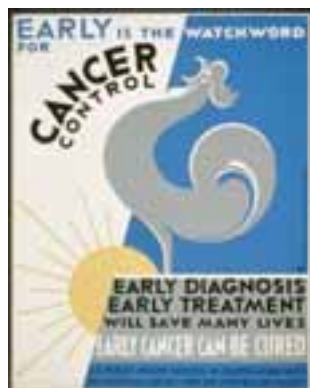
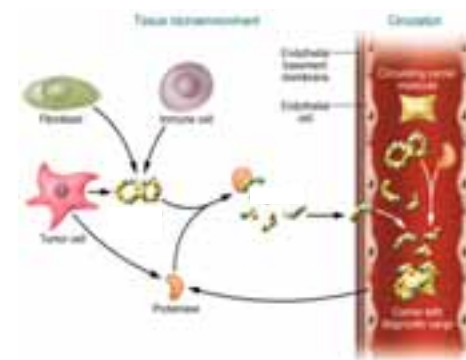
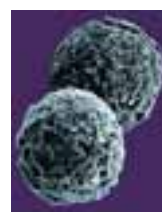




Technology For Proteomics Translation to Clinical Research Studies

Lance A. Liotta MD PhD
George Mason University

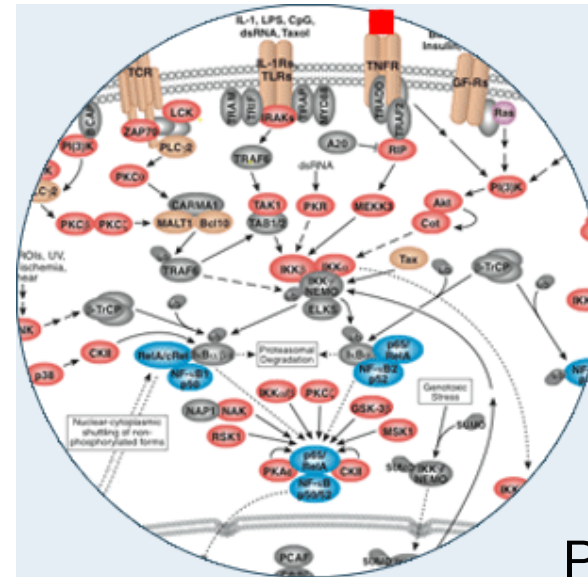


- A. Novel one step preservative for tissue phosphoproteins**
- B. Protein Microarrays: 200 signal pathway phosphoproteins**
 - Translation to clinical research trials**
 - The universal tissue preservative: obviate frozen storage**
- C. Smart nanoparticles for one step in-solution molecular size sieving, affinity capture, biomarker preservation and amplification of effective sensitivity.**

There is a need to measure the state of activity of the actual drug targets (the proteins) in a patient's individual cancer.

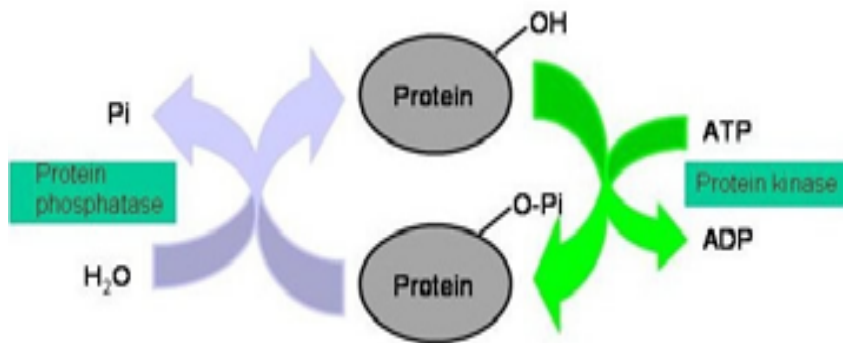


Patient A



Patient B

Proteomics is the missing link for designing individualized therapies



Concurrent phosphorylation of kinases and kinase substrates indicates functional linkage

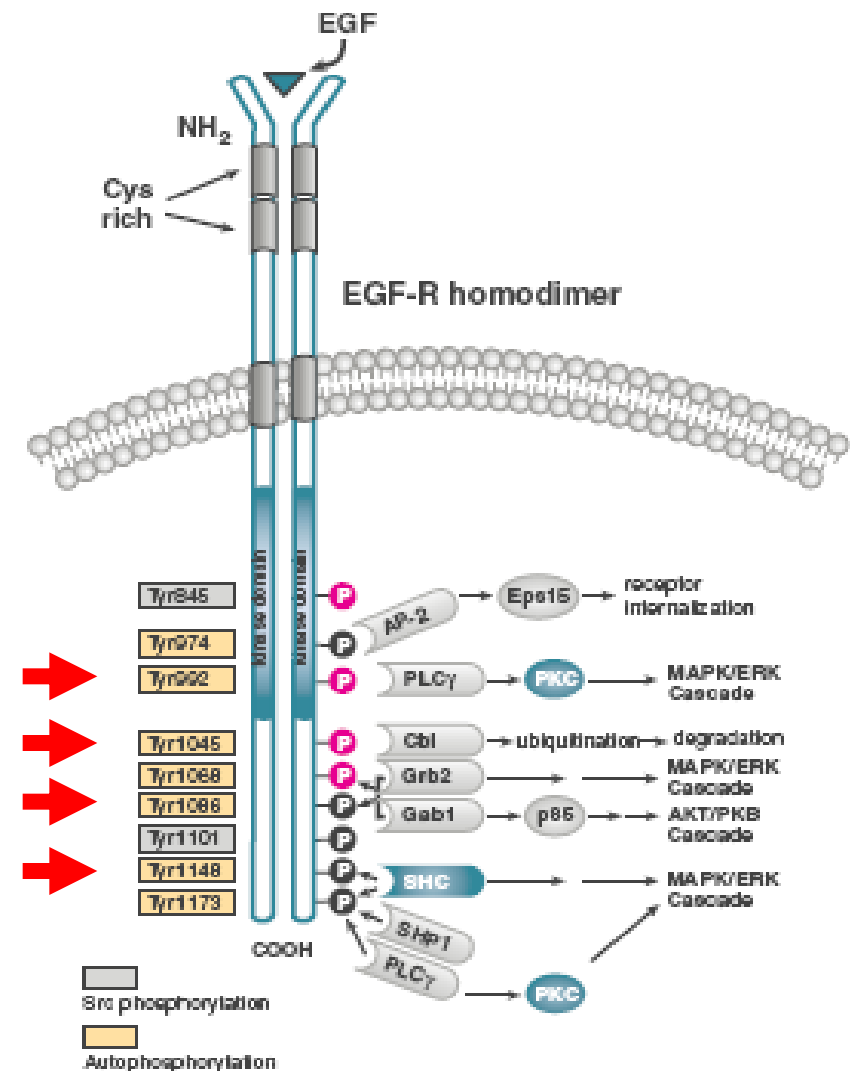
“Proteins carry the epigenetic marks and information”

Emma Whitelaw, DISCOVER Nov. 2006

Genetic or epigenetic defects are selected during cancer progression because they cooperate to orchestrate alterations in protein networks generating a survival advantage for the target cell.

Post-translational modifications, such as phosphorylation, reflect the activity state of cellular signaling networks.

Patterns of phosphorylation indicate docking events and infer protein-protein interactions.



Pre-analytical Variables: The tissue is alive!

"It's Alive!!!"

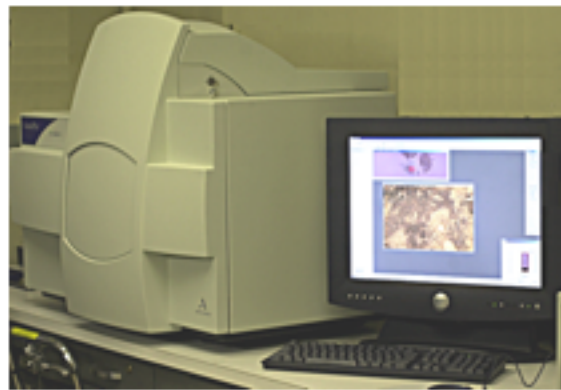
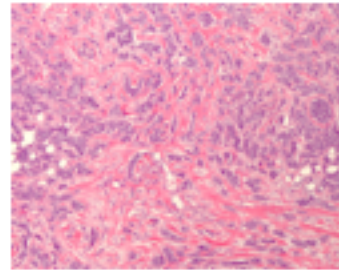


- The tissue is alive and reactive post excision
- During the post excision delay time, tissue signal pathway biomarkers fluctuate upward and downward as the tissue undergoes hypoxia, metabolic acidosis, wounding, hypotension, hypoglycemia, and dehydration.
- Fixation chemistries of the future that stabilize ex vivo reactive biomarkers
- Universal preservation of tissue biomarkers without freezing

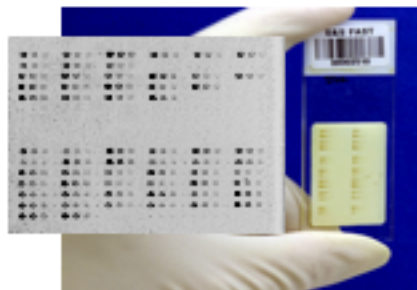
Patient



Biopsy



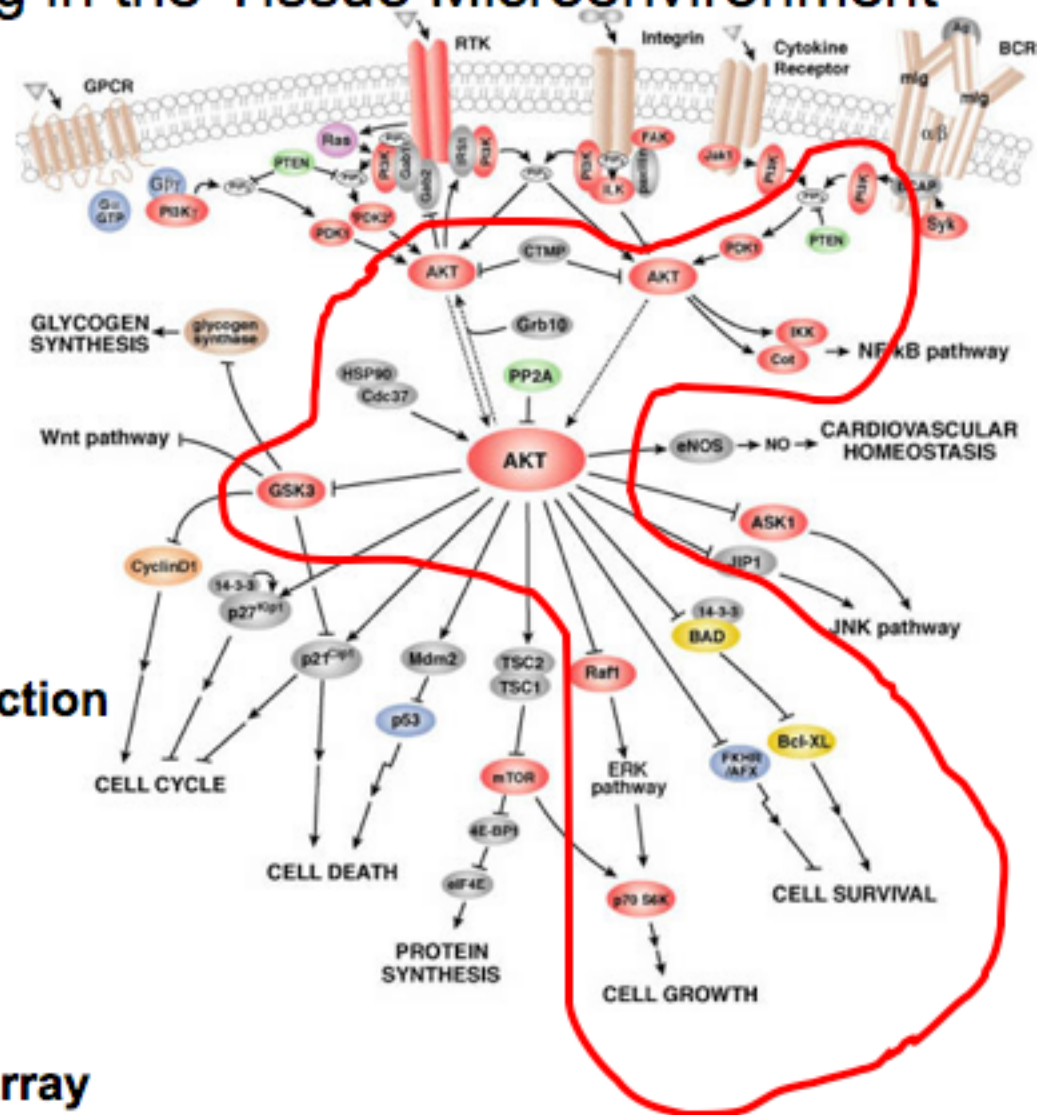
Microdissection



PROTEIN MICROARRAYS

Akt/PKB Signaling Pathways

Circuit Mapping in the Tissue Microenvironment



Protein microarray

State of phosphorylated
cell signaling proteins

Thousands of times more
sensitive compared to IHC

Minimization of pre-analytical variables: novel phosphoprotein preservative



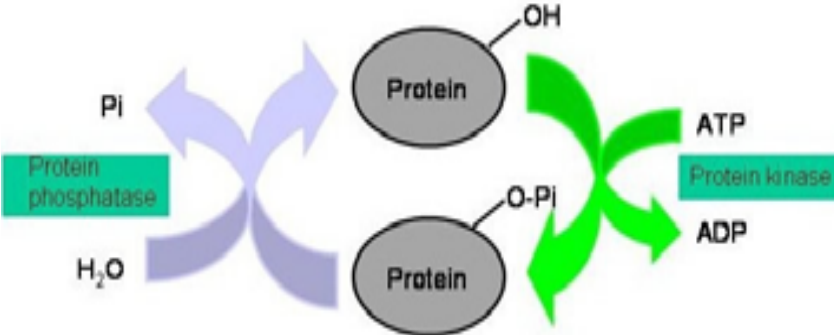
**Molecular & Cellular
PROTEOMICS**

HOME HELP FEEDBACK SUBSCRIPTIONS ARCHIVE SEARCH TABLE OF CONTENTS

Originally published In Press as [doi:10.1074/mcp.M700596-MCP200](https://doi.org/10.1074/mcp.M700596-MCP200) on July 30, 2008.

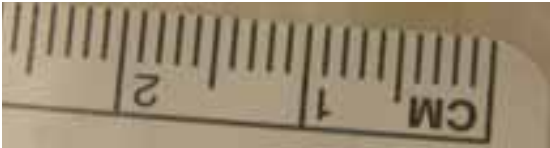
July 30, 2008.

[Journal of Biochemistry and Molecular Biology, Inc.](#)



Issue Phosphoprotein Stability in the Clinical Tissue Process*

John H. Edmiston[§], Michael Heiby[‡], Mariaelena Pierobon^{‡,¶}, Manuela Sciro^{‡,**},
Banks[§], Jianghong Deng[‡], Amy J. VanMeter[‡], David H. Geho^{‡,¶},
Lance A. Liotta[‡], Emanuel F. Petricoin, III[‡] and Lance A. Liotta[‡]



**Core needle biopsies shipped in preservative without freezing
Retention of morphology and antigenicity for FS and LCM**



Ginny Espina

Comparison of *in vivo* effects of molecular targeted therapy before and after therapy.

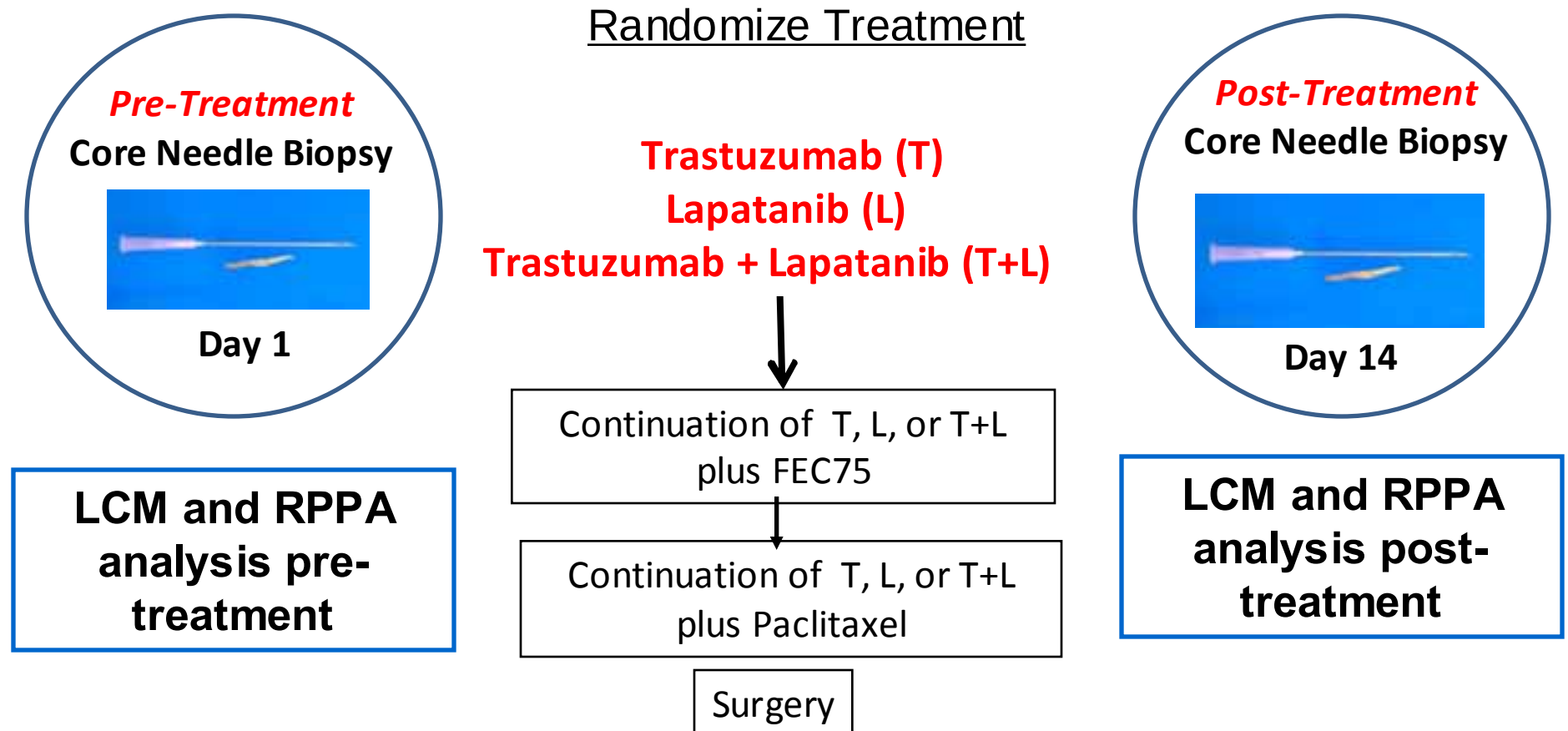
Establish workflow for multi-site trial w/o freezing for sample shipment.

US Oncology 05-074

Phase II Randomized Trial of Neoadjuvant Trastuzumab and/or Lapatinib plus Chemotherapy

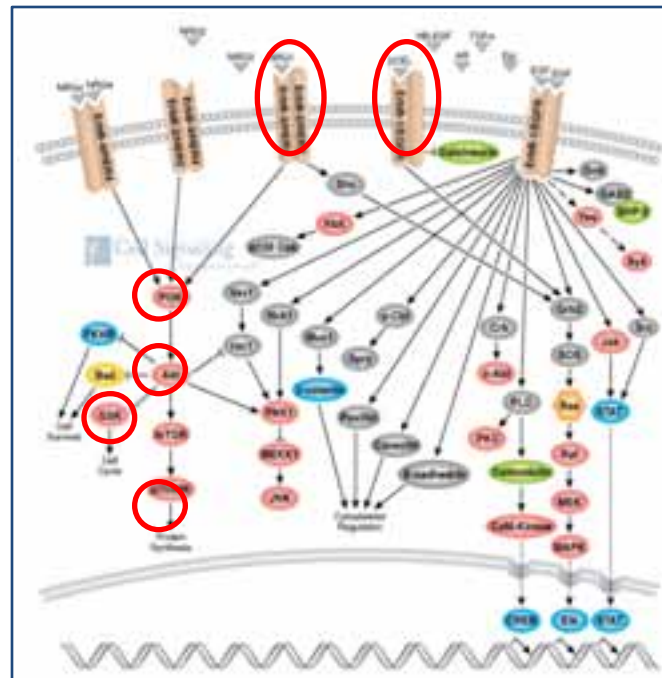
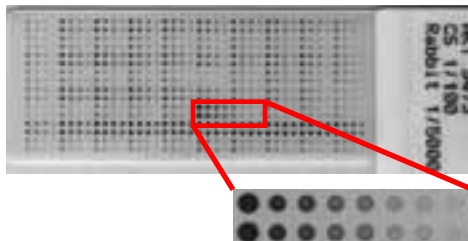
Target enrollment: 99 Enrolled to date: 82

Eligibility: Her2+ invasive breast cancer, Stage II/III, adequate cardiac function



Phase II Randomized Trial of Neoadjuvant Trastuzumab and/or Lapatinib plus Chemotherapy (Sequential FEC75 and Paclitaxel) in Women with ErbB2 (HER2/neu) Over-expressing Invasive Breast Cancer

Laser Capture Microdissection Pre and Post Treatment Core Needle Biopsies



<i>Nucleus Proteins</i>	<i>Membrane Proteins</i>	<i>Cytoplasmic Proteins</i>
Catenin(beta) Ser33/37/Thr41	CD24	AKT Ser473
Fox 01/03Thr24/Thr32	CD44	AKT Thr308
	CD133	AKT
	E-Cadherin	Atg5
	EGFR Tyr992	Beclin
	EGFR Tyr1068	β actin
	EGFR Tyr1148	ERK Thr202/Tyr204
	EGFR Tyr1173	GSK3α/β Ser21/Ser9
	EGFR Tyr 1045	GSK3α/β Tyr279/Tyr216
	EGFR	LC3B
	Fibronectin	Mek ½ Ser217/Ser221
	Her 2	MMP9
	Her2 P95/P185	MMP14
	Her2 Tyr1248	mTOR Ser2448
	Her3 Tyr1289	Musashi
	IGF1R Tyr1131/Tyr1146	NFκB S536
	Integrin α1β5	P70S6K Thr389
	IRS-1 Ser 612	PI3K
		P38 MAPK
		Thr180/Tyr182
		PTEN Ser380
		PTEN
		Smad2 Ser465/Ser467
		Stat3 Tyr705
		Stat3 Ser727
		Stat5 Tyr694
		TIMP2
	Met Y1234/1235	

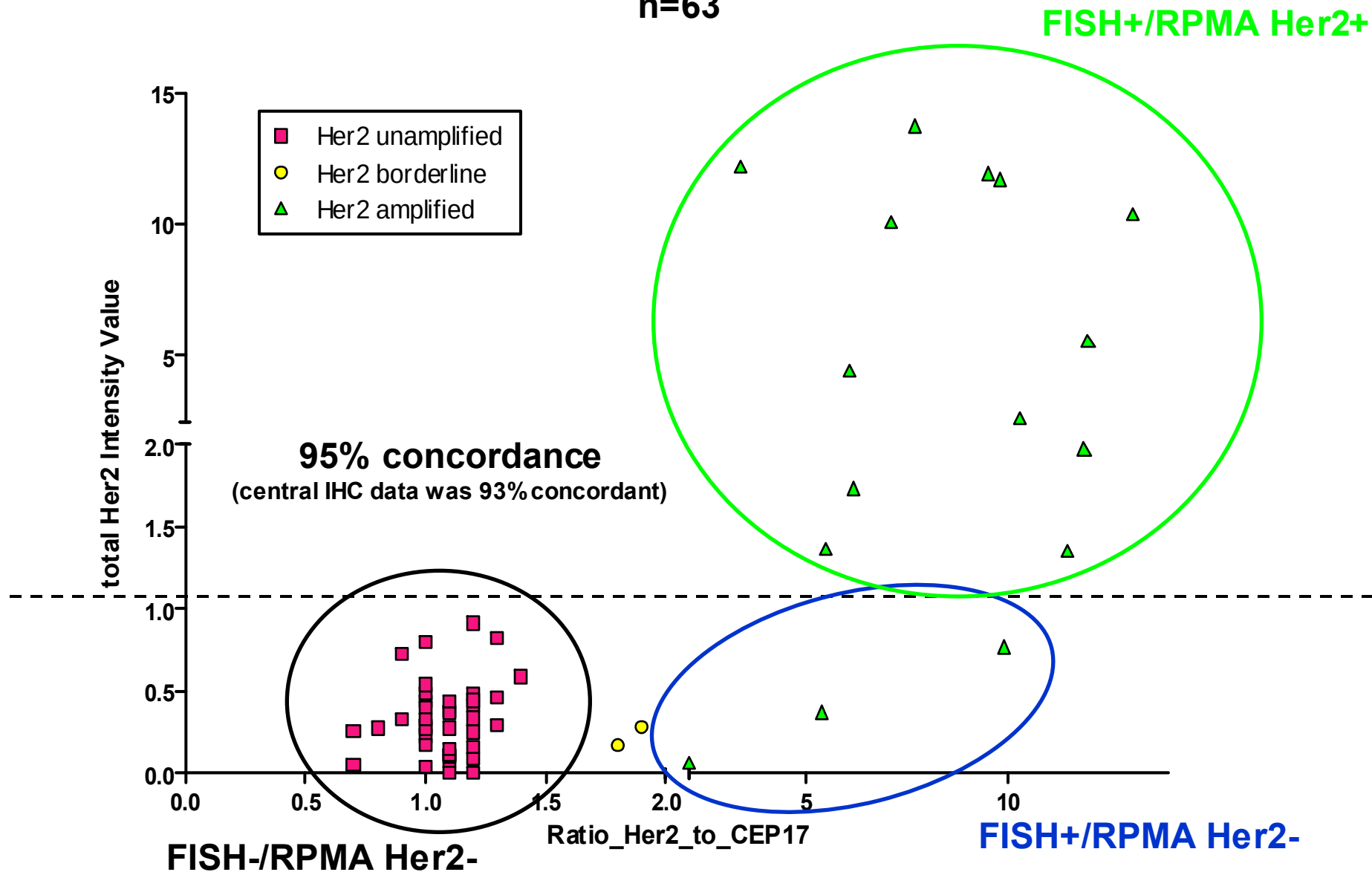
Reverse Phase Protein Microarrays

Sensitivity: <500 cells
Built-in calibrators and controls
Precision: CV 3-10%
CAP CLIA compliant

**Gallagher R, et
al AACR 2009
abstract #2756**

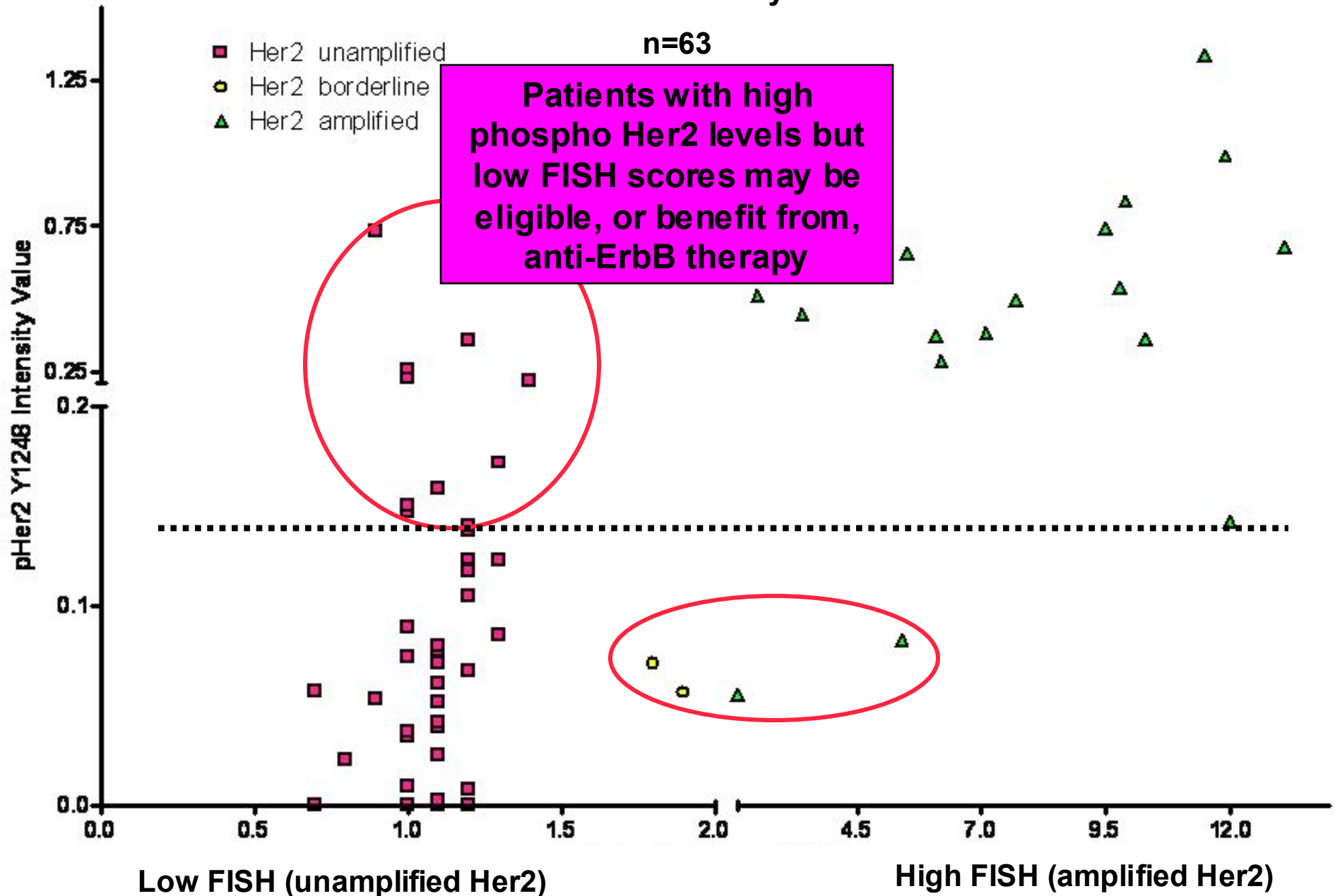
Concordance of Total Her2 RPMA Measurements with Her2 Central FISH Data

n=63



Are patients with elevated **phospho**Her2 likely to benefit from ErbB inhibition?

Her2 FISH v. Her2 Tyr1248



Clinical research trials using phosphoprotein signal pathway profiling for stratification of tyrosine kinase inhibitors

A. Breast Cancer: (USO, Inova TKI: EGF/HER2 combination therapy
Status: Started Sept 2007 Target Completion Dec 2009

B. Multiple Myeloma: (Hem Oncol Assoc, Inova) targeted inhibitor screening
Status: Started May 2007 Target Completion May 2010

C. Breast Cancer Carcinoma in Situ: DCIS cancer stem cells
Status: Started Sept 2007 Target Completion Dec 2009

D. Breast Cancer Carcinoma in Situ: Treatment
Status: Will start in Dec 2009 Target Completion Dec 2011

E. Colon Cancer Liver Metastasis: Stratified combination therapy
Status: Started Aug 2009 , Target Completion Dec 2010

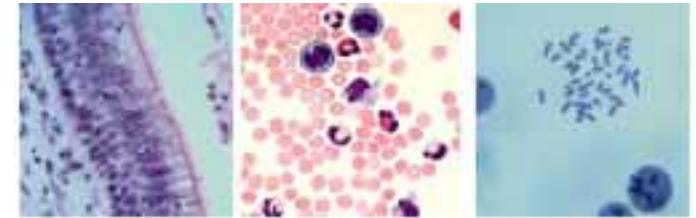
F. Breast Cancer Stage IV: Side Out sponsored trial individualized therapy based on genomic and proteomic analysis
Status: Will start in Dec 2009 Target Completion 2010



Core needle biopsy



**Multipurpose Molecular
Preservation, Histologic
Fidelity, and RT Storage**



Highest quality cytology and
histomorphology for pathologic
diagnosis and immunohistology



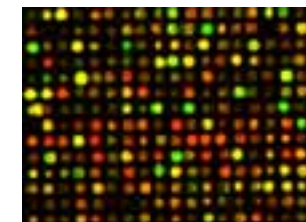
Paraffin
Block



Laser Capture
Microdissection



Protein Microarray



Genomics Microarray

Archive



**One Step Molecular
Preservation and Fixation**

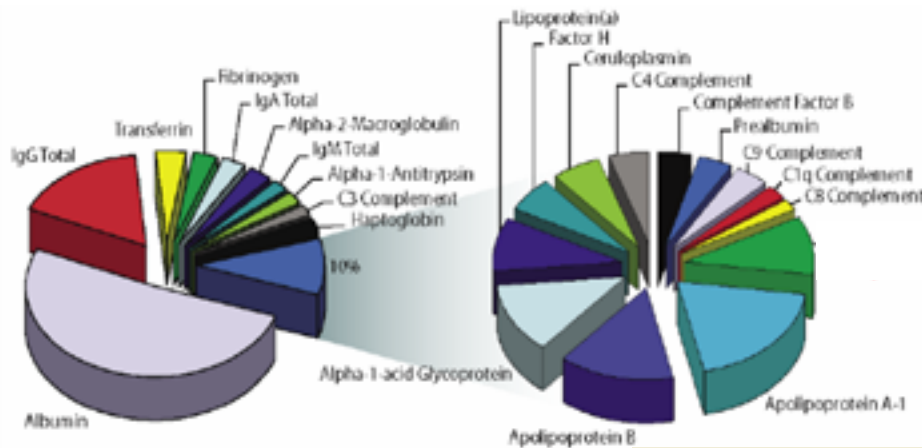
- a) Preserves morphology for histopathologic diagnosis
- b) Stabilizes proteins, phosphoproteins and PTMs
- c) Stabilizes RNA, miRNA, and DNA
- d) Can be frozen for frozen section diagnosis or used for Flow Cytometry
- e) Paraffin embedding for indefinite storage at room temperature

Roadblocks to Body Fluid Biomarker Discovery

Biomarkers exist in very low concentration: Significantly below the detection limits of mass spectrometry

Obscured by abundant resident blood proteins such as albumin

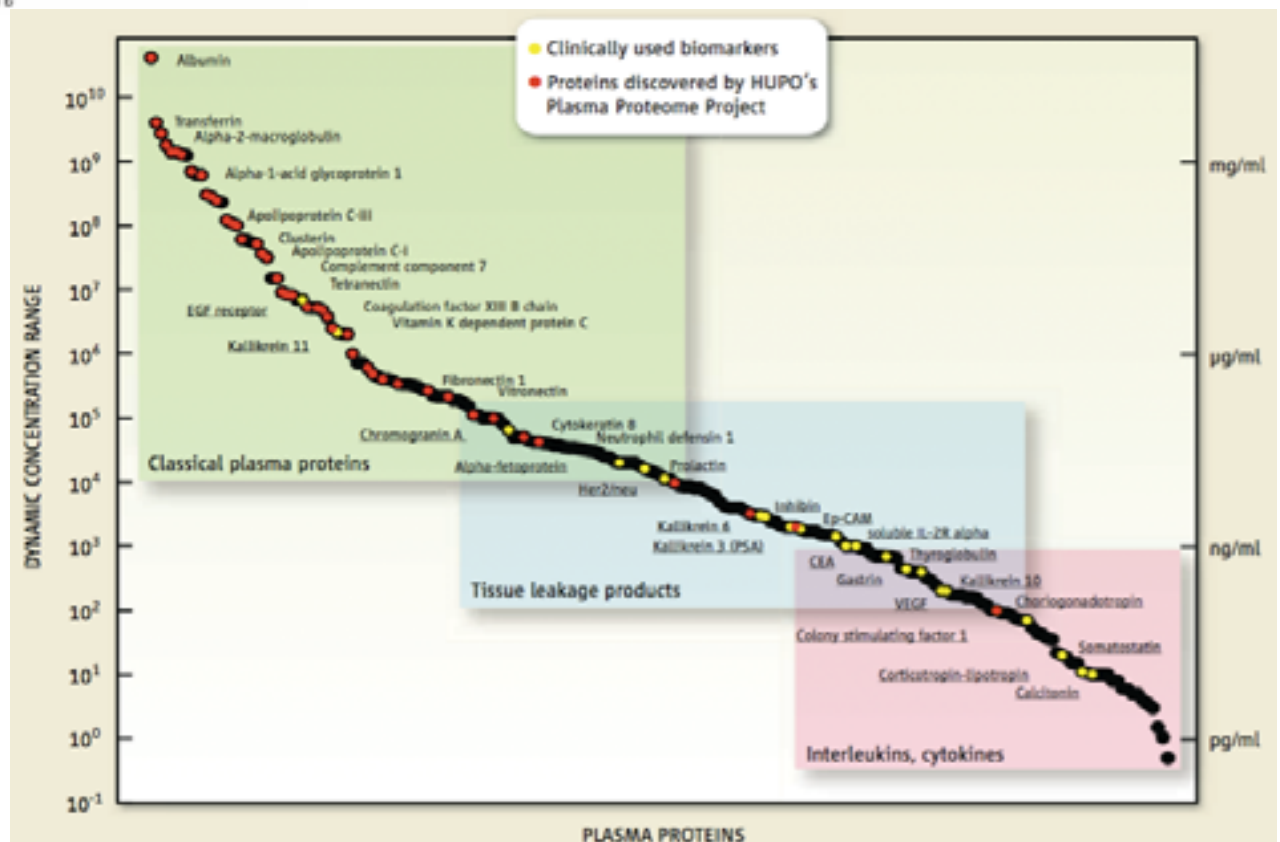
Rapidly degraded by enzymes post collection



-22 proteins constitute 99% blood protein mass.

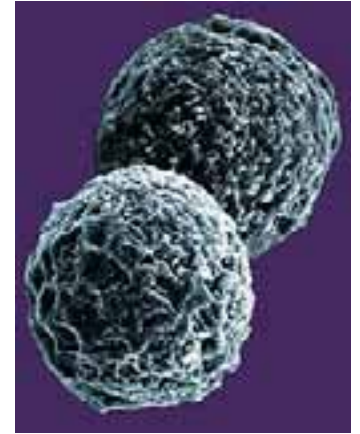
-A stage I cancer with a diameter of less than 0.5 cm. : biomarker conc: **picogram/ml**

- Current mass spec methods can not detect less than **10 nanograms/ml** ms/MRM does not have sufficient sensitivity, precision or dynamic range

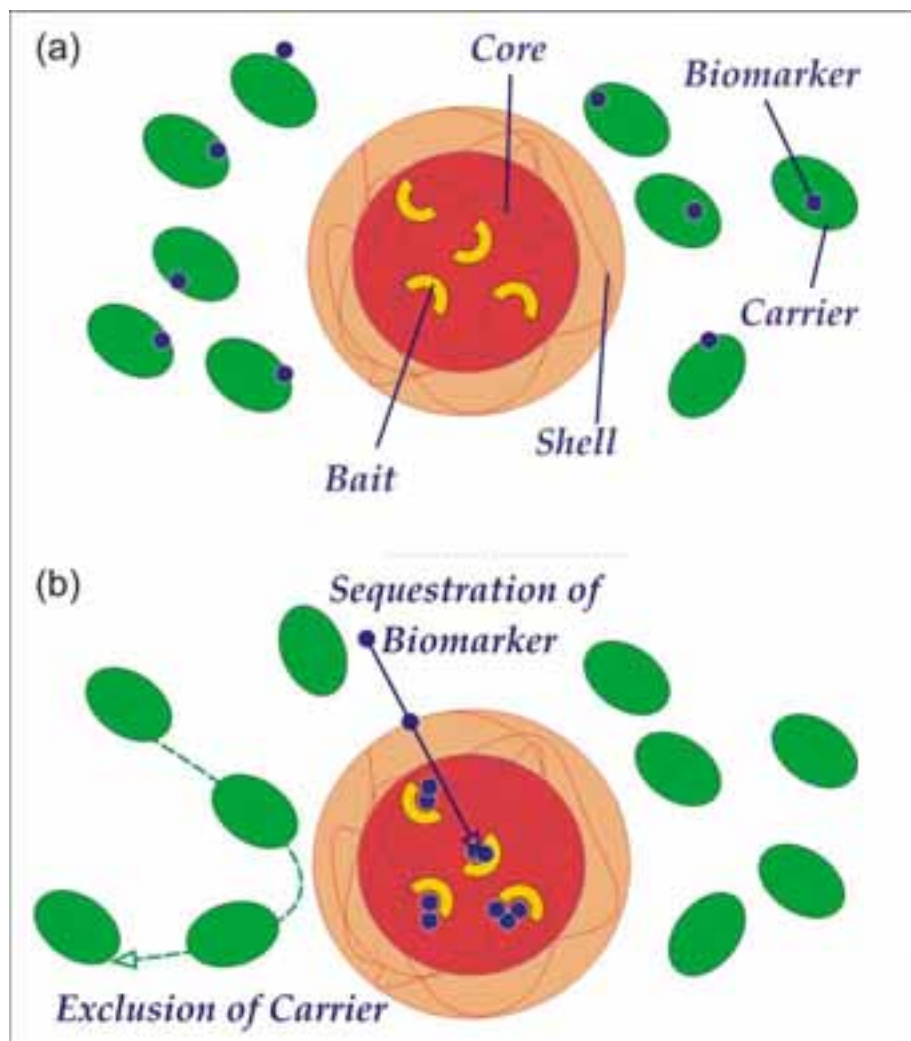


Novel technology to overcome biomarker physiologic barriers

Smart” Core Shell Affinity Bait Nanoporous Particles



- **Three independent functions within minutes, in one step, in solution:**
 - a) **Molecular size sieving**
 - b) **Affinity capture of all solution phase target molecules**
 - c) **Complete protection of harvested proteins from enzymatic degradation**
 - d) **Amplify the effective concentration of very low abundance molecules**

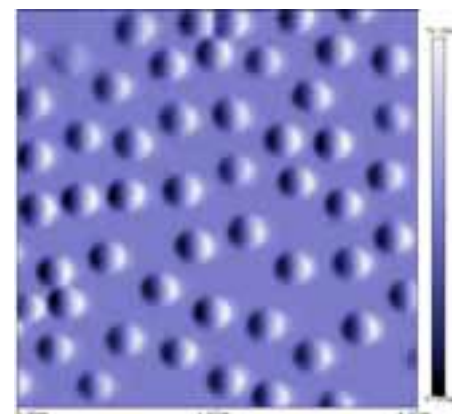


Smart Hydrogel Particles: Biomarker Harvesting: One-Step Affinity Purification, Size Exclusion, and Protection against Degradation

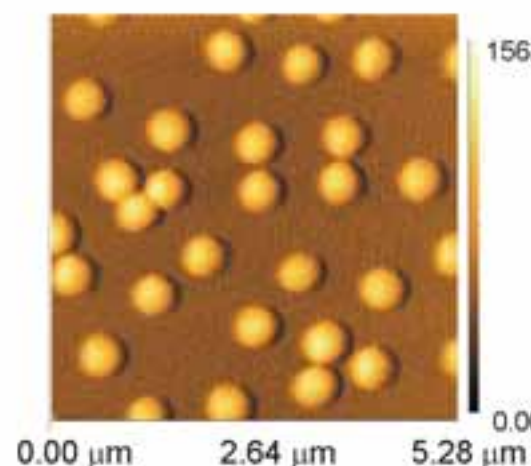
Alessandra Luchini,^{1,2} David H. Geho,² Barney Bishop,³ Duy Tran,² Cassandra Xia,² Robert L. Dufour,² Clinton D. Jones,^{1,2} Virginia Espina,² Alexis Patanarut,³ Weidong Zhou,² Mark M. Ross,² Alessandra Tessitore,² Emanuel F. Petricoin III,² and Lance A. Liotta²

NANO
LETTERS

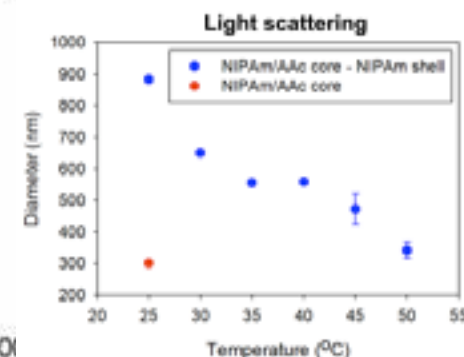
2008
Vol. 8, No. 1
350–361



Alessandra Luchini

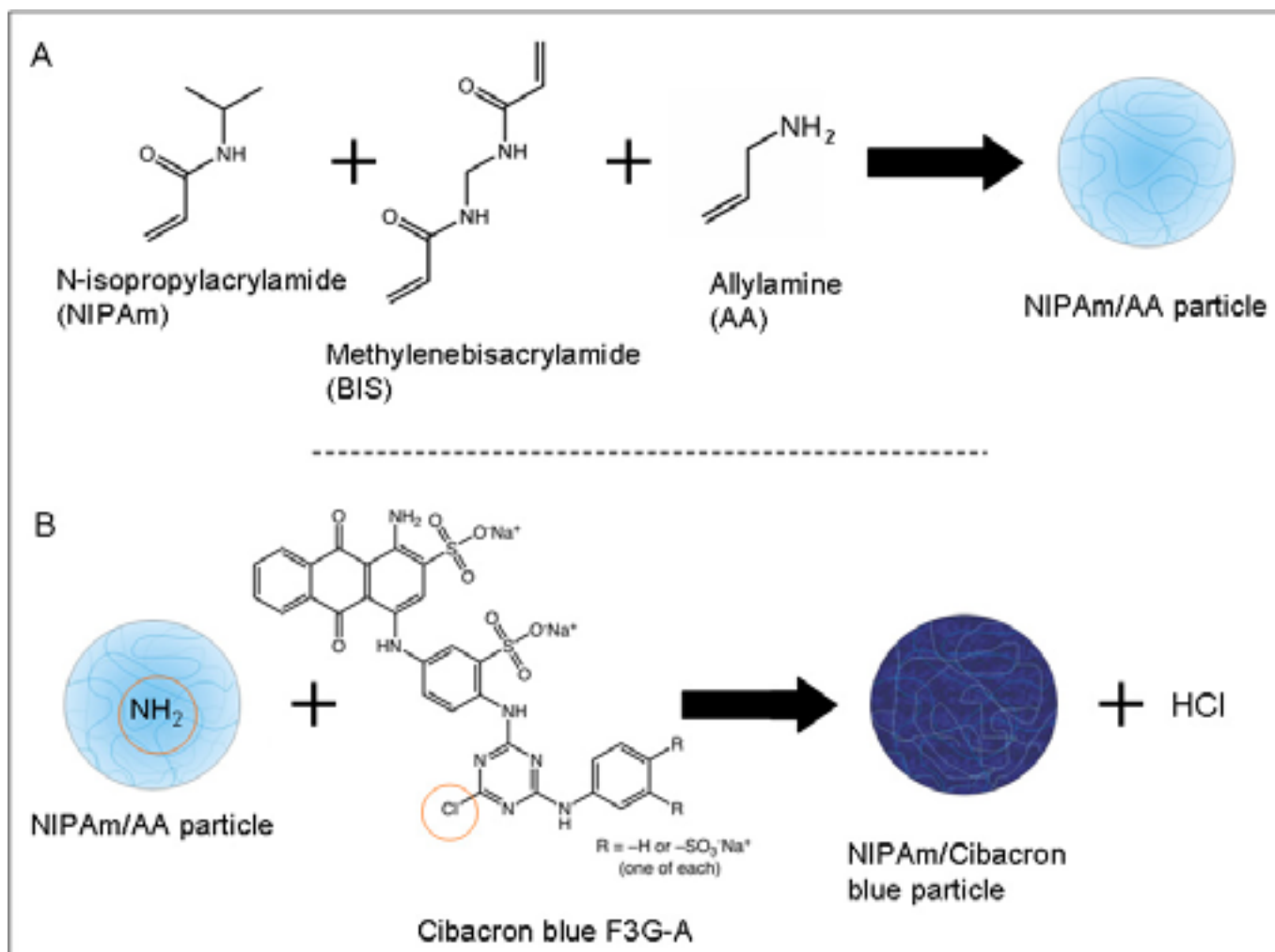


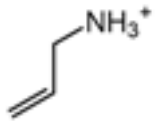
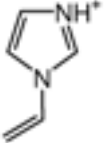
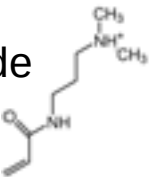
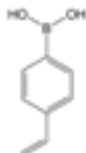
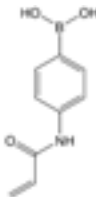
156.63 nm



- Particles can be produced in large quantities
- Stable at room temperature indefinitely
- Low cost
- Uniform in size (0.7 micron)
- Reproducibility among batches

Hydrogel NIPAm/ Core Synthesis Bait Covalent Binding

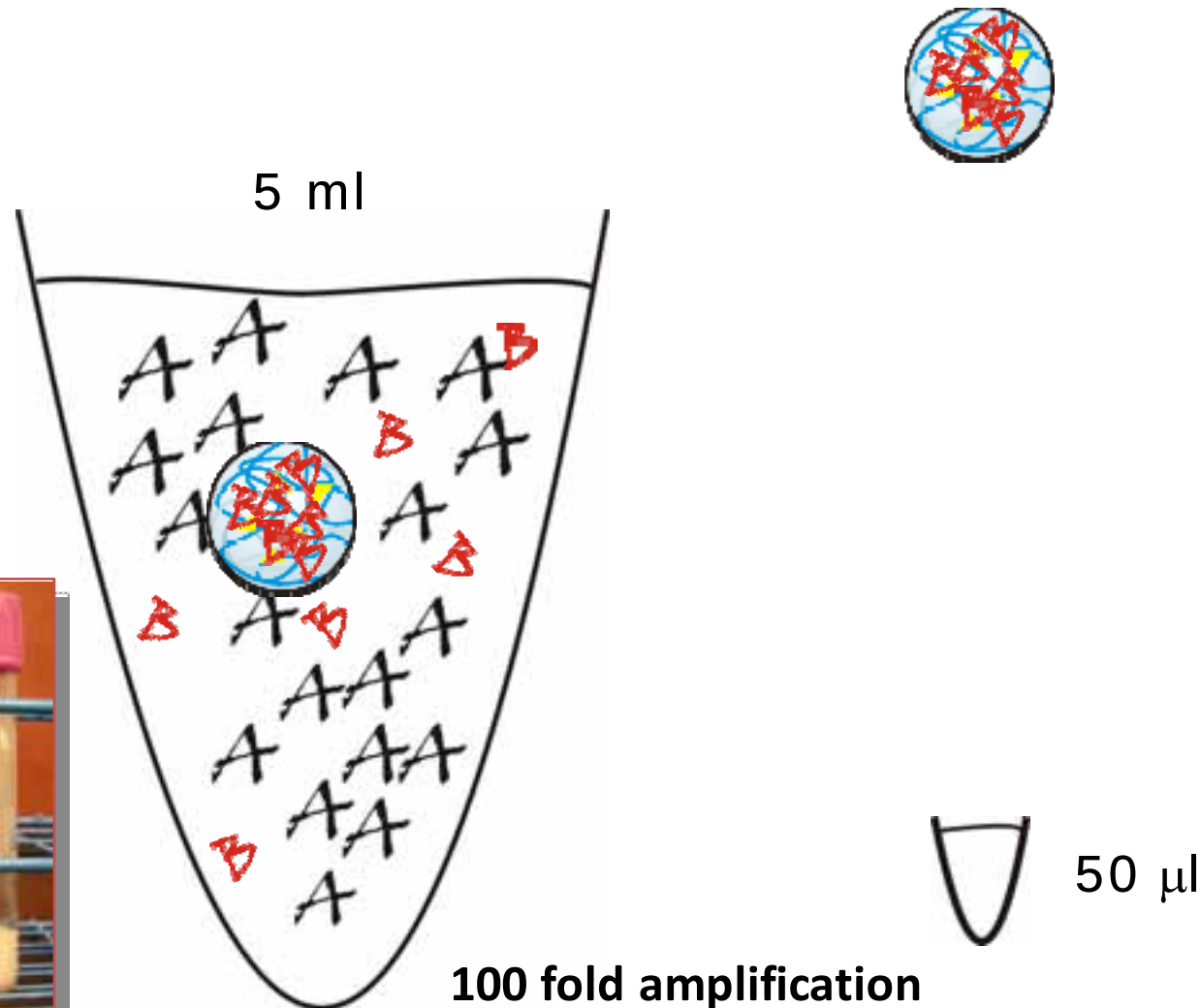


Bait	Target
 Acrylic acid	Cationic proteins and polypeptides
 allylamine  1-vinylimidazole  N,N'-dimethylamino propyl]methacrylamide	Anionic proteins and polypeptides
 Cibacron blue F3G A  Procion Red H8BN	Proteins and polypeptides
 Cyclodextrins	Small molecules, cholesterol
 p-vinylphenyl boronic acid  N-acryoyl-<i>m</i>-aminophenyl boronic acid	Polysaccharides, glycopeptides, RNA
TiO ₂ nanoparticles incorporated in NIPAm beads	Phosphopeptides

In-solution harvesting

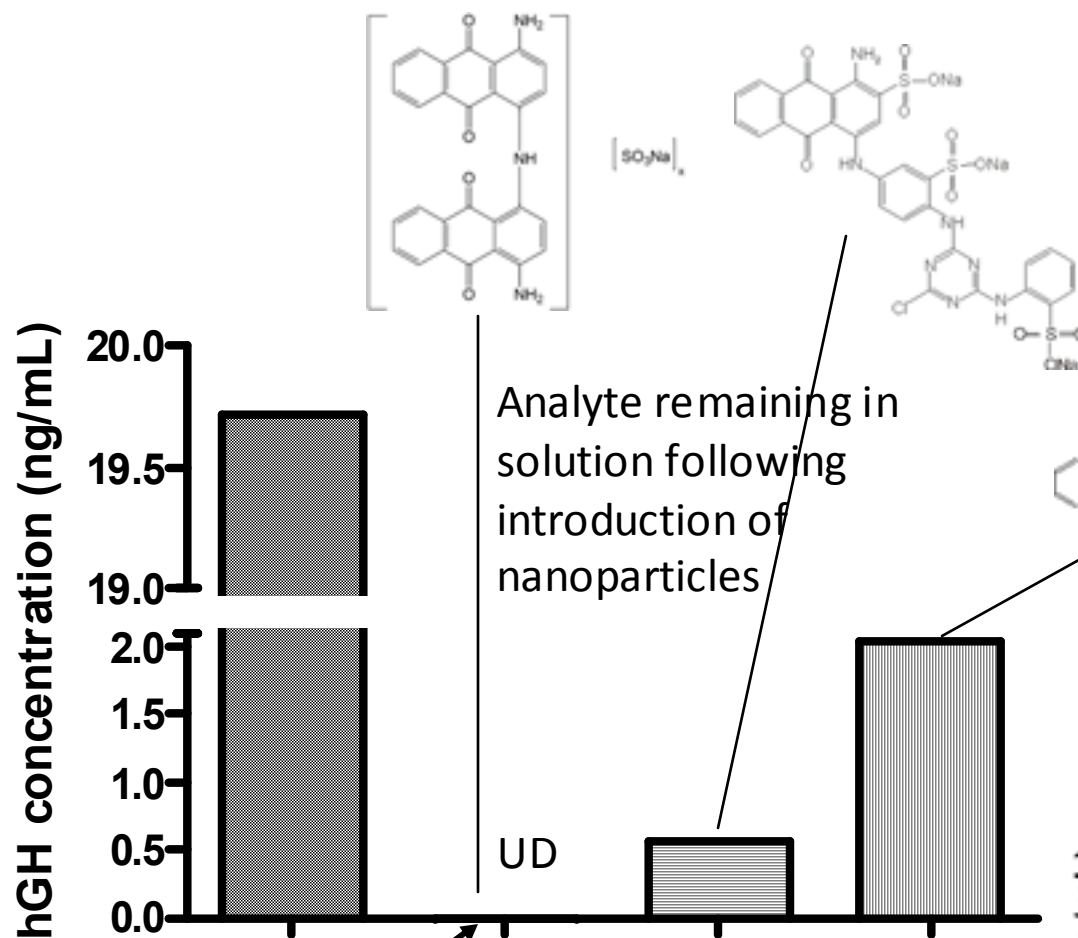
Smart particles amplify the biomarker concentration

Nanoparticles in vacutainer blood collection tubes



Capture Efficiency >99.95%

Bait specific high affinity protein biomarker binding achieves virtually 100% sequestration of all solution phase analyte molecules. Elution yield is 100%



Analyte remaining in solution following introduction of nanoparticles

UD

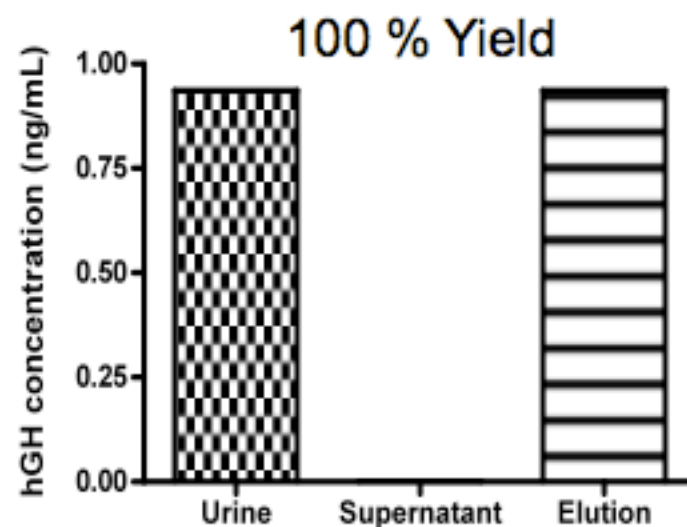
Urine

Acid Black 48

Cibacron Blue F3G-A

Reactive Blue 2

Immulite detection limit
50 picograms/mL



100 % Yield

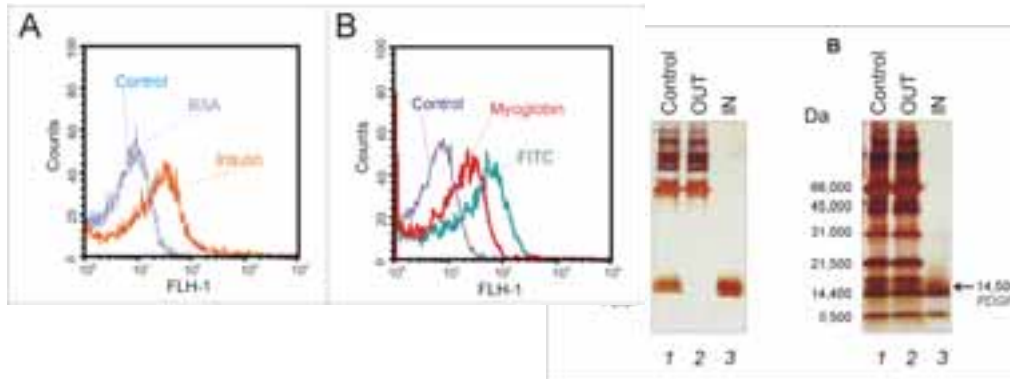
Urine

Supernatant

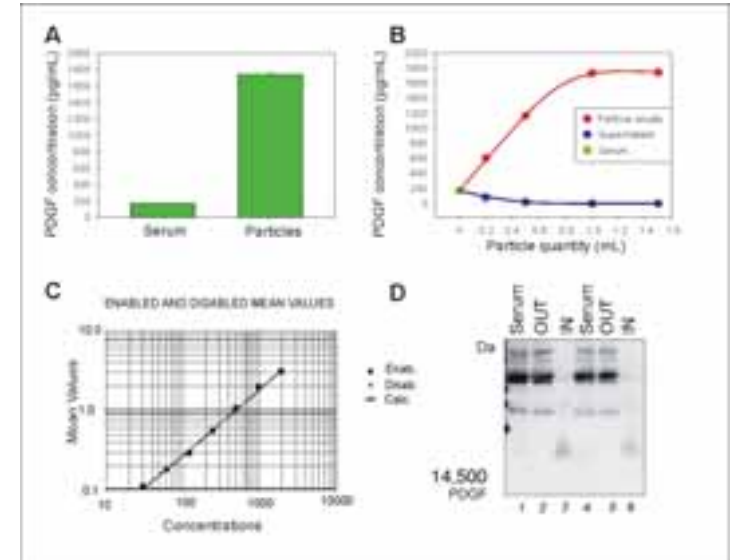
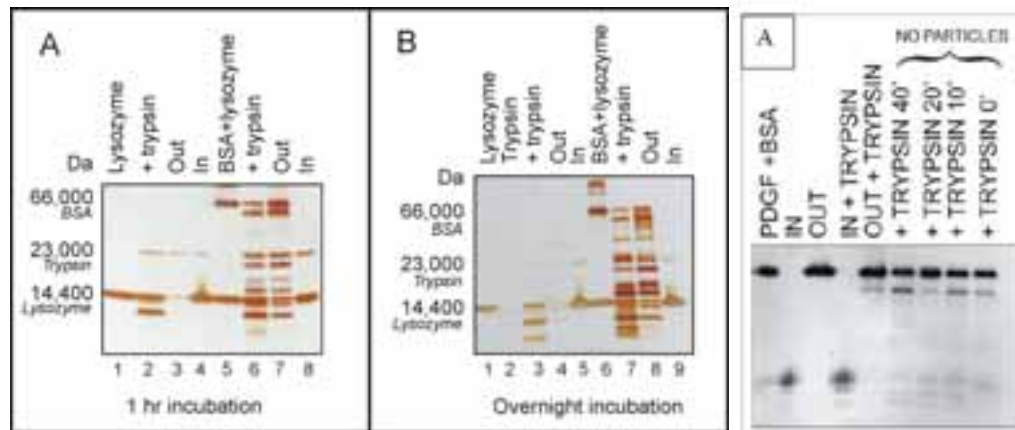
Elution

Size sieving & complete albumin exclusion

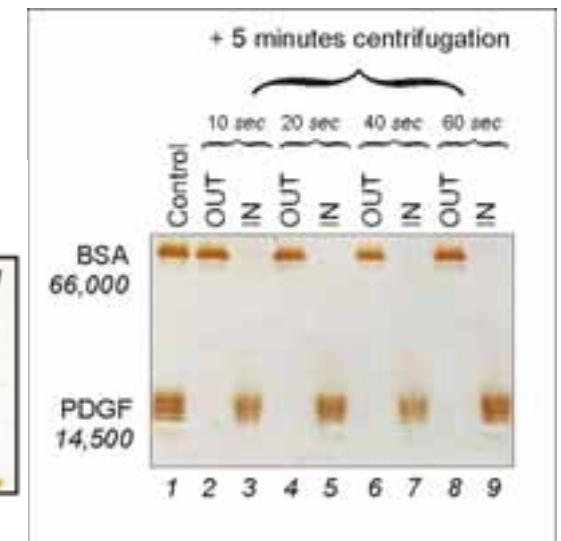
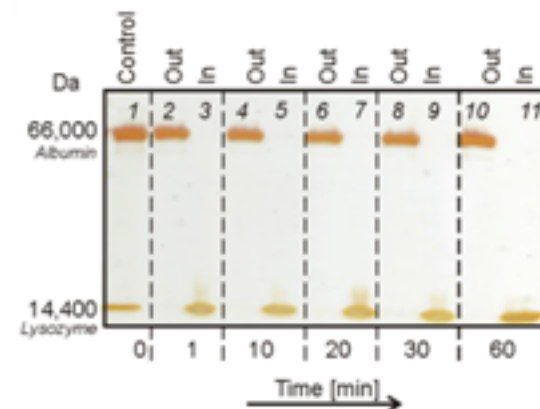
Concentration: High Precision %C.V. <3.0

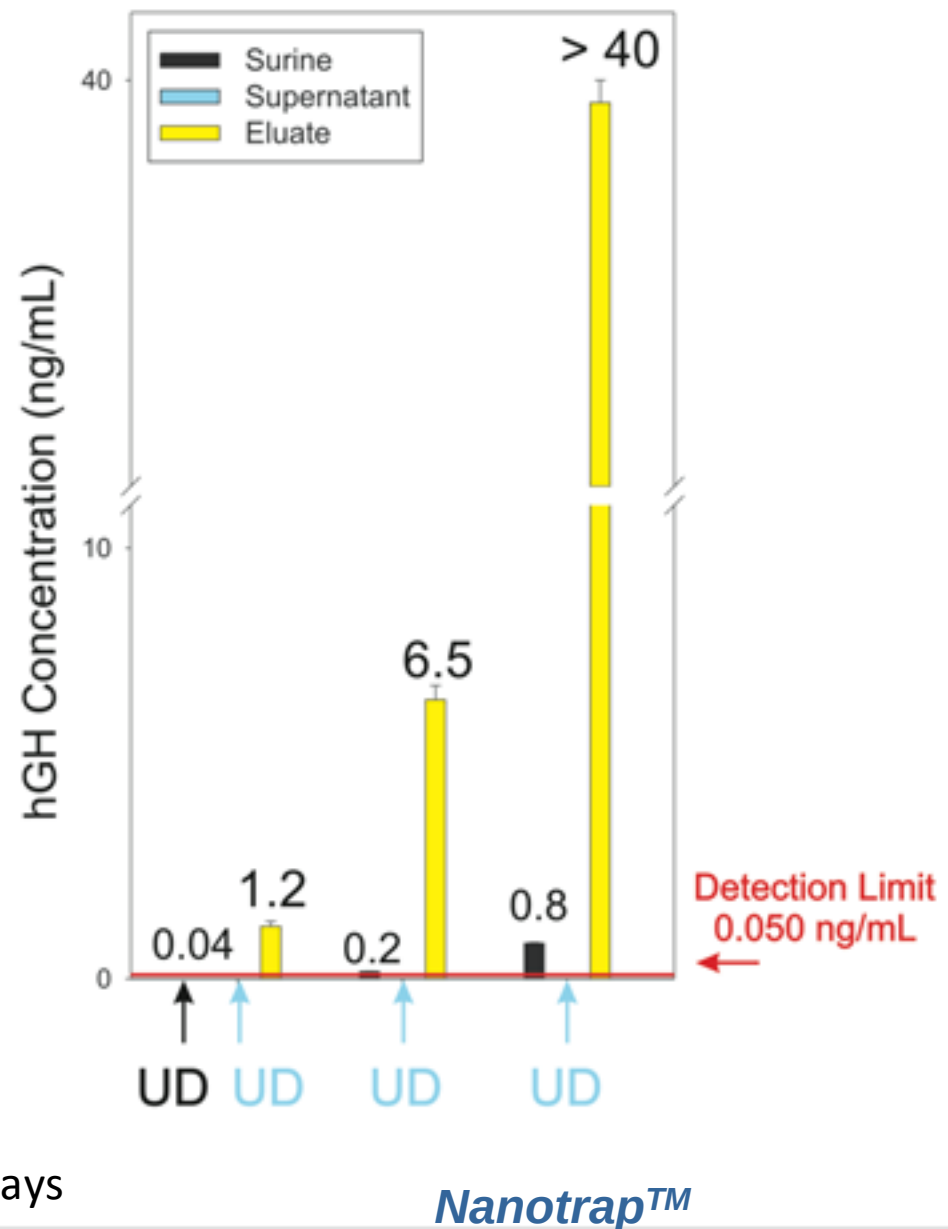


Complete protection from degradation



Rapid 100 %uptake: minutes





Human Growth Hormone
Levels in the blood: 1-10 ng/mL

Levels in the urine: 1000 fold less than serum

Below the detection limits of clinical immunoassays

Nanoparticles amplify the effective concentration of hGH
50 fold to achieve routine measurement in urine

CERES NANOSCIENCE
License of ISS/GMU IMAT IP

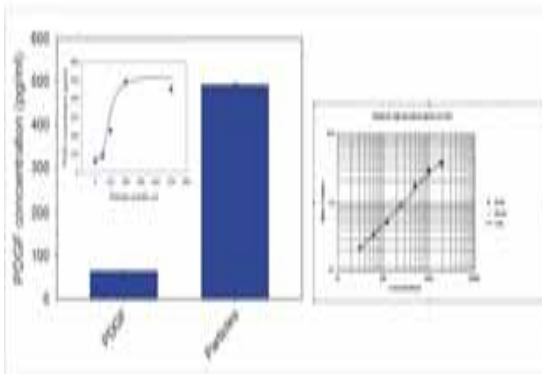
AMPLIFICATION OF LOW ABUNDANCE LABILE BIOMARKER PROTEINS BY SMART

NANOPARTICLES

100 fold Effective

Amplification of Mass Spec Peptide Sensitivity

Amplification of Clinical ELISA Sensitivity: Linear Measurement of serum PDGF for levels that are 100 fold below the ELISA detection limit.



Dilution	Particle Eluate Peptide	Peptide (Hits) [Ions]	Starting Solution Peptide	Peptide (Hits) [Ions]
1:600	K.KATVTLEDHLACK.C	17/24	K.KATVTLEDHLACK.C	17/24
	K.KATVTLEDHLACK.C	19/24	K.KATVTLEDHLACK.C	19/24
	K.ATVTLEDHLACK.C	14/22	K.KATVTLEDHLACK.C	14/22
	K.ATVTLEDHLACK.C	15/22	K.TRTEVFEISR.R	15/22
	K.TRTEVFEISR.R	14/18	K.ATVTLEDHLACK.C	14/18
	K.ATVTLEDHLACK.C	14/22	K.TRTEVFEISR.R	14/22
	K.TRTEVFEISR.R	15/18	R.SLGSLTIAEPAM*IAECK.T	15/18
	K.TRTEVFEISR.R	17/18	R.SLGSLTIAEPAMIAECK.T	17/18
	K.ATVTLEDHLACK.C	16/22	R.TNANFLVWPPCVEVQR.C	16/22
	K.TRTEVFEISR.R	14/18	R.TNANFLVWPPCVEVQR.C	14/18
	R.PTQVQLRPVQVR.K	12/22	R.TNANFLVWPPCVEVQR.C	12/22
	K.TRTEVFEISR.R	16/18	R.TNANFLVWPPCVEVQR.C	16/18
	R.NVQCRPTQVQLRPVQVR.K	26/64	R.TNANFLVWPPCVEVQR.C	26/64
	R.TEVFEISR.R	13/14		13/14
	R.SLGSLTIAEPAMIAECK.T	21/32		21/32
	R.SLGSLTIAEPAMIAECK.T	28/64		28/64
	R.TNANFLVWPPCVEVQR.C	18/30		18/30
	R.TNANFLVWPPCVEVQR.C	17/30		17/30
	R.TNANFLVWPPCVEVQR.C	20/30		20/30
	R.TNANFLVWPPCVEVQR.C	21/30		21/30
	R.TNANFLVWPPCVEVQR.C	21/30		21/30
	R.TNANFLVWPPCVEVQR.C	21/30		21/30
	R.TNANFLVWPPCVEVQR.C	20/30		20/30
1:6000	K.KATVTLEDHLACK.C	13/24	PDGF was not detected	
	K.ATVTLEDHLACK.C	14/22		
	K.TRTEVFEISR.R	13/18		
	R.TEVFEISR.R	12/14		
	R.TNANFLVWPPCVEVQR.C	16/30		
1:60000	K.KATVTLEDHLACK.C	15/24	PDGF was not detected	
	K.ATVTLEDHLACK.C	17/22		
	R.TEVFEISR.R	13/14		
1:600000	PDGF was not detected		PDGF was not detected	

MS nanoparticle harvesting Low Abundance Serum Biomarker Candidates

Longo et al PlosOne 09

Reference	P (pro)	Sf	Score	MW	Accession	Peptide
hemopexin [Homo sapiens]	4.44E-16	1.84	20.23	51643.3	11321561	2
lysozyme precursor [Homo sapiens]	2.11E-14	4.54	50.25	16526.3	4557894	6
apolipoprotein H precursor [Homo sapiens]	1.38E-13	12.33	130.31	38286.7	4557327	17
hypothetical protein LOC649897 [Homo sapiens]	2.91E-13	2.92	30.26	22058.9	91206438	20
apolipoprotein C-III precursor [Homo sapiens]	3.26E-13	6.59	70.25	10845.5	4557323	15
platelet factor 4 variant 1 [Homo sapiens]	5.80E-13	3.80	40.24	11545.3	4505735	8
C-type lectin domain family 3, member B [Homo sapiens]	8.72E-13	6.16	70.28	22552.3	4507557	7
alpha-2-HS-glycoprotein [Homo sapiens]	1.36E-12	5.33	60.23	39299.7	4502005	16
pro-platelet basic protein precursor [Homo sapiens]	1.37E-12	8.29	90.28	13885.4	4505981	64
coagulation factor XII precursor [Homo sapiens]	2.02E-12	6.65	70.26	67774.1	4503629	7
properdin P factor, complement [Homo sapiens]	4.81E-12	2.86	30.24	51242.0	4505737	5
histidine-rich glycoprotein precursor [Homo sapiens]	5.01E-12	9.24	100.24	59540.9	4504489	12
clusterin isoform 1 [Homo sapiens]	1.25E-11	7.39	80.28	57795.7	42716297	10
pancreatic ribonuclease precursor [Homo sapiens]	3.20E-11	2.84	30.30	17632.7	38201682	3
vitronectin precursor [Homo sapiens]	1.11E-10	2.85	30.19	54271.2	88853069	3
gelsolin isoform b [Homo sapiens]	1.21E-10	6.54	70.23	80590.6	38044288	9
platelet-derived growth factor beta isoform 1, preproprotein [Homo sapiens]	3.79E-10	7.09	80.22	27266.1	4505681	25
platelet-derived growth factor beta isoform 2, preproprotein [Homo sapiens]	3.79E-10	7.09	80.22	25486.2	15451786	25
insulin-like growth factor 1 (somatomedin C) [Homo sapiens]	6.67E-10	0.96	10.21	17014.3	11024682	2
transthyretin [Homo sapiens]	7.20E-10	6.34	70.24	15877.1	4507725	7
alpha 1 type XVIII collagen isoform 1 precursor [Homo sapiens]	9.46E-10	0.97	10.23	153745.9	13385620	1
fibrinogen, alpha polypeptide isoform alpha preproprotein [Homo sapiens]	8.68E-09	4.45	50.23	69713.8	11761629	5
selenoprotein P precursor [Homo sapiens]	1.04E-08	0.96	10.24	141646.7	62530391	1
lipocalin 2 (oncogene 24p3) [Homo sapiens]	1.32E-08	3.78	40.21	22573.7	38455402	4
serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 [Homo sapiens]	3.60E-08	1.91	20.24	46707.1	50363219	2
platelet factor 4 (chemokine (C-X-C motif) ligand 4) [Homo sapiens]	4.38E-08	4.76	50.24	10837.9	4505733	10
coagulation factor II precursor [Homo sapiens]	7.52E-08	0.97	10.21	69992.2	4503635	1
RAP1A, member of RAS oncogene family [Homo sapiens]	9.02E-08	0.97	10.20	20973.7	4506413	1
paraoxonase 1 [Homo sapiens]	2.54E-07	0.96	10.17	39706.3	19923106	1
orosomucoid 1 precursor [Homo sapiens]	3.28E-07	1.88	20.20	23496.8	9257232	2
peptidoglycan recognition protein 1 [Homo sapiens]	4.60E-07	0.91	10.17	21716.7	4827036	1
chemokine (C-C motif) ligand 14 isoform 1 precursor [Homo sapiens]	5.02E-07	2.79	30.22	10671.3	14589961	3
thrombospondin repeat containing 1 isoform 1 [Homo sapiens]	8.60E-07	0.71	10.12	116470.7	38016904	1

ISS Sponsored GMU Translational Research Program

Istituto Superiore di Sanità – Rome
George Mason University Virginia USA

Professor Enrico Garaci
Ruggero DeMaria
Claudio Belluco



Participating Centers:

- IEO - Milan
- INT – Milan
- IST Genova
- CRO - Aviano
- IRE - Rome
- IRCCS Oncol. - Bari
- Univers. - Brescia
- Ospedale Maggiore - Milan
- Surgery and Pediatric Depts. - Padova
- S. Camillo Hosp - Rome



NCI USA- Italy Bilateral Agreement

ITALY-USA PROGRAM ON ONCOPROTEOMICS NATIONAL SERUM

BANK

PROSPECTIVE SERUM COLLECTION - EXPECTED

TUMOR TYPE	CANCER	CONTROL	TOTAL
Breast	4095	830	4925
Colorectal	1060	950	2010
Lung	1300	520	1820
Ovary	315	360	675
HCC	205	150	355
Leukemia	300	-	300
TOTAL	7275	2810	Target: 10085 at present 4474
Prostate (3600 serum samples expected: at present 858)			

NanoTrap MRM

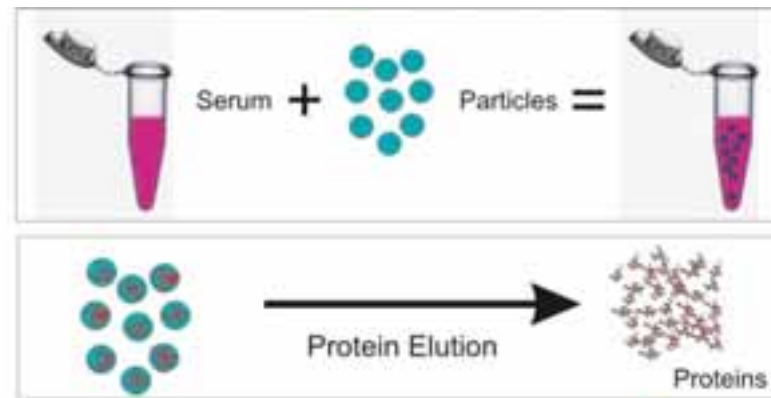
Biomarker Discovery
Workflow Employing
Nanoparticle capture

High Throughput,

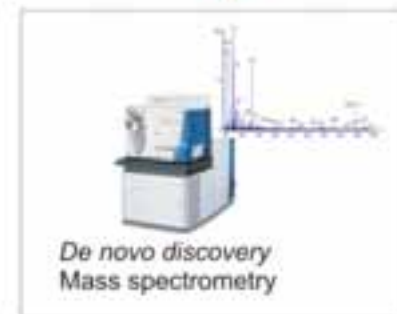
High Sensitivity Discovery,

Preservation

and Validation



Biomarker Discovery



Biomarker Validation



Antibody

No antibody



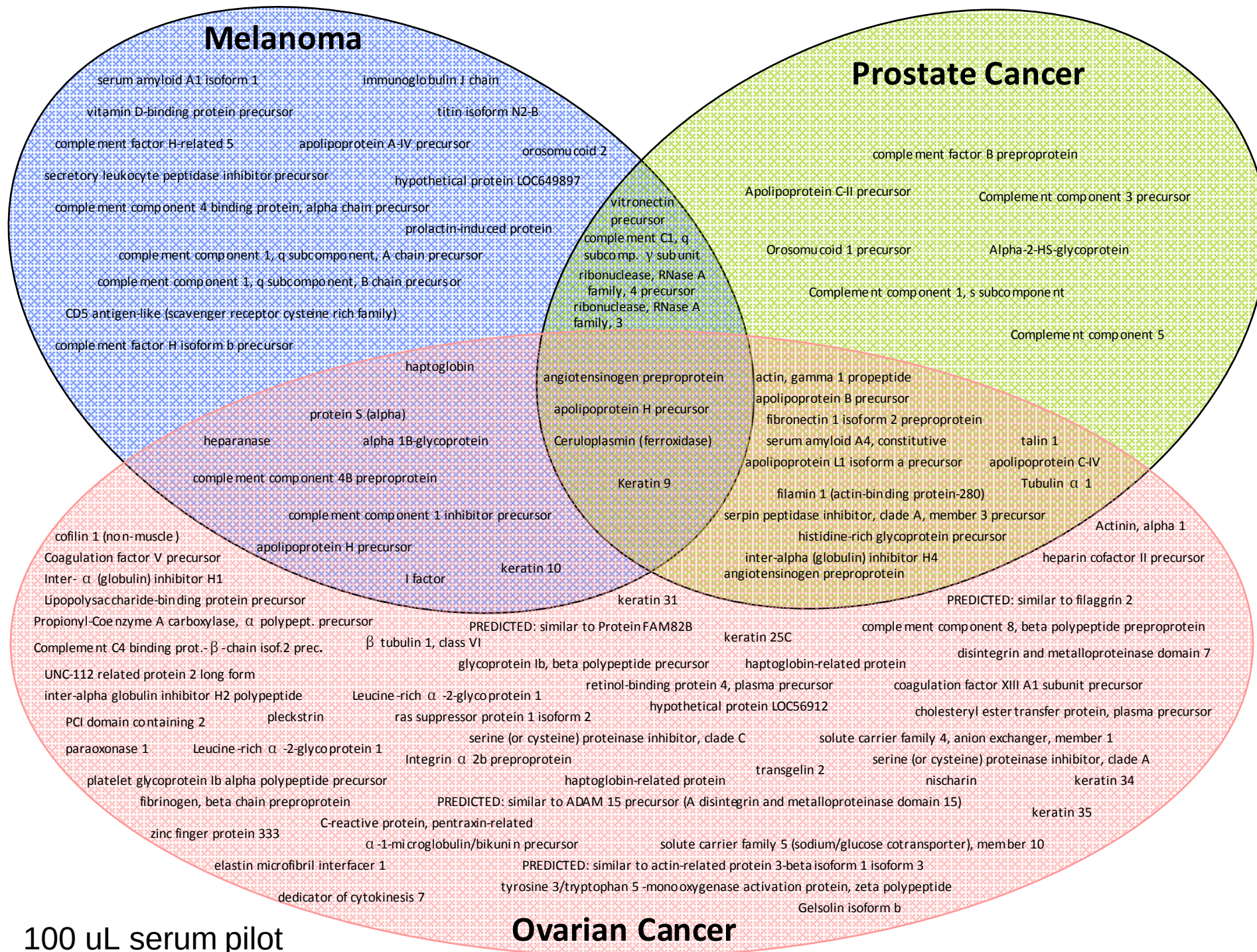
Immunological assay
Immulite 1000



Reverse Phase
Protein Microarray (RPPM)



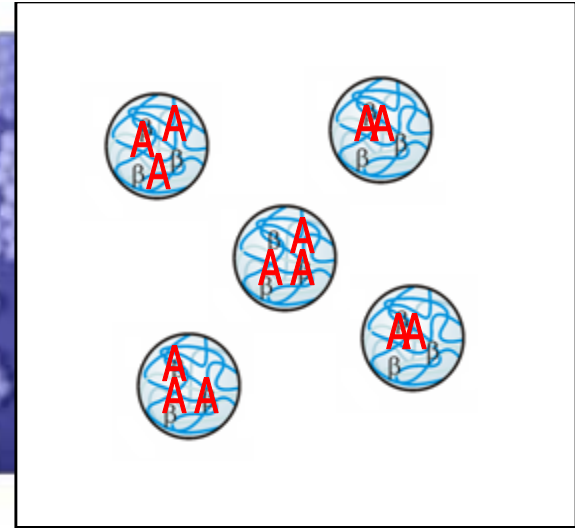
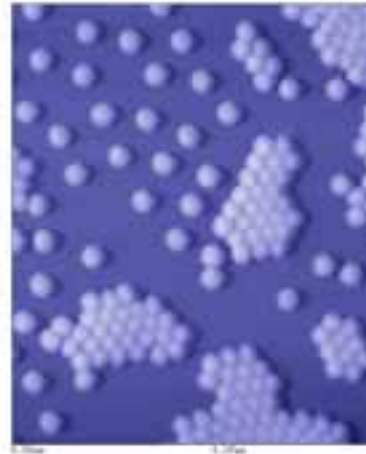
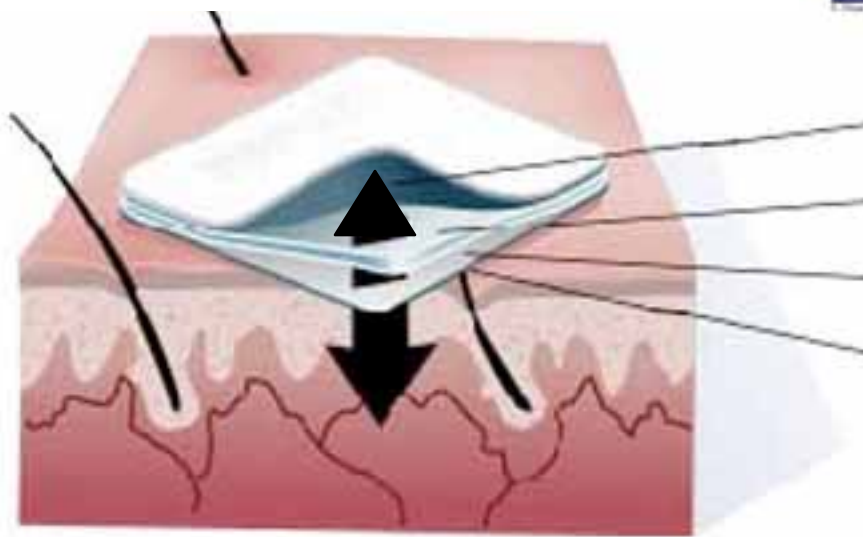
Multiple Reaction
Monitoring (MRM)



100 uL serum pilot

Smart Nanoparticles for Biomarker Harvesting

Example application to skin patch for diagnostic marker (proteins and metabolites) harvesting



Water resistant cover

Harvesting Nanoparticles

Porous membrane

Permeation enhancer

- User friendly non invasive
- Amplifies low abundant markers over time of patch duration
- Protects biomarkers from degradation
- Mail-in room temperature shipping





Center for Applied Proteomics and Molecular Medicine

Co-Directors: Lance Liotta and Emanuel Petricoin

IMAT Technology Development

Phosphoprotein

Tissue Preservative

Virginia Espina

Claudius Mueller

Alex Reeder

Kirsten Edmiston

Lindsay Wescott

Sally Rucker

Smart Nanoparticles

Alessandra Luchini

Virginia Espina

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Sponsors of Breast Cancer Research at GMU



GlaxoSmithKline/US Oncology



DOD Breast Cancer Research Program



NIH/National Cancer Institute



Susan G. Komen Foundation



Collaborating Organizations

University of California San Francisco



Inova Health System (Inova Fairfax Hospital)



Side Out Foundation



“Smart” Nanoporous Particles / Biomarker publications

Simpson, M. et al Serum S100A6 Concentration Predicts Peritoneal Tumor Burden In Mice With Epithelial Ovarian Cancer And Is Associated With Advanced Stage in Patients. PloS one 2009 in press

Longo, C., et al., *Core-shell hydrogel particles harvest, concentrate and preserve labile low abundance biomarkers*. PloS one, 2009. 4(3): p. e4763-4790.

Fredolini, C., et al., *Concentration and Preservation of Very Low Abundance Biomarkers in Urine, such as Human Growth Hormone (hGH), by Cibacron Blue F3G-A Loaded Hydrogel Particles*. Nano Research, 2008. 1: p. 502-518.

Luchini, A., et al., *Smart hydrogel particles: biomarker harvesting: one-step affinity purification, size exclusion, and protection against degradation*. Nano letters, 2008. 8(1): p. 350-61.

Luchini, A., and Liotta L., *Nanoparticle Technology: Addressing the fundamental roadblocks to protein biomarker discovery*. Journal of Materials Chemistry, 2009. 19: p. 5071 - 5077.