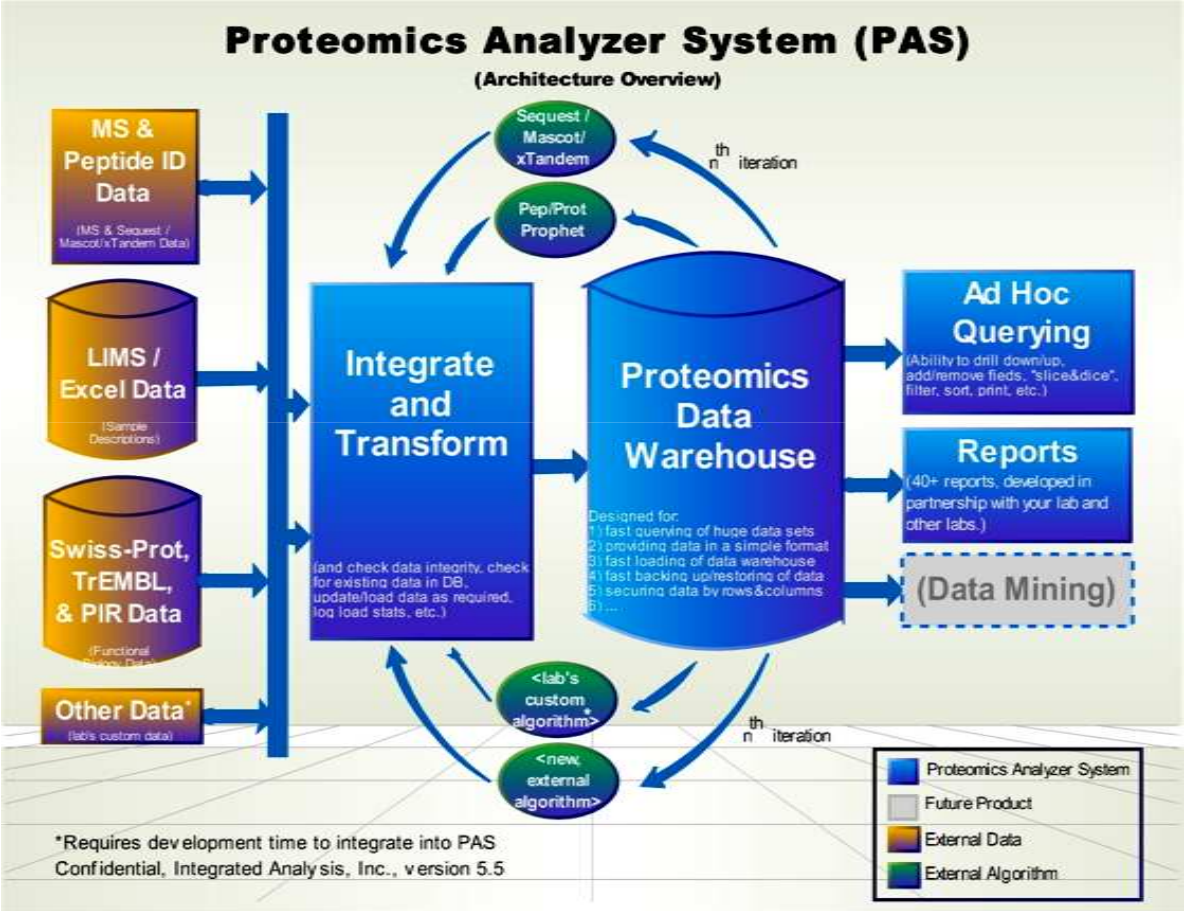
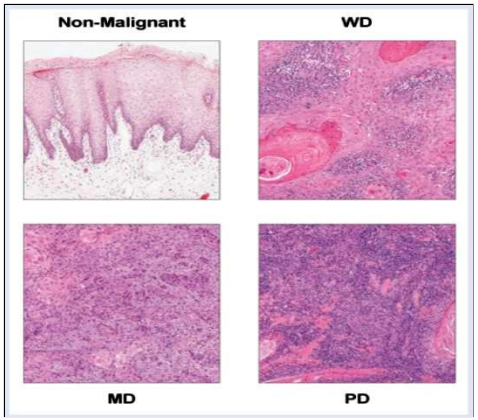
Proteomics Bioinformatics Platform

Oracle Database

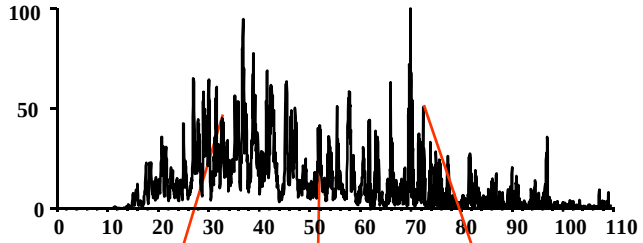


Quantitative Proteomics Study

Non-label Spectral Count Quantitation – Oral Cavity Cancer



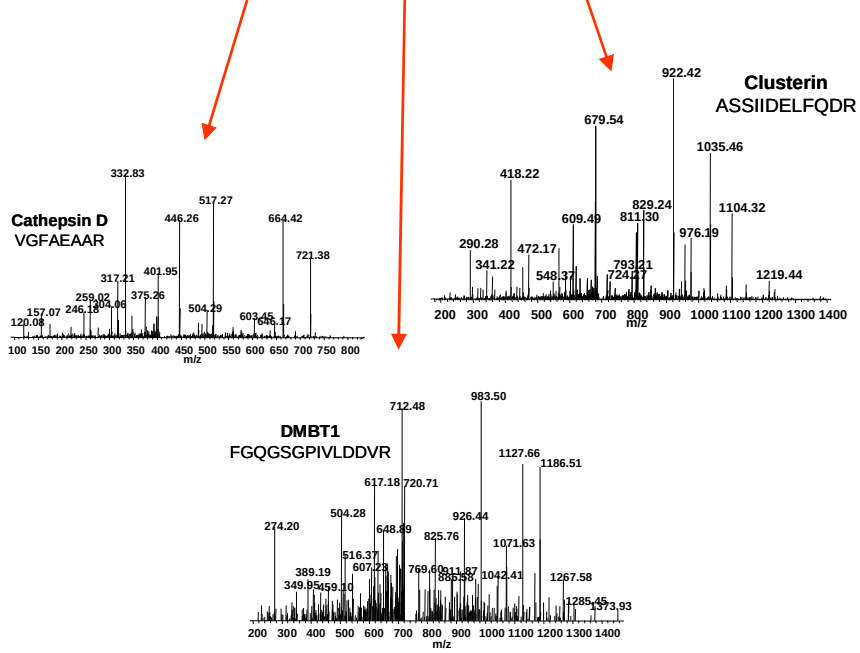
Liquid Tissue®



Spectral Count Quantitation

Non-label Quantitation Based on # of Unique Peptides per Protein

Accession	Protein	Sum T	Norm T	Well T	Mod T	Poor T
P13647	Keratin, type II cytoskeletal 5 (Cytokeratin 5)	681	21.73	31.42	23.64	23.20
P02533	Keratin, type I cytoskeletal 14 (Cytokeratin 14)	449	7.80	42.54	29.18	20.49
P08779	Keratin, type I cytoskeletal 16 (Cytokeratin 16)	387	8.27	41.09	30.23	20.41
P19013	**Keratin, type II cytoskeletal 4 (Cytokeratin 4)**	**179**	**77.65**	**3.91**	**12.29**	6.15
P04264	Keratin, type II cytoskeletal 1 (Cytokeratin 1)	146	18.49	22.60	28.77	30.14
Q04695	Keratin, type I cytoskeletal 17 (Cytokeratin 17)	76	6.58	40.79	31.58	21.05
P05783	Keratin, type I cytoskeletal 18 (Cytokeratin 18)	13	0.00	23.08	15.38	61.54
P08729	Keratin, type II cytoskeletal 7 (Cytokeratin 7)	11	0.00	0.00	90.90	10.10
P15924	**Desmoplakin (DP) (250/210 kDa paraneoplastic pemphigus antigen)**	**385**	**15.32**	**43.90**	**18.44**	**22.34**
Q13835	Plakophilin 1 (Band-6-protein) (B6P)	95	16.84	30.53	21.05	31.58
P32926	Desmoglein 3 precursor (130 kDa pemphigus vulgaris antigen) (PVA)	65	15.38	29.23	30.77	24.62
Q9Y446	Plakophilin 3	35	28.57	28.57	31.43	11.43
Q02487	Desmocollin 2A/2B precursor (Desmosomal glycoprotein II and III)	7	0.00	42.86	28.57	28.57
Q76E58	Epiplakin	1	0.00	0.00	100.00	0.00
Q14764	Major vault protein (MVP) (Lung resistance-related protein)	18	5.56	33.33	38.89	22.22
P07476	Involucrin	57	8.77	56.14	26.32	8.77
P08670	**Vimentin**	**79**	**0.00**	**36.71**	**44.30**	**18.99**
P16144	Integrin beta-4 precursor (GP150) (CD104 antigen)	4	0.00	0.00	25.00	75.00
P09211	Glutathione S-transferase P (EC 2.5.1.18) (GST class-pi) (GSTP1-1)	22	4.55	31.82	22.73	40.91
P04792	Heat-shock protein beta-1 (HspB1) (Heat shock 27 kDa protein)	83	4.82	28.92	31.33	34.94
P11142	Heat shock cognate 71 kDa protein (Heat shock 70 kDa protein 8)	20	5.00	30.00	25.00	40.00
Q86U54	CD151 antigen (CD151 protein)	1	0.00	0.00	100.00	0.00
O43490	Prominin 1 precursor (Prominin-like protein 1) (CD133 antigen)	1	0.00	0.00	0.00	100.00



Patel et al. *Clinical Cancer Research* 14(4):1002-1014, 2008

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Quantitative Proteomics Study

Candidate Biomarkers

Cell Cycle Regulation Proteins

Accession	Protein	Peptides (number)	Norm (%)	Well D (%)	Mod D (%)	Poorly D (%)
P04083	Annexin A1	120	22	35	21	23
P06748	Nucleophosmin (NPM)	21	14	29	14	43
O43707	Alpha-actinin 4	16	0	44	19	38
P42224	Signal transducer/activator of transcription 1-alpha/beta	14	0	57	29	14
P78371	T-complex protein 1, beta subunit	13	15	31	31	23
P06703	Calcyclin	12	0	67	25	8
P31146	Coronin-1A (Coronin-like protein p57)	11	0	27	45	27
Q9BPX3	Condensin subunit 3 antigen)	10	0	50	20	30
P27797	Calreticulin	8	0	50	13	38
O14757	Serine/threonine-protein kinase Chk1	7	57	14	0	29
P27348	14-3-3 protein tau	7	0	29	43	29
Q16543	Hsp90 co-chaperone Cdc37	6	0	67	0	33
Q13561	Dynactin complex 50 kDa subunit	5	0	100	0	0
P50991	T-complex protein 1, delta subunit	5	0	40	60	0
P35232	Prohibitin	5	0	20	20	60

Patel et al. *Clinical Cancer Research* 14(4):1002-1014, 2008

Quantitative Proteomics Study

Candidate Biomarkers

Signal Transduction Proteins

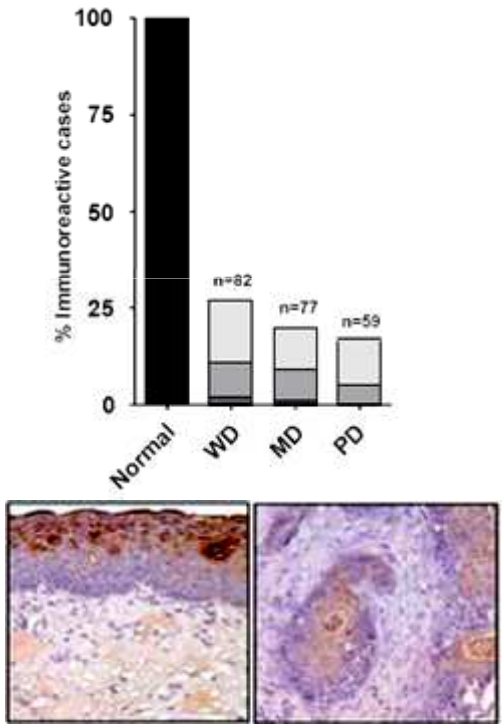
Accession	Protein	Peptides (number)	Norm (%)	Well D (%)	Mod D (%)	Poorly D (%)
P11021	78 kDa glucose-regulated protein	36	3	47	28	22
P42224	Signal transducer/activator of transcription 1-alpha/beta	14	0	57	29	14
Q08881	Tyrosine-protein kinase ITK/TSK	11	18	36	27	18
P63000	Ras-related C3 botulinum toxin substrate 1	6	67	0	0	33
P35232	Prohibitin	5	0	20	20	60
P16144	Integrin beta-4	4	0	75	25	0
P00533	Epidermal growth factor receptor (ErbB-1)	4	0	50	0	50

Patel et al. *Clinical Cancer Research* 14(4):1002-1014, 2008

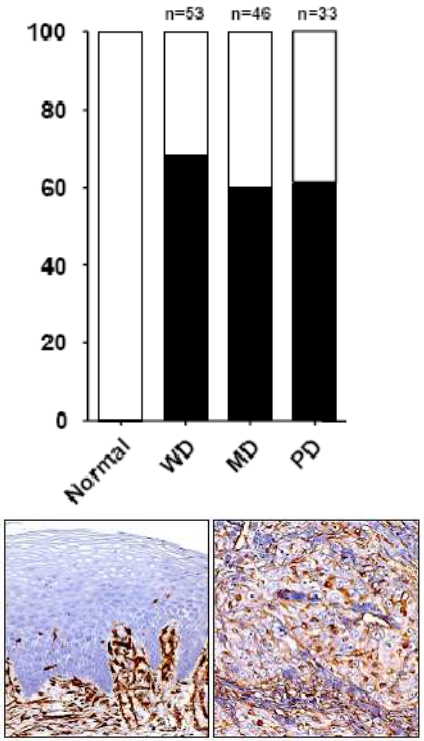
Quantitative Proteomics Study

Confirmation of Candidate Biomarkers by TMA

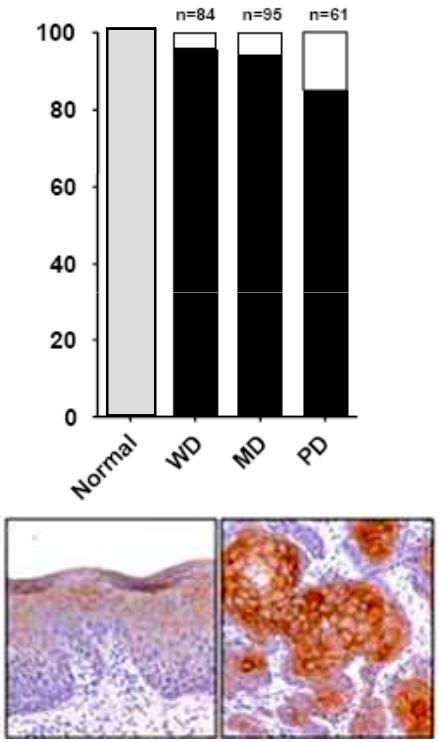
Cytokeratin 4



Vimentin



Desmoplakin



Patel et al. *Clinical Cancer Research* 14(4):1002-1014, 2008

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Demonstrated Applications

Recent Diagnostic Biomarker Discovery Project
Endometrial Cancer Biomarkers

Cancer

Sample #		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
Protein Name																																		
Glutathione S-transferase P	P09211	5	5	6	6	6	5	7	4	7	2	5	5	2	1	1	3	3	3	7	11	1	8	9	1	0	12	3	6	3	4	8	5	2
Transgelin-2	P37802	7	5	6	5	5	6	5	9	7	6	4	5	2	1	0	2	3	3	6	6	1	6	7	4	2	5	0	6	5	1	1	6	0
6-phosphogluconate dehydrogenase	P52209	6	5	4	6	5	7	2	4	3	3	6	4	1	3	1	4	2	2	6	11	1	4	1	0	0	9	0	5	1	11	3	4	0
Vinculin	P18206	11	17	5	11	11	21	22	12	11	7	14	14	3	9	4	10	8	9	6	9	0	4	16	1	0	4	1	3	1	2	0	4	0
ELAV-like protein 1	Q15717	2	2	1	1	4	2	2	2	1	0	1	1	1	1	1	2	1	2	3	3	1	2	0	0	0	4	1	2	0	1	2	1	0
Transmembrane protein Tmp21	P49755	3	3	1	1	2	1	0	2	1	1	1	1	0	0	0	1	0	0	1	2	0	1	1	1	1	1	1	1	2	1	3	1	1
Galectin-3-binding protein	Q08380	3	4	6	3	1	0	2	1	1	5	2	1	0	1	0	1	1	2	2	5	0	5	2	0	1	5	0	2	3	4	5	0	4
MARCKS-like protein 1	P49006	1	2	2	0	1	2	0	1	1	1	1	1	2	2	1	2	2	2	1	1	1	3	0	0	0	1	0	0	1	0	2	1	0
Tenascin	P24821	0	9	1	3	6	2	21	6	6	0	1	4	2	3	2	5	1	1	1	2	0	2	5	0	0	4	0	0	6	1	0	1	0
14-3-3 protein eta	Q04917	2	1	2	1	2	1	2	2	1	3	1	1	1	1	1	2	1	1	1	2	1	0	0	0	0	1	0	1	0	1	1	1	0
Proteasome activator 28-alpha	Q06323	3	1	4	1	2	4	1	1	3	2	1	1	0	0	0	4	1	2	1	3	0	5	2	1	1	0	1	2	3	0	2	2	0
Glucosidase 2 subunit beta	P14314	3	4	5	2	3	6	5	4	5	3	3	4	0	2	1	3	2	1	2	3	0	5	3	0	0	5	0	2	1	0	5	1	0

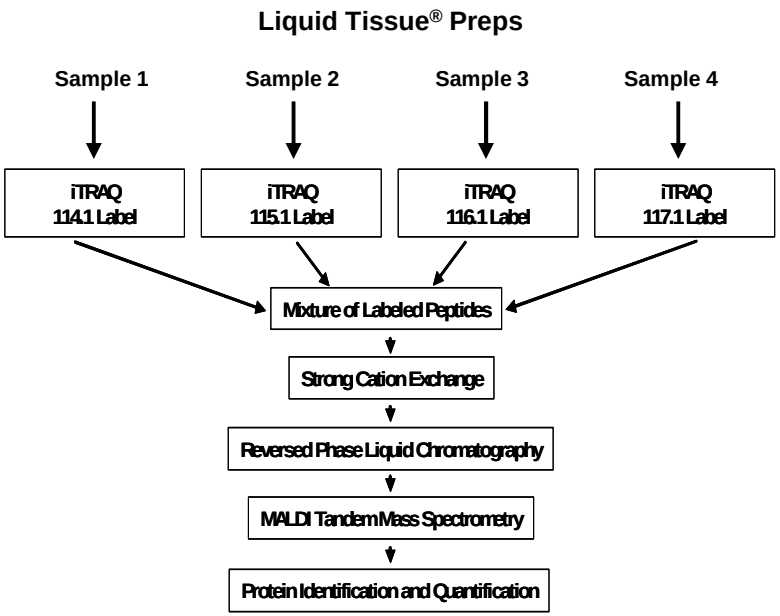
Normal

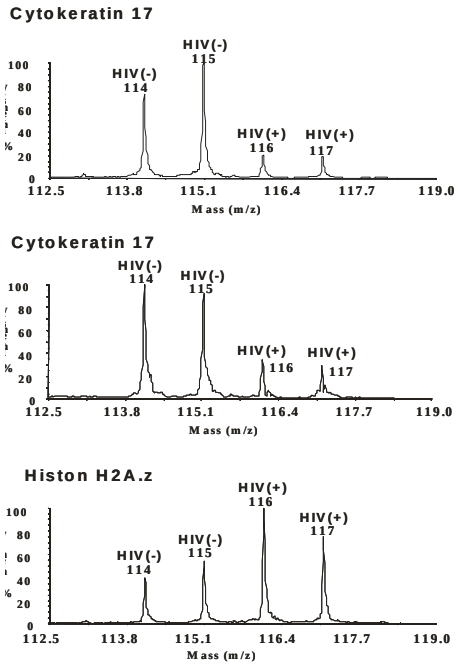
Sample #	34	35	36	37	38	39	40	41	42	43	44	45
Protein Name												
Glutathione S-transferase P	1	1	0	1	2	3	2	1	0	2	0	0
Transgelin-2	1	0	2	0	0	3	3	0	0	1	0	0
6-phosphogluconate dehydrogenase	0	0	1	0	1	1	1	2	0	0	0	0
Vinculin	4	1	0	0	2	0	2	3	0	1	0	0
ELAV-like protein 1	1	1	0	1	0	1	0	0	0	0	0	0
Transmembrane protein Tmp21	1	0	0	0	0	0	0	0	0	0	0	0
Galectin-3-binding protein	1	1	0	1	0	0	0	0	0	0	0	0
MARCKS-like protein 1	0	0	0	0	0	0	0	0	0	0	0	0
Tenascin	0	0	0	0	0	0	0	0	0	0	0	0
14-3-3 protein eta	0	0	0	0	1	1	0	0	0	0	0	0
Proteasome activator 28-alpha	0	0	0	0	0	1	1	2	0	0	0	0
Glucosidase 2 subunit beta	0	1	0	1	1	0	0	1	0	0	0	1

2-3 peptides	low abundance
3-5 peptides	Med abundance
>6 peptides	High abundance

Demonstrated Applications

Differential Expression by iTRAQ

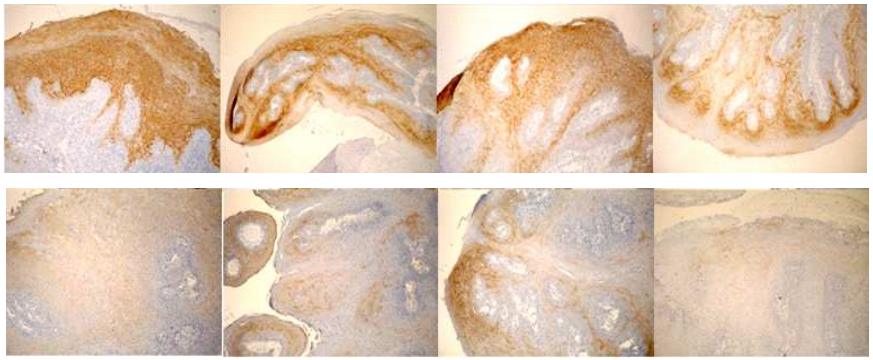




Cytokeratin 17 by IHC

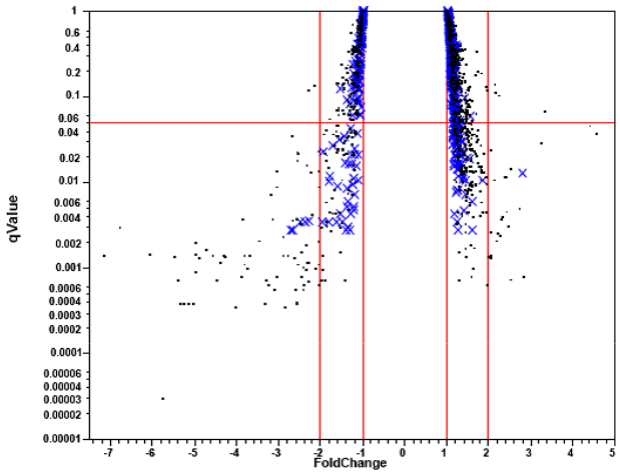
HPV(+)HIV(-)

HPV(-)HIV(+)



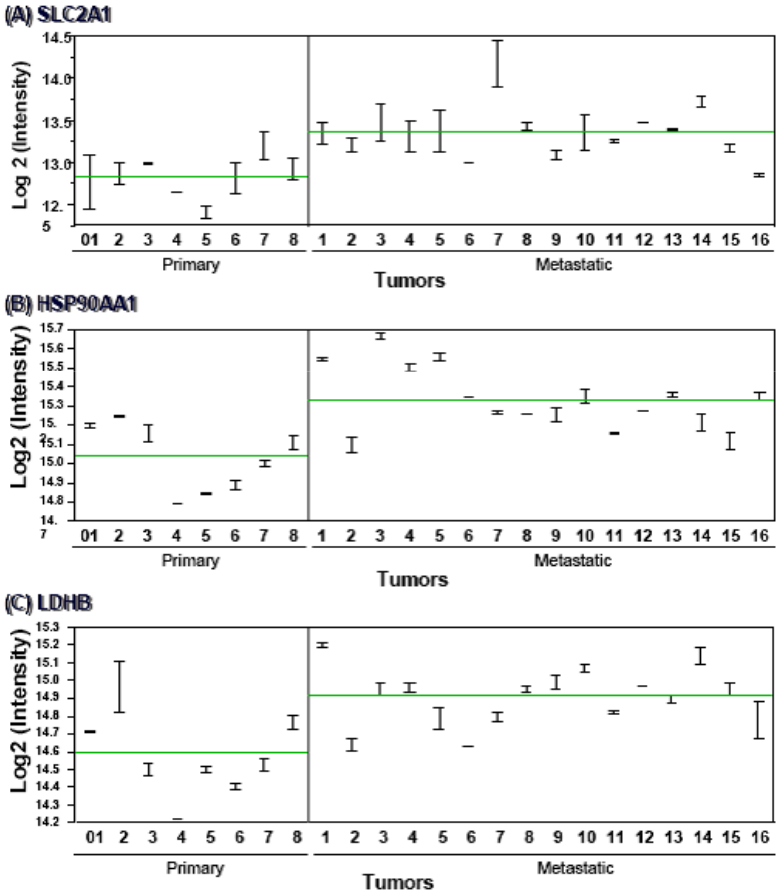
Demonstrated Applications

Recent Diagnostic Biomarker Discovery Project
Melanoma Biomarkers



Accession number	Gene Symbol	Annotation	# of peptides sequenced	q-value
Q59GX2	SLC2A1	Solute carrier family 2, facilitated glucose transporter member 1	2	0.007
P07900	HSP90AA1	Heat shock protein HSP 90-alpha	17	0.008
P07195	LDHB	L-lactate dehydrogenase B chain	12	0.008
Q6FHV0	MIF	Macrophage migration inhibitory factor	4	0.012
Q9UDE9	LDHA	L-lactate dehydrogenase A chain	17	0.041

These proteins have been previously reported to be overexpressed in metastatic melanoma.

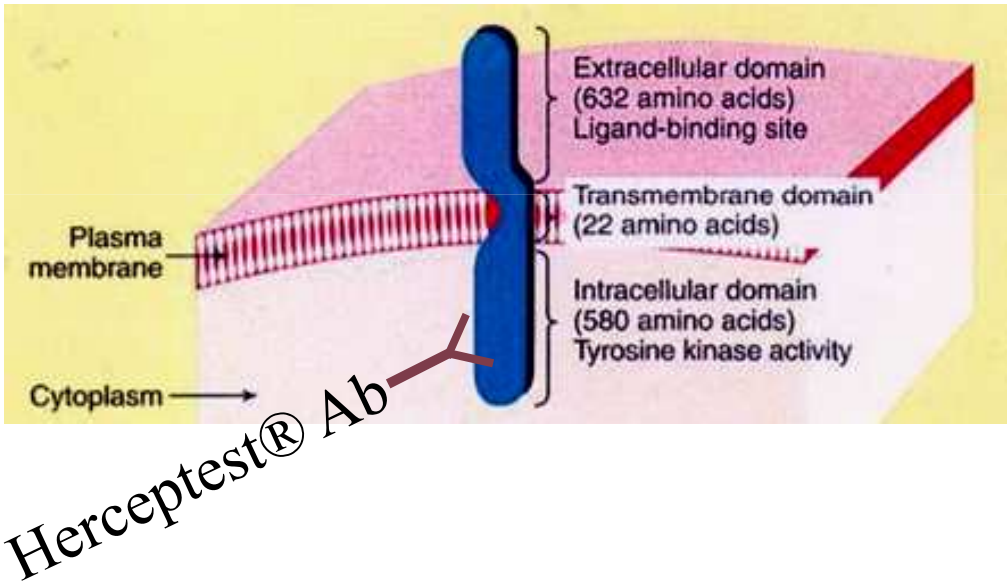


Huang et al. *in preparation* (John Wayne Cancer Institute)

Expression Pathology

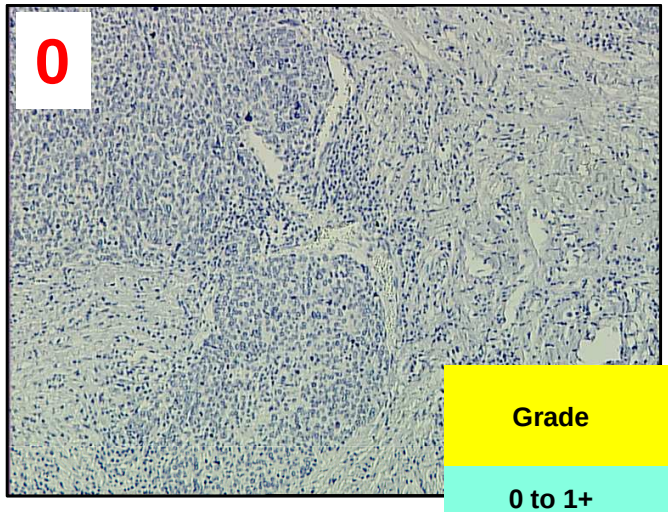
Targeted Protein Quantitation

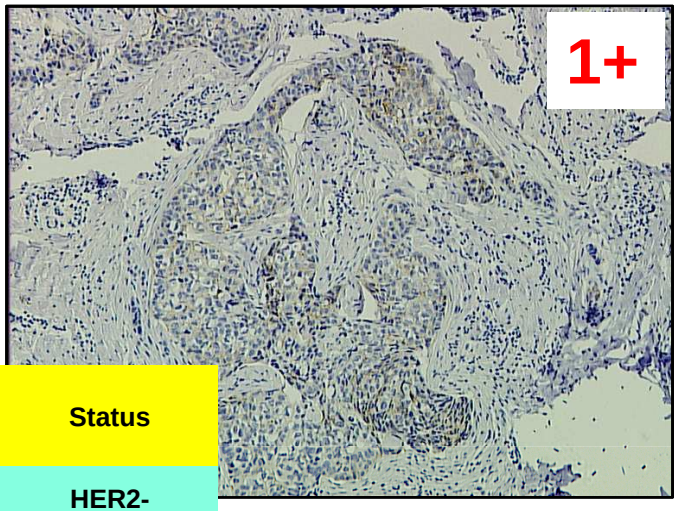
Her2 Protein



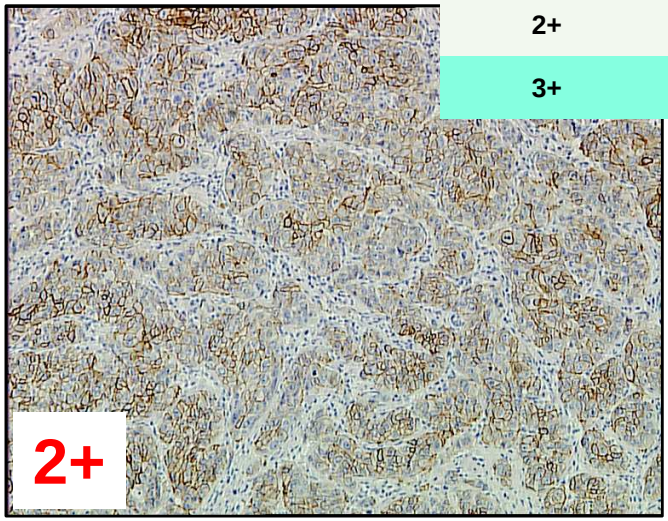
Targeted Protein Quantitation

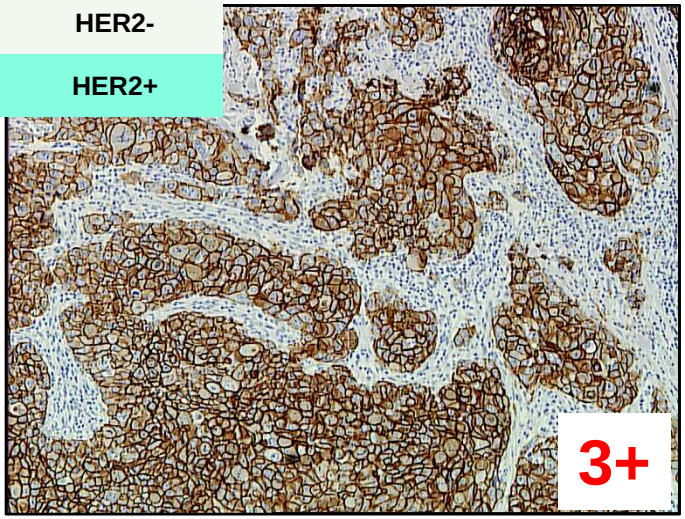
Her2 Quantitation





Grade	HER2 Protein Expression	Status
0 to 1+	Normal	HER2-
2+	Moderate*	HER2-
3+	High	HER2+

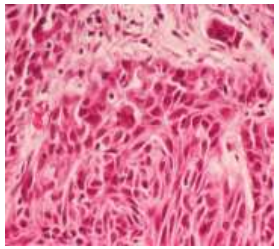




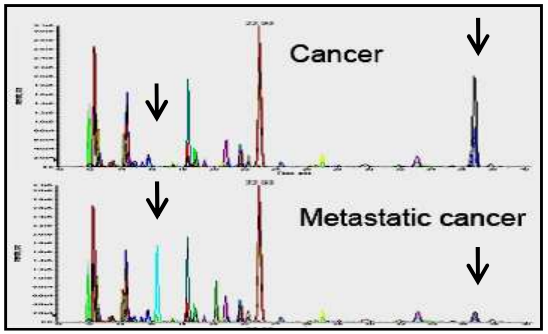
Targeted Protein Quantitation

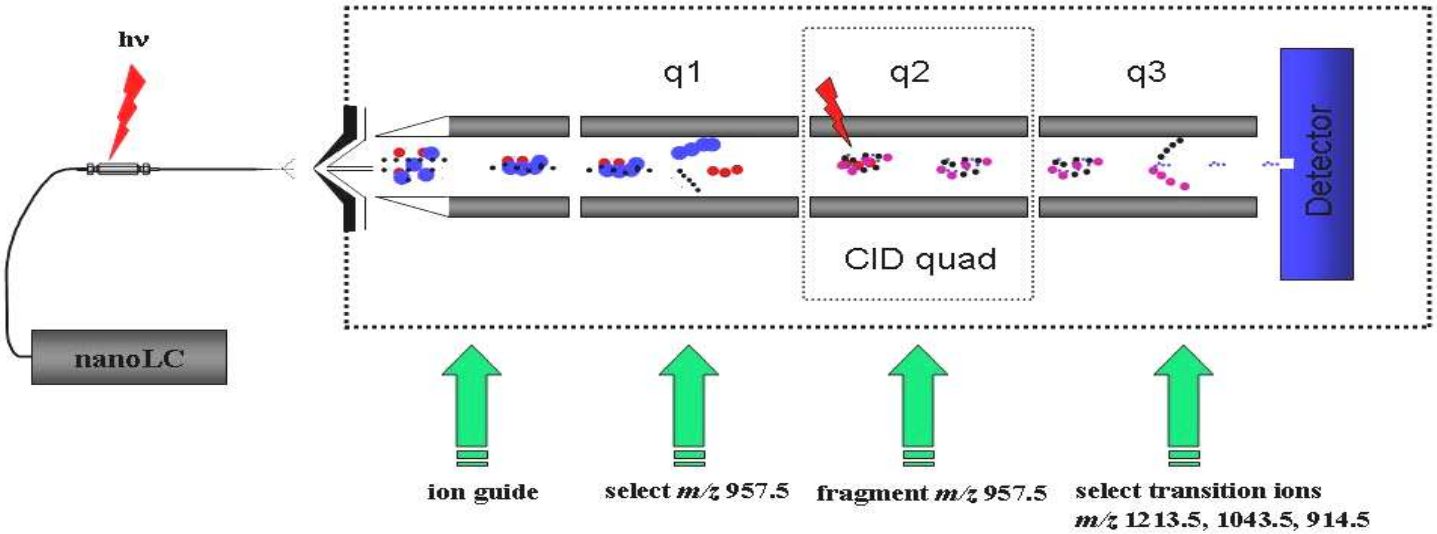
Selected Reaction Monitoring (SRM)
Multiple Reaction Monitoring (MRM)

Formalin Fixed Tissue



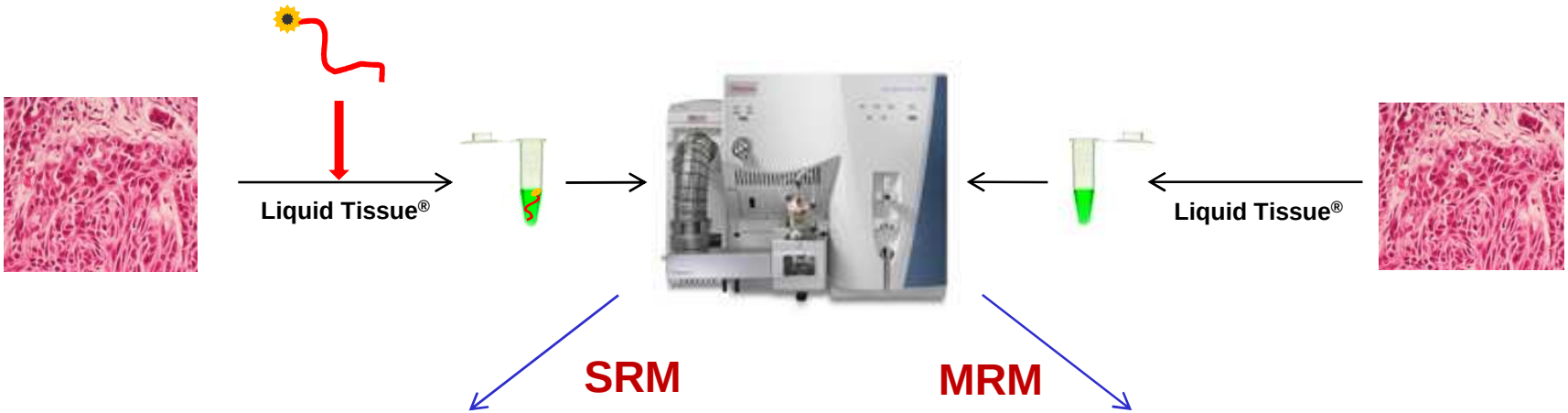
Liquid Tissue®

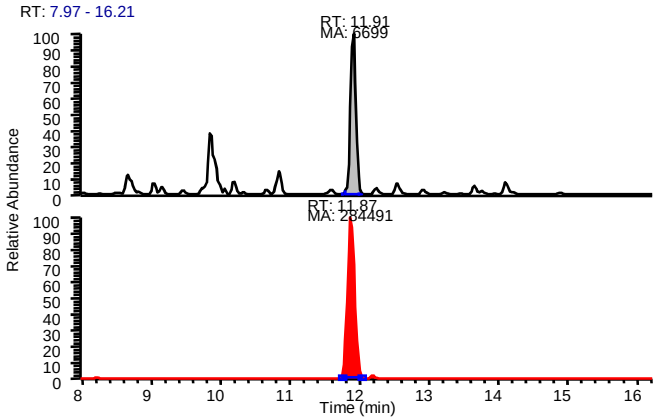


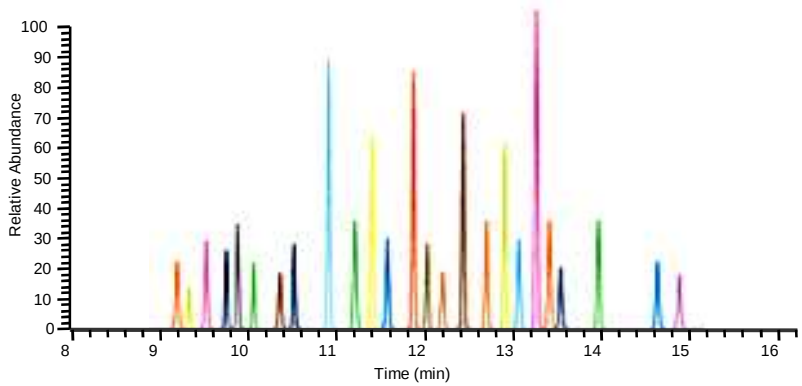


Targeted Protein Quantitation

Selected Reaction Monitoring (SRM)
Multiple Reaction Monitoring (MRM)

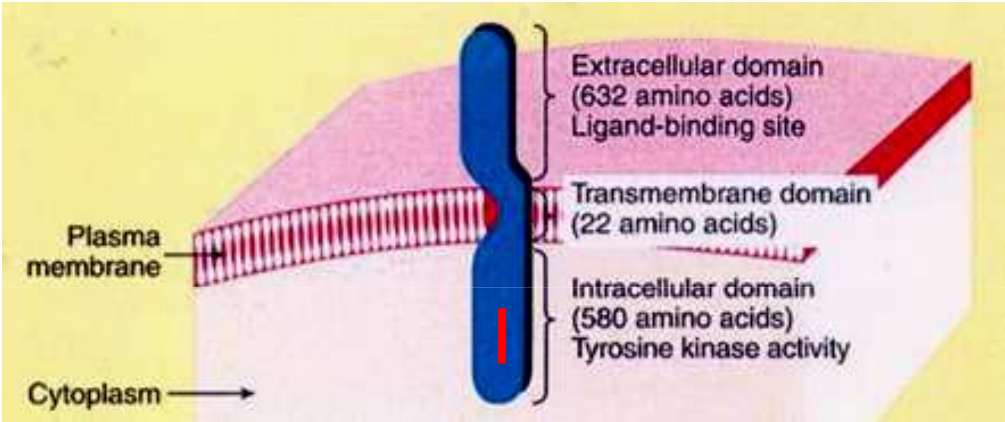






Targeted Protein Quantitation

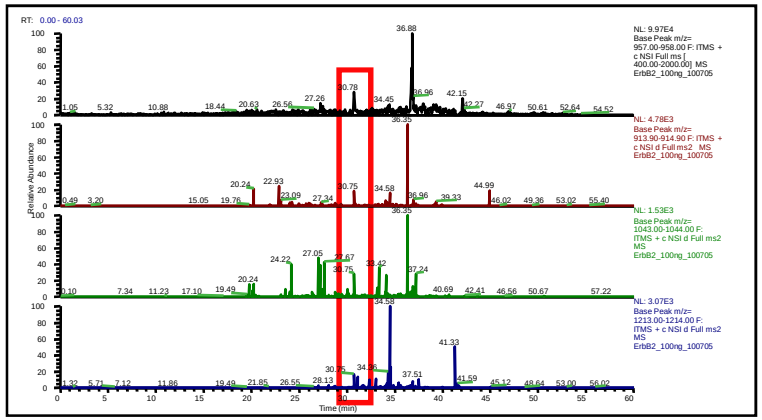
Her2 Protein



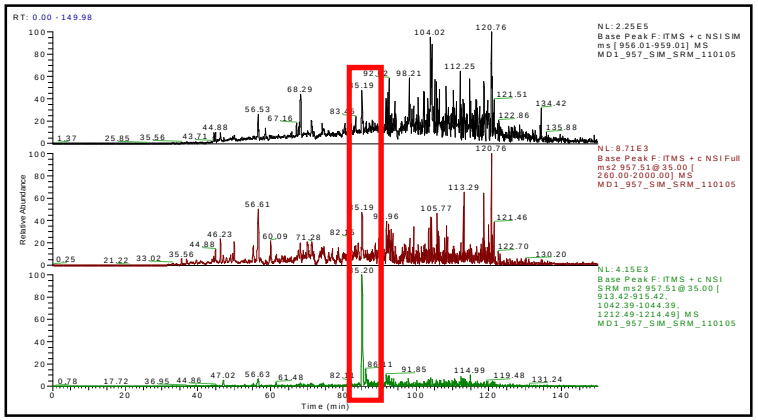
Targeted Protein Quantitation

Her2 Protein

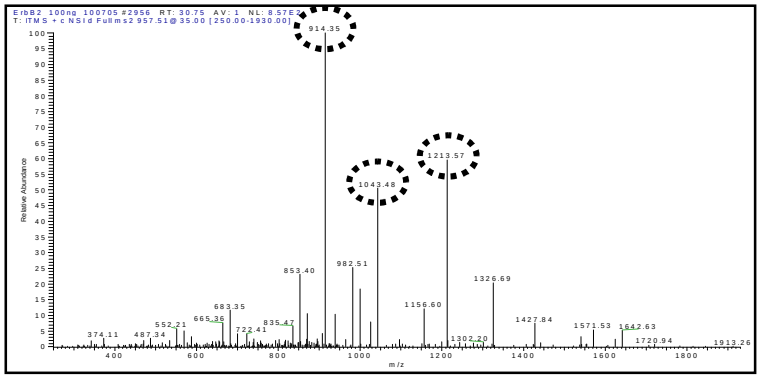
Tryptic Digest of Her2 Protein



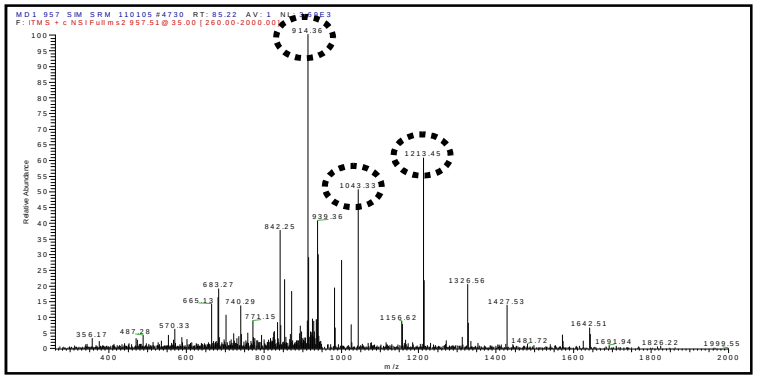
Peptide Observable in Tumor Tissue



Monitor Transition Ions



Transition Ions Observable in Tumor Tissue



Targeted Protein Quantitation

Quantitative Selected Reaction Monitoring (Q/SRM)

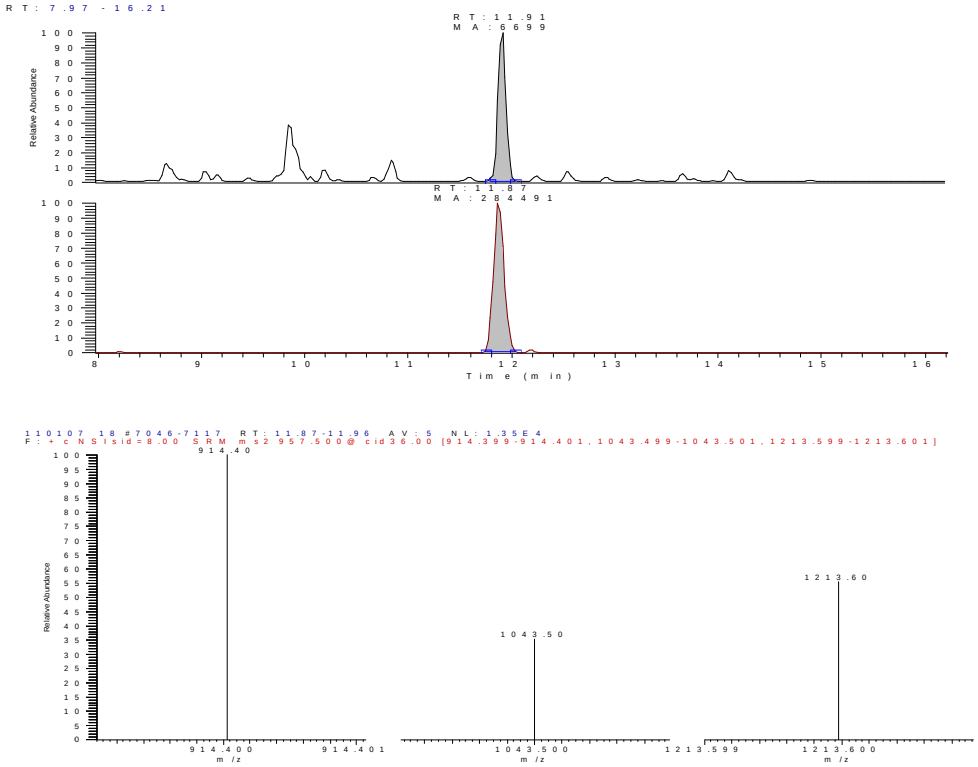
GGGDLTLGLEPSEEEAPR[C13N15]

Expression Pathology

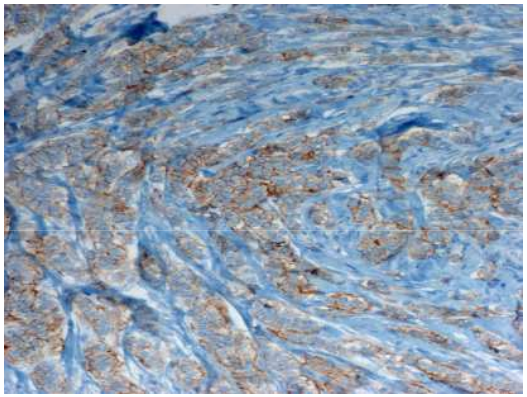
Targeted Protein Quantitation

Her2 Quantitation in Breast Cancer – Sample 1

250amol Her2/µg protein



IHC Score: 1+



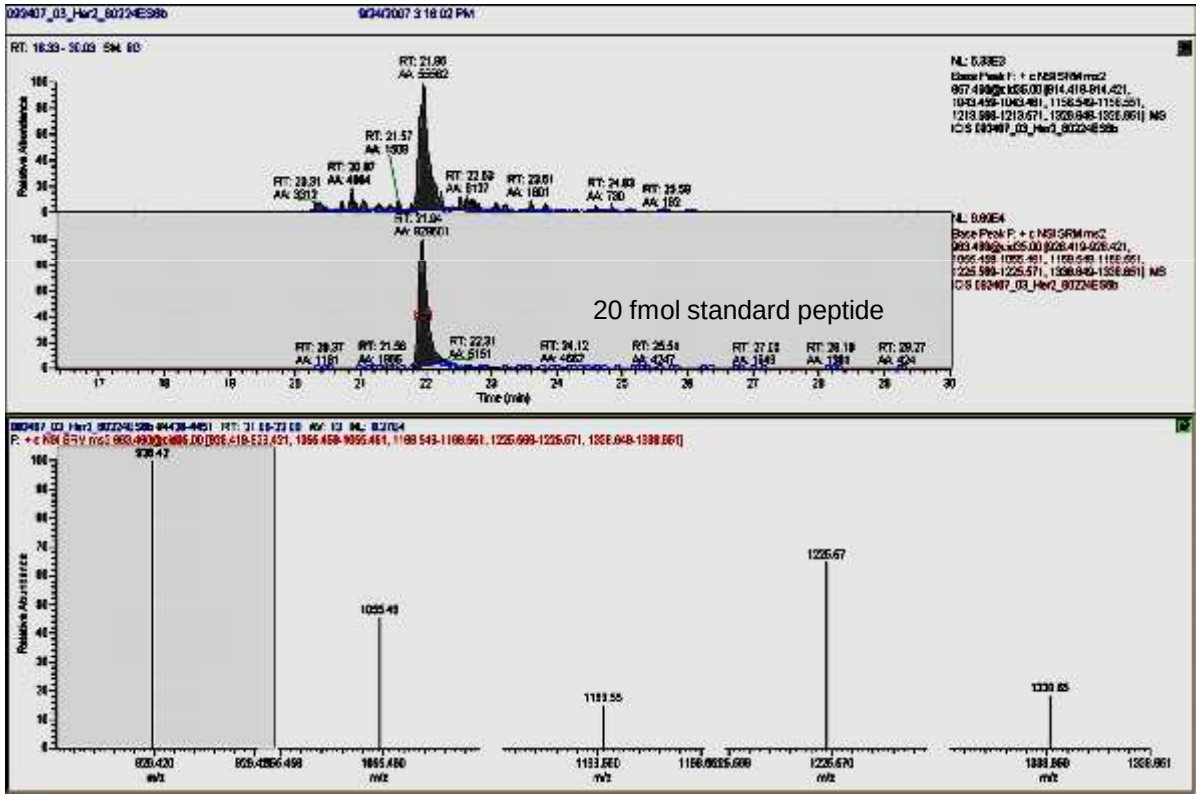
Expression Pathology

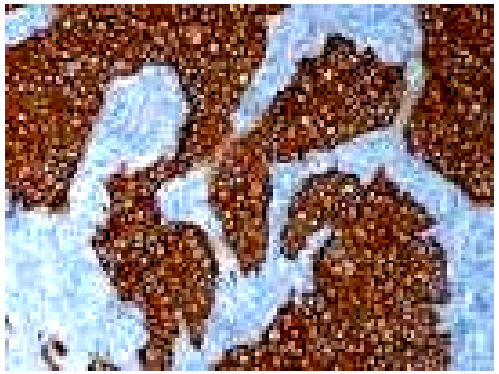
Targeted Protein Quantitation

Her2 Quantitation in Breast Cancer – Sample 2

4.79fmol Her2/µg protein

IHC Score: 3+

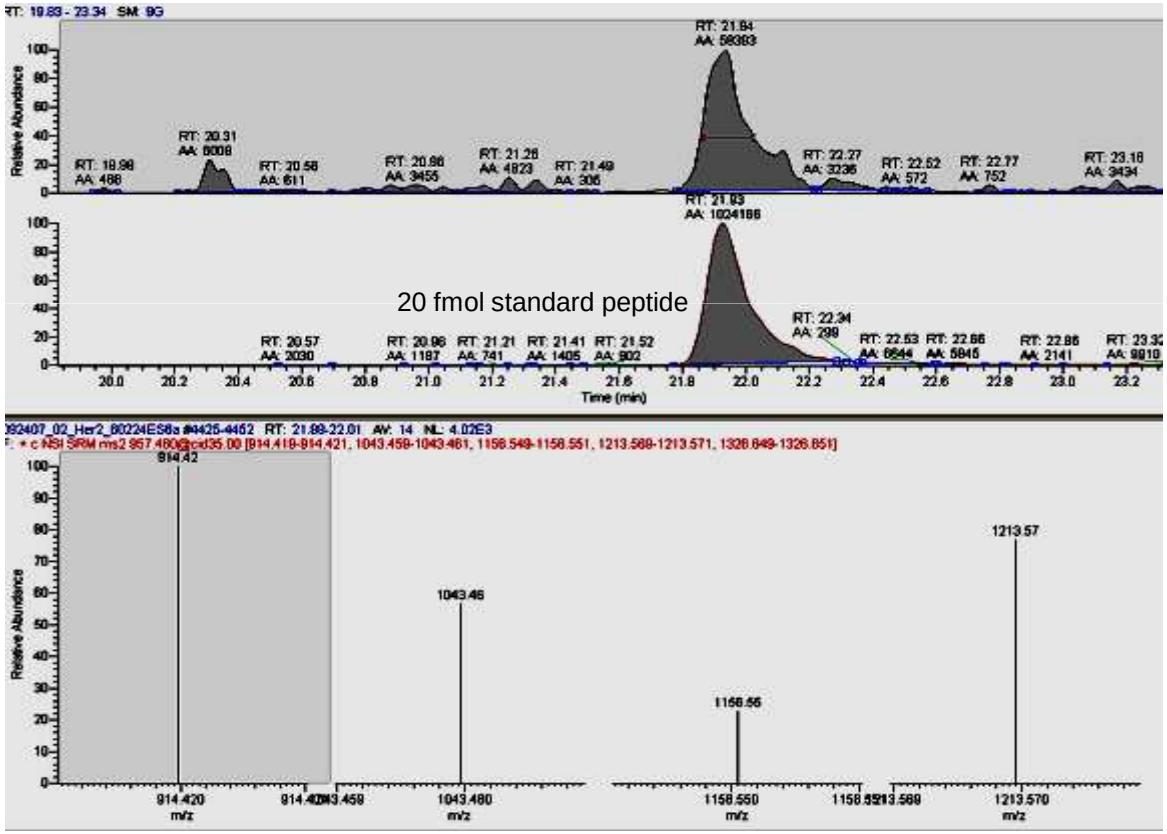




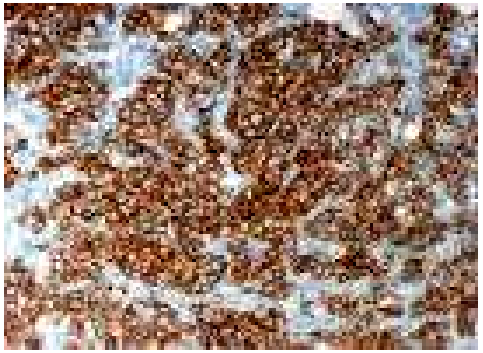
Targeted Protein Quantitation

Her2 Quantitation in Breast Cancer – Sample 3

4.56fmol Her2/ug protein

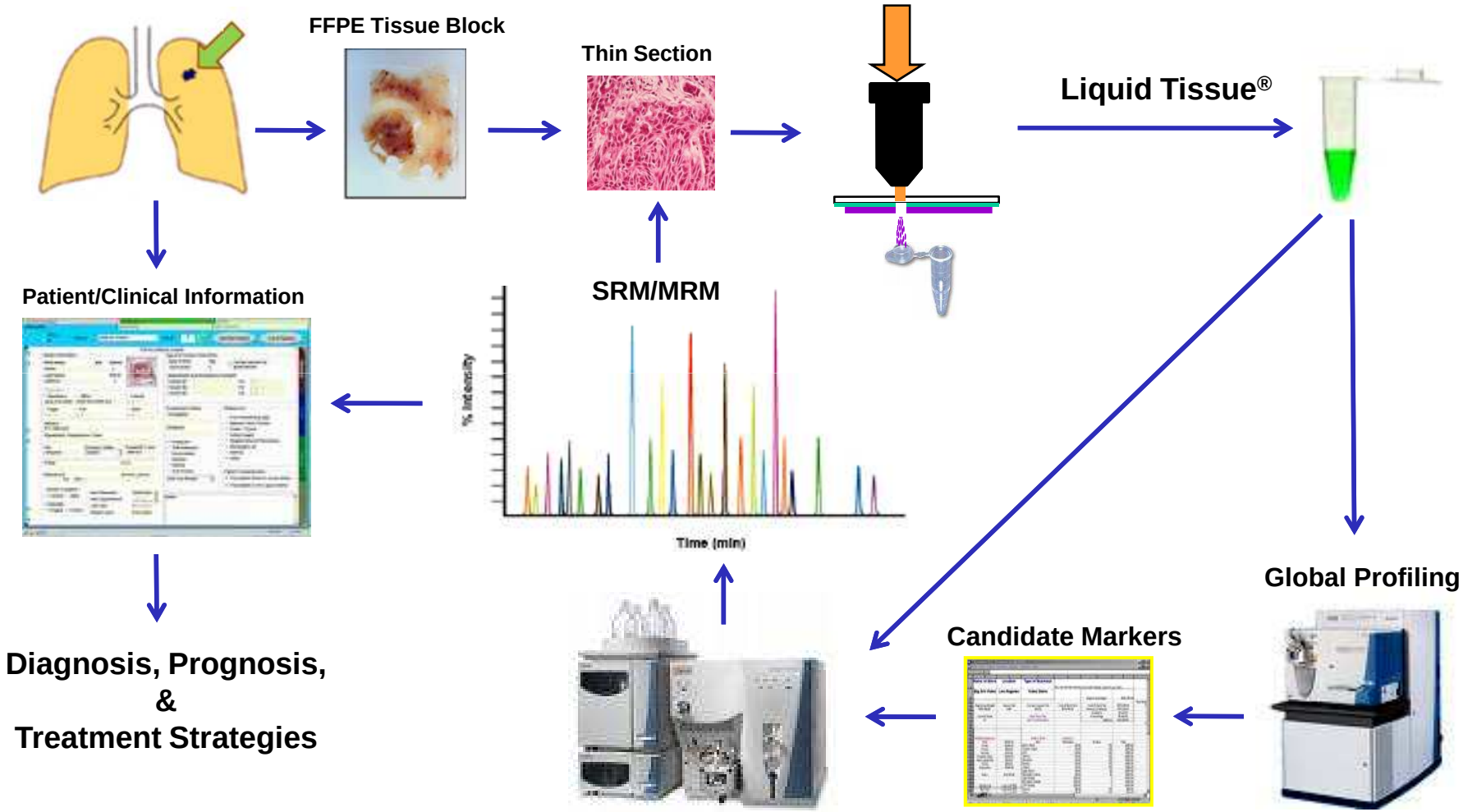


IHC Score: 3+



Biomarker Discovery Project

Markers of Lung Metastasis

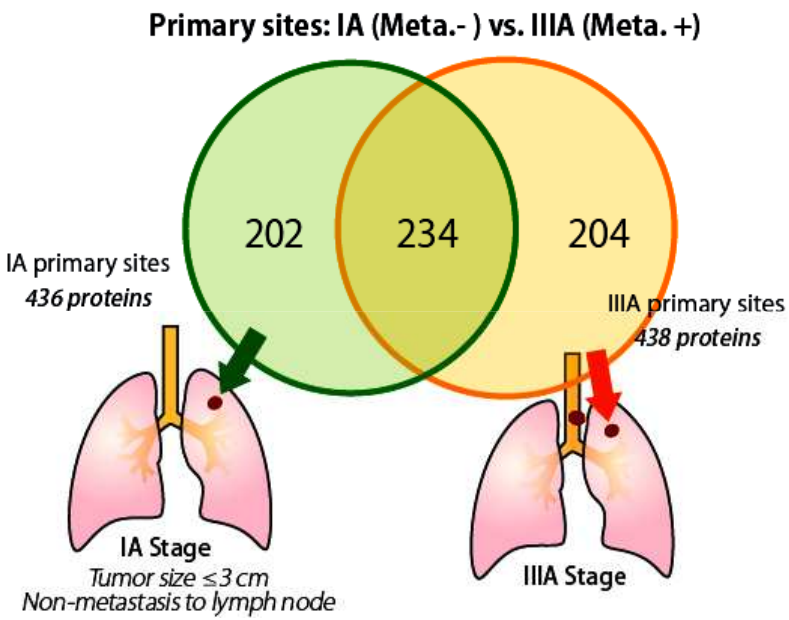


Expression Pathology AMR BIOSYS TECHNOLOGIES, INC

Expression Pathology

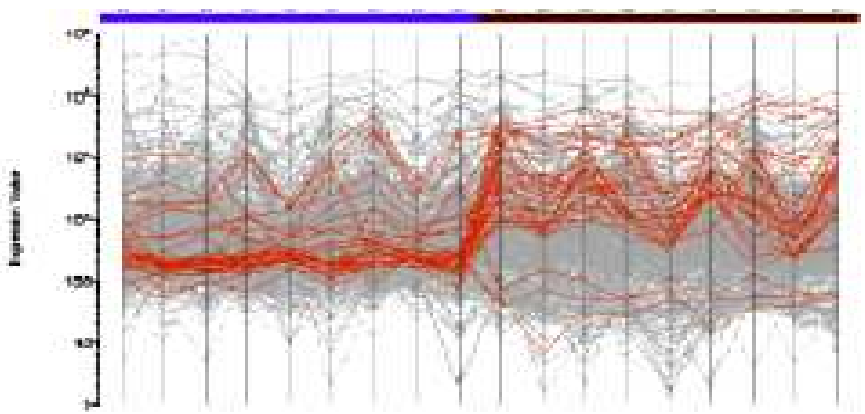
Biomarker Discovery Project

Markers of Lung Metastasis – Global Profiling Results



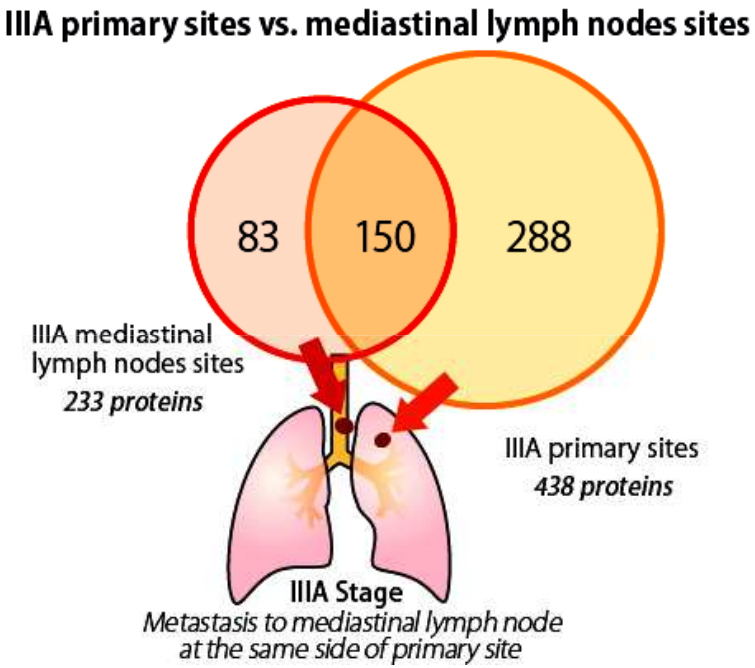
Across 9 IA stage and 9 IIIA stage:

- >14,000 peak clusters analyzed
- 75 peaks significant (p-value ≤ 0/01)



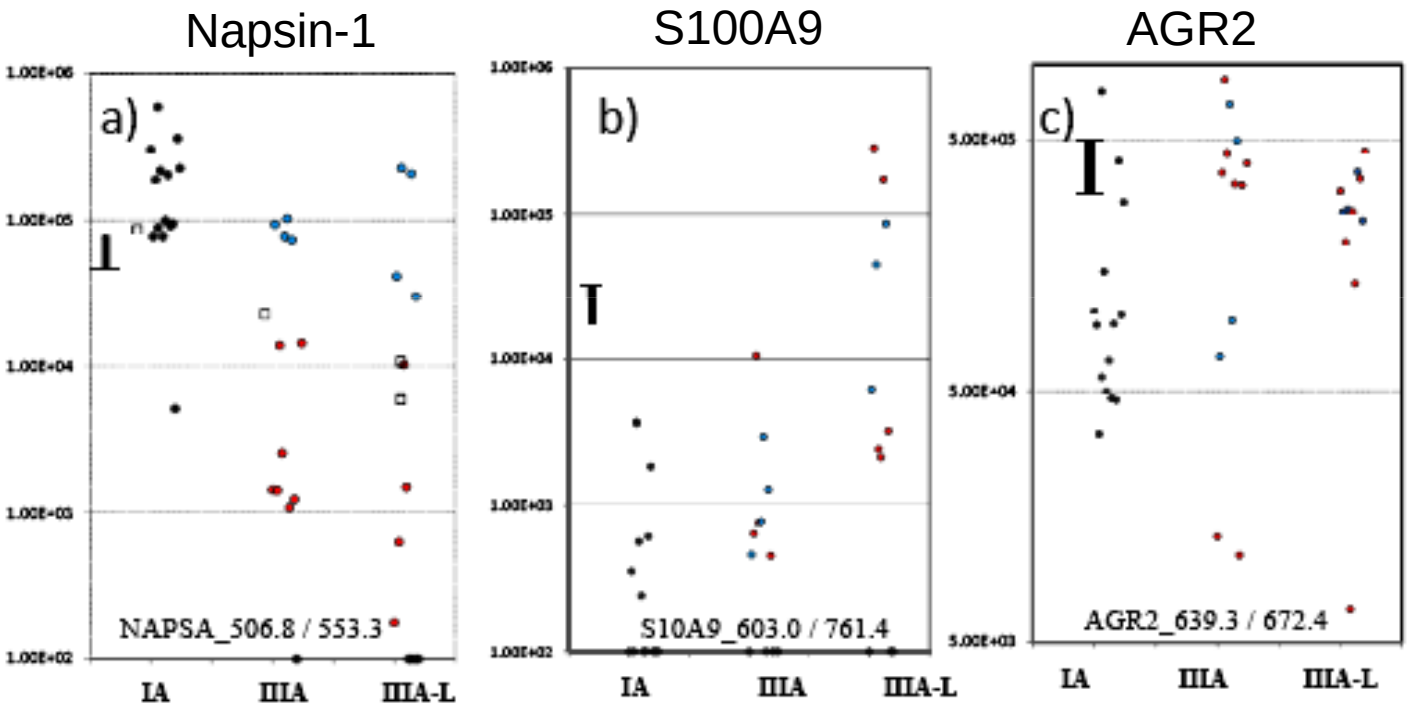
Biomarker Discovery Project

Markers of Lung Metastasis – Global Profiling Results



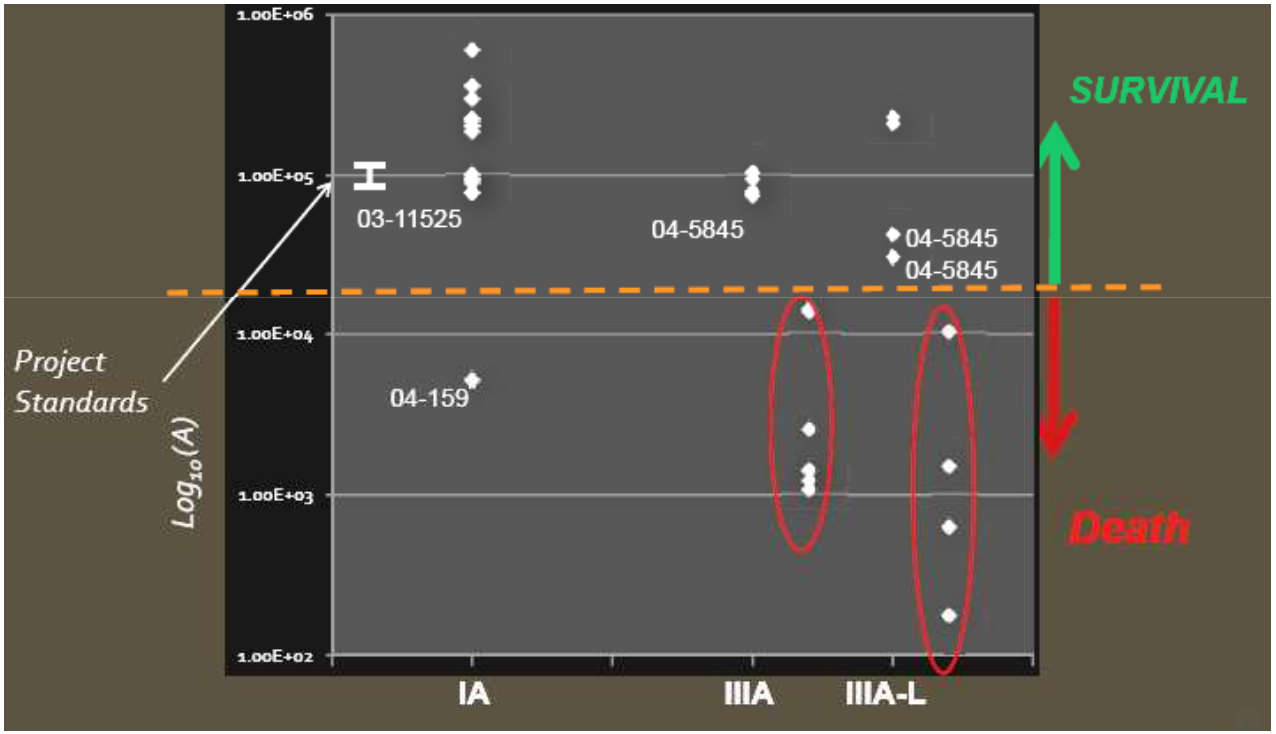
Biomarker Discovery Project

SRM Analysis of Individual Proteins
Correlation to Survival



Biomarker Discovery Project

Decreased Expression of Napsin A Generally Correlates with Progression Appears to be Prognostic for Survival!



Diagnostic Opportunities
Tissue Protein Analysis System

Combine EPI technologies with multiplex labeled MRM to develop integrated quantitative tissue protein assay platform

- Existing protein immunoassays (HER2, ER, PR…)
- Tumor signal pathways (HER2, EGFR…)
- Metastasis panels (breast, prostate, colorectal, lung…)
- Companion Diagnostic Assays (HER2, EGFR, Abl, Kit…..)
- Proprietary new biomarkers

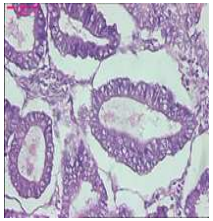


Image-Directed DIRECTOR™ Laser Microdissection

Liquid Tissue® Sample Processing

Mass Spectrometry

Quantitative Patient Reports of Tissue Protein Levels

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