Highly Sensitive Targeted Quantification of Low-Abundance Proteins, Isoforms, and Post-Translational Modifications

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Protein post-translational modifications (PTMs)

DNA encodes functions

↓

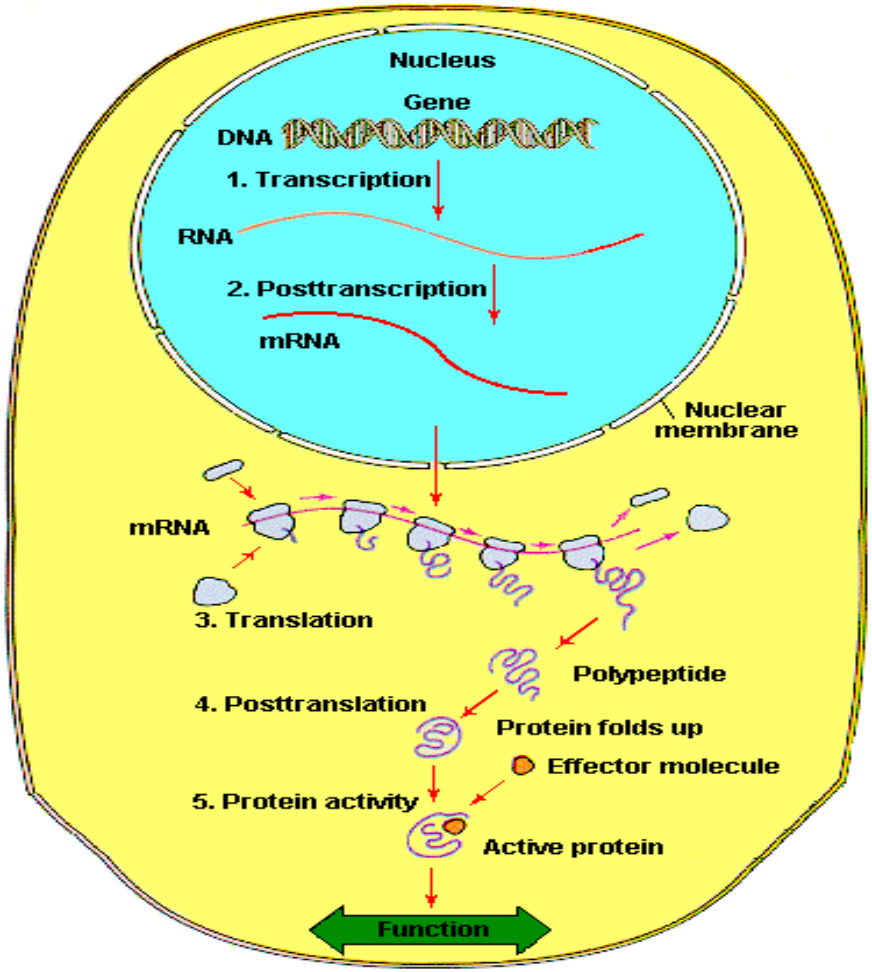
mRNA transfers functions

↓

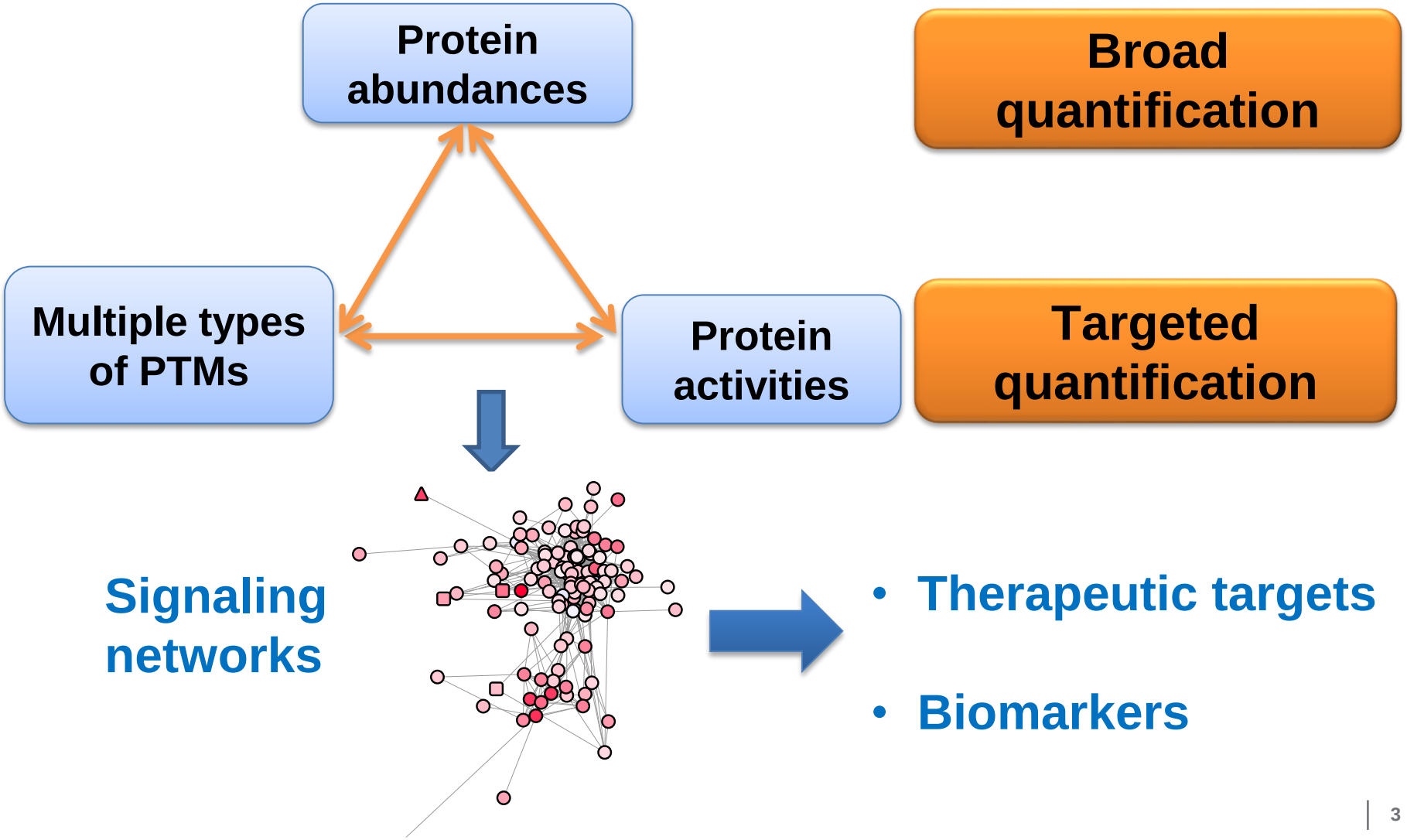
Proteins fulfill functions

↕

PTMs regulate functions



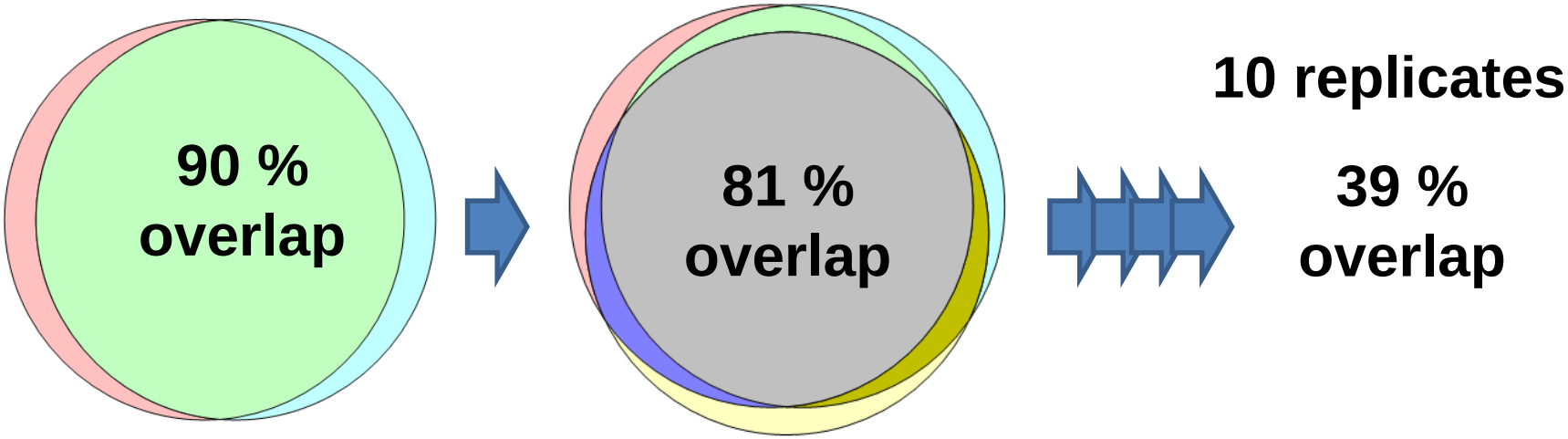
Integrative functional proteomics



From global discovery to targeted quantification

Limitations of global discovery:

- **Not reproducible** in terms of low overlap between large number of biological replicates or missing data.



- **Limited sensitivity** for detecting low-abundance proteins

Targeted mass spectrometry for quantification

- ~100% reproducible detection (Only selected candidates will be screened)
- Better sensitivity and no missing data
- High specificity and absolute quantification
- **Multiplexed** (~100 proteins per analysis) and affinity-reagent free
- ***Versatile in quantifying proteins, isoforms, PTMs***

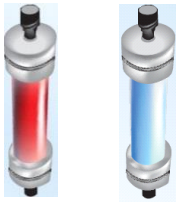
Sensitivity challenge in targeted proteomics

10-100 µL human blood plasma/serum

Lower limit of detection

100-1000 ng/mL — **LC-SRM**

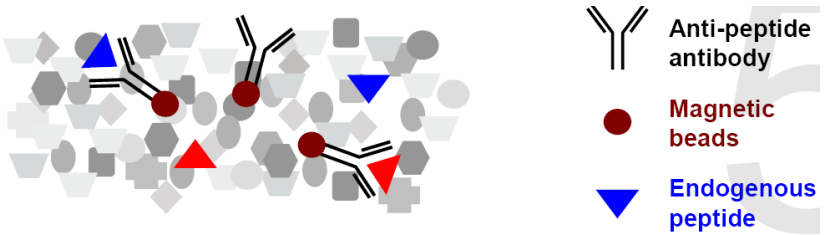
1-10 ng/mL



IgY-14 **SCX**

1-100 pg/mL

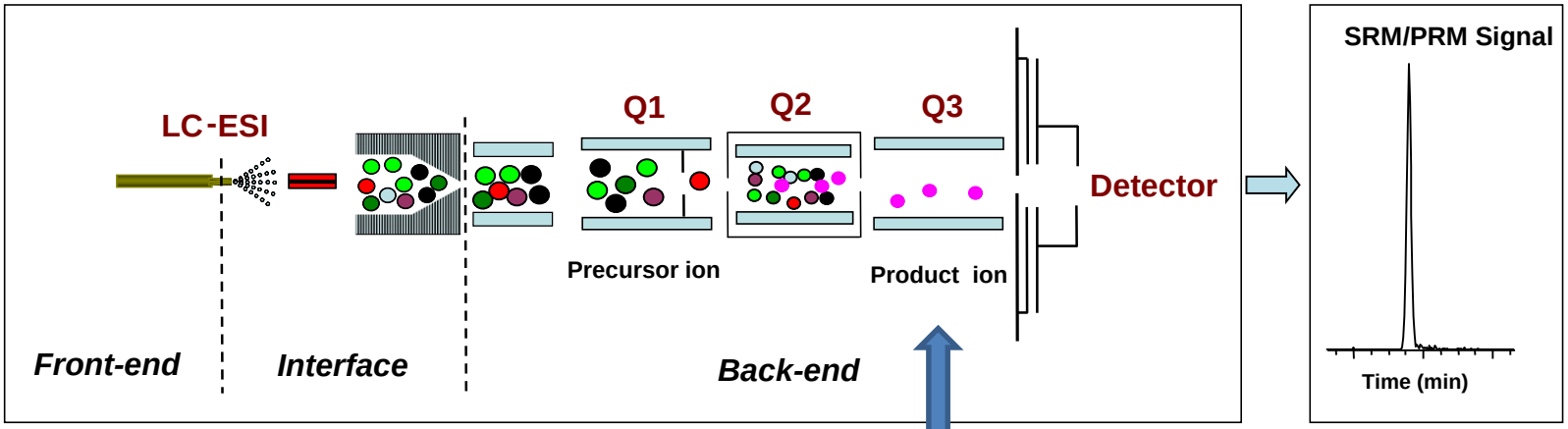
in human plasma/serum

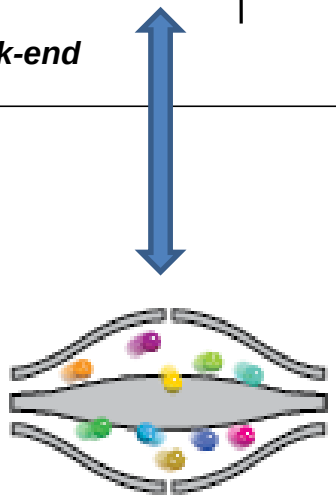


SISCAPA

Keshishian et al. ***Mol Cell Proteomics*** 2007, *6*, 2212-2229.
Whiteaker et al. ***Mol Cell Proteomics*** 2012, 11(6):M111.015347.
Kuhn et al. ***Mol Cell Proteomics*** 2012, 11(6):M111.013854.

Targeted quantification using selected reaction monitoring (SRM) or parallel reaction monitoring (PRM)

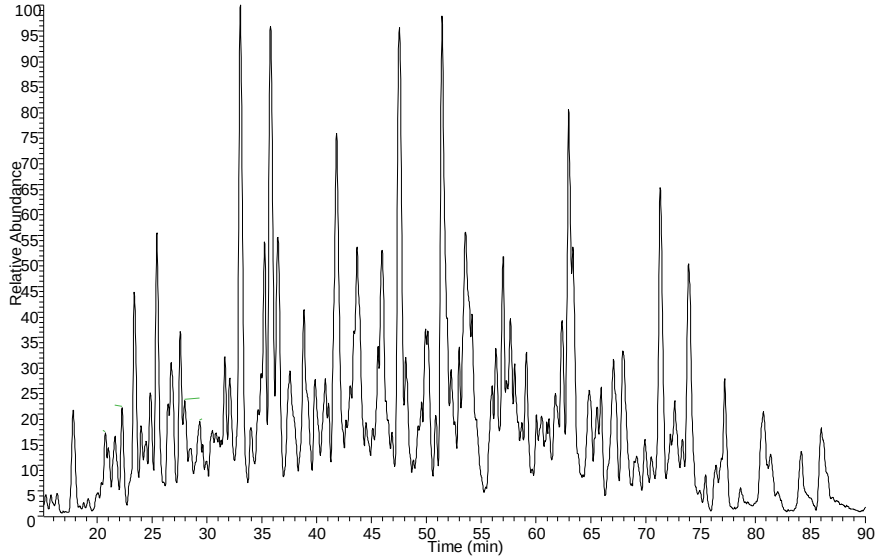


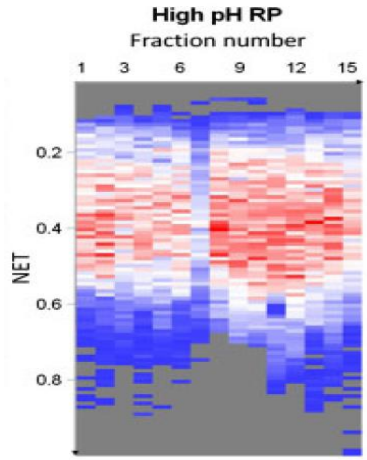


PRM

Orbitrap in
Q Exactive

Front-end: High pH RPLC extensive fractionation (peak capacity ~500)





Orthogonality

High pH RPLC

Versus

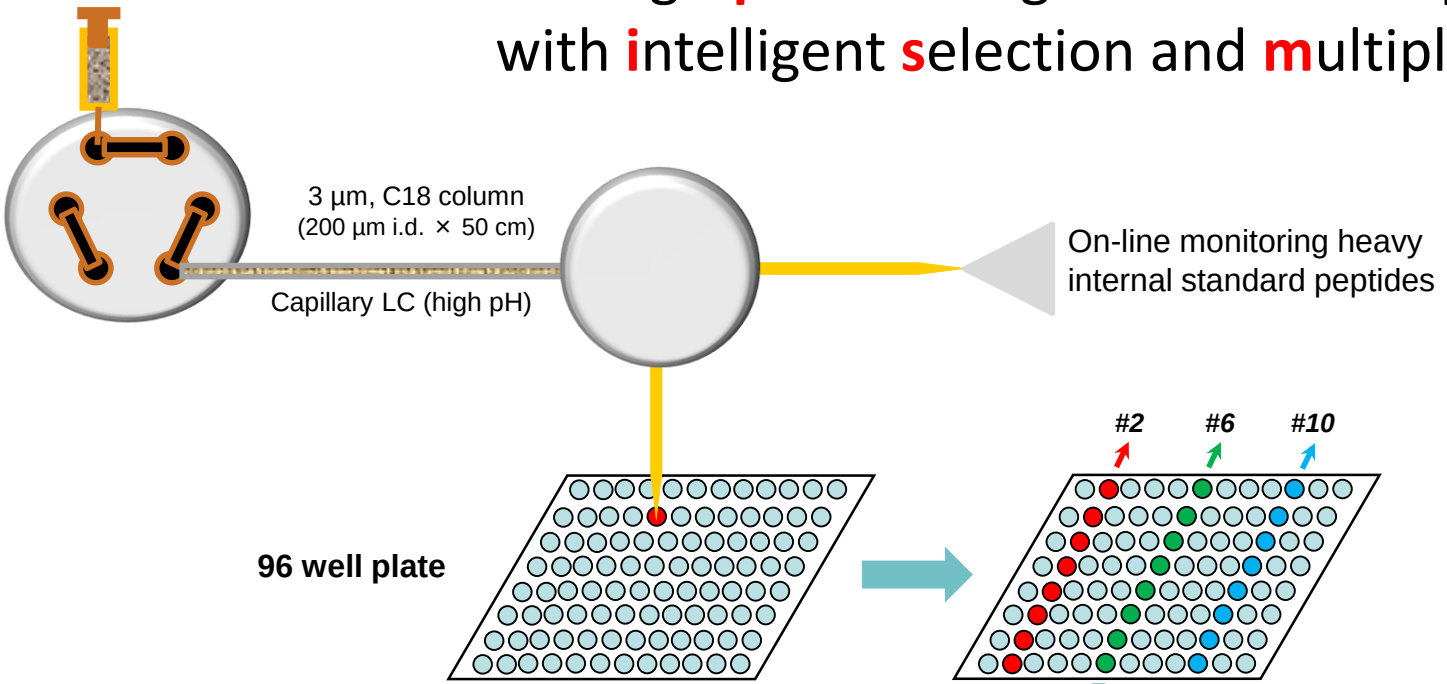
Low pH RPLC

- **High-resolution and highly reproducible separation is ideal for focusing and enriching target analytes**
- **But too many fractions limit the overall throughput**
- **Need an intelligent fractionation strategy**

Wang *et al.* *Proteomics*. 2011, 11, 2019-26.

The PRISM strategy

PRISM: high-**p**ressure high-**r**esolution separations with **i**ntelligent **s**election and **m**ultiplexing



***Intelligent* Selection of targeted peptides**

Fraction multiplexing (based on partial orthogonality between low and high pH RPLC)

LC-SRM/PRM

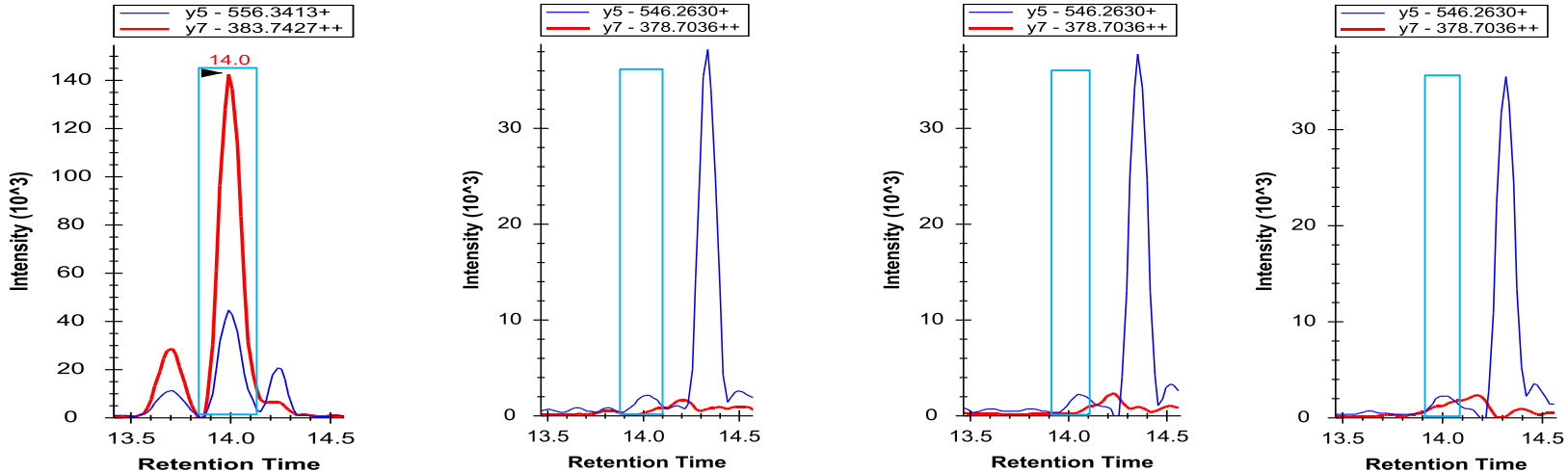
Shi *et al., PNAS,* 2012, 109, 15395-400.

Enhanced sensitivity with PRISM-SRM

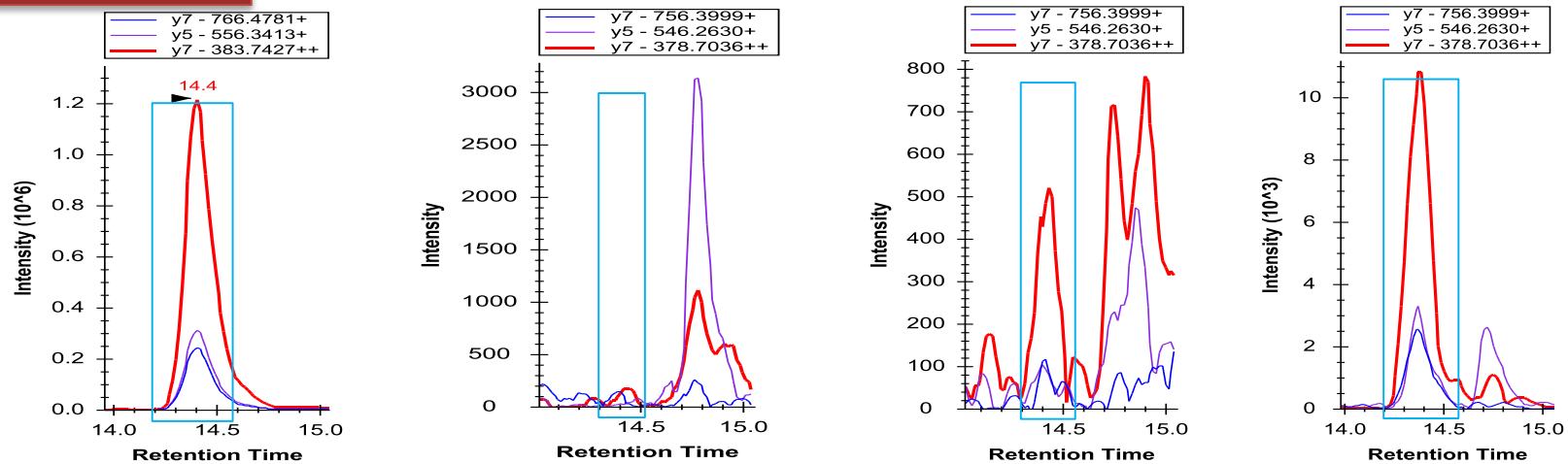
IgY14 only

Bovine carbonic anhydrase: DFPIANGER ($509.8^{2+}/378.7^{2+}$)

Heavy | 0.0 ng/mL | 50 pg/mL | 1 ng/mL

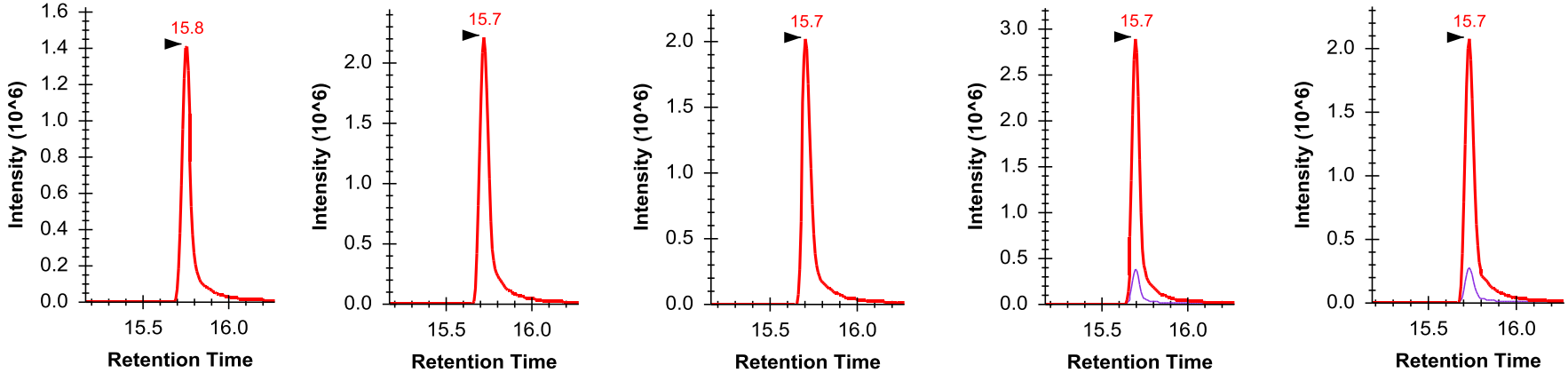


IgY14 + PRISM

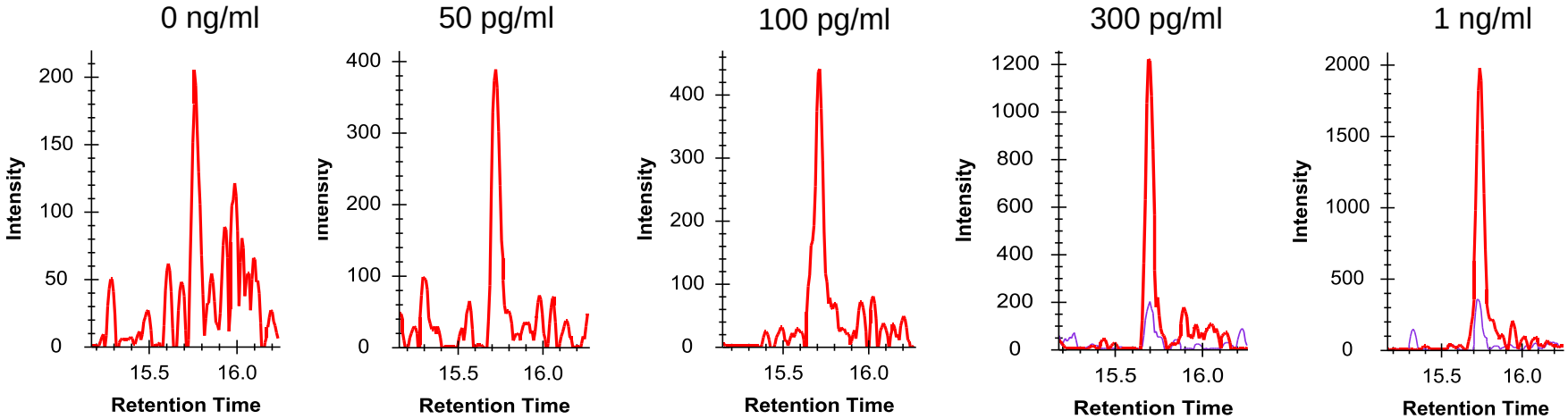


PSA calibration curve experiment

LSEPAELTDAV($^{13}C_6$,$^{15}N_2$)**K** (internal standard at 2 fmol/μL)



LSEPAELTDAVK (prostate-specific antigen) 636.8/943.5 (red), 636.8/846.5 (blue).



XICs of transitions monitored for peptide LSEPAELTDAVK derived from PSA spiked into human female serum with IgY14 depletion coupled with PRISM.

Shi et al. PNAS. 2012, 109, 15395-400.

Detection of endogenous cancer-associated proteins in non-depleted human sera

KLK6 — LSELIQPLPLER

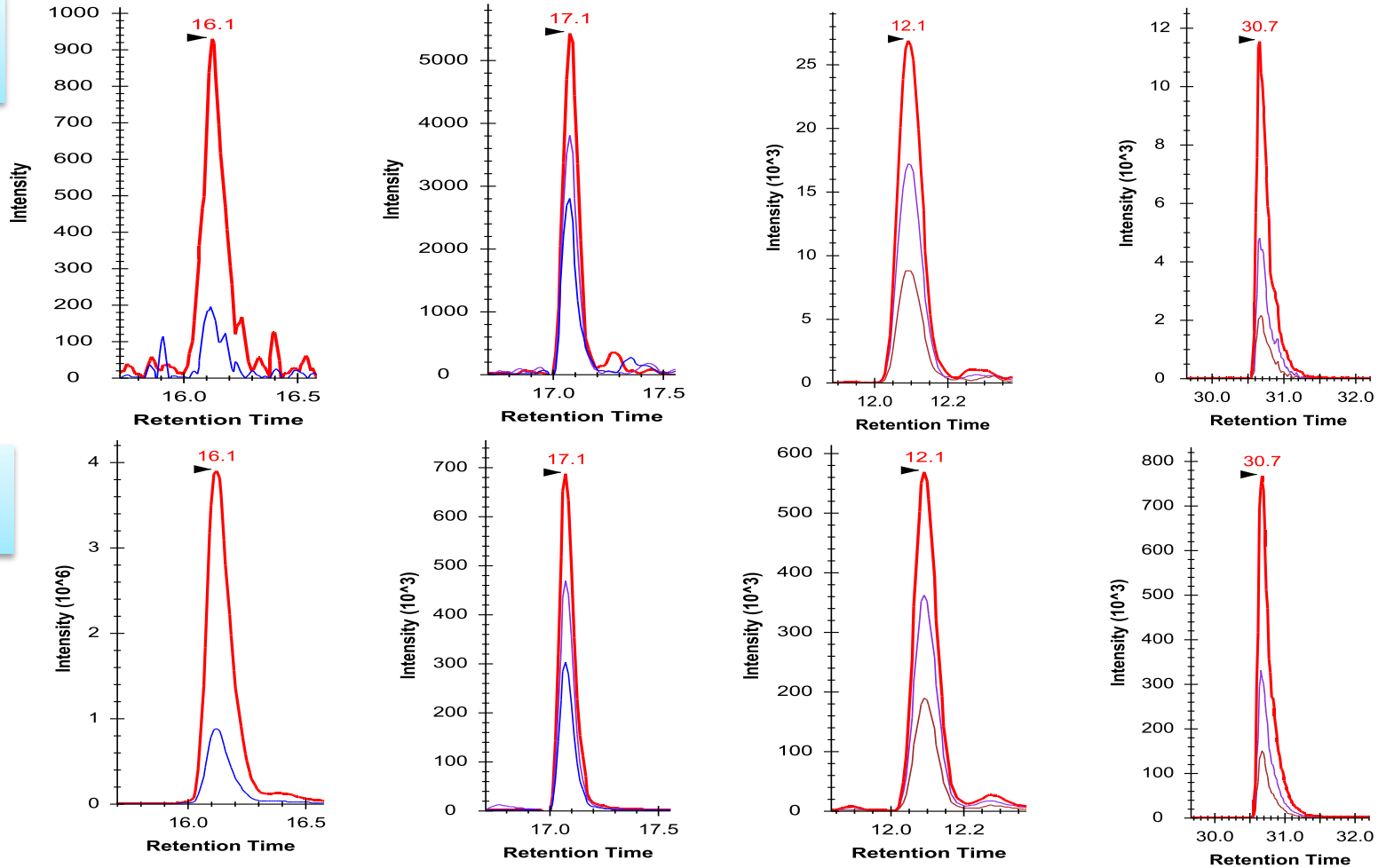
EGFR — LTQLGTFEDHFLSLQR

MMP9 — AVIDDAFAR

POSTN — AAAITSDILEALGR

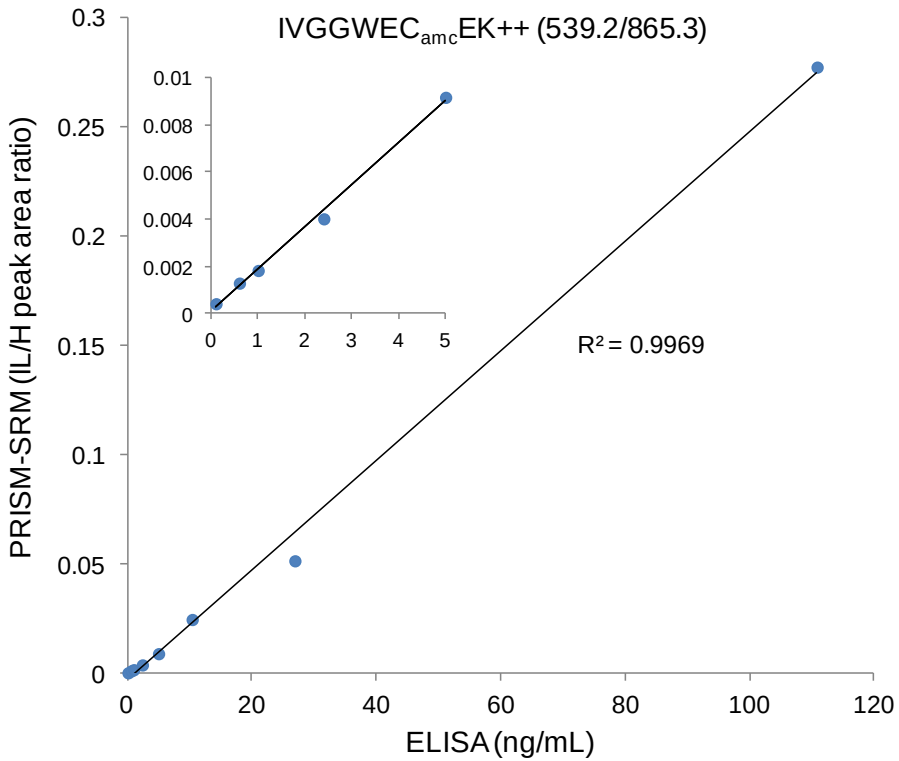
Detection in serum

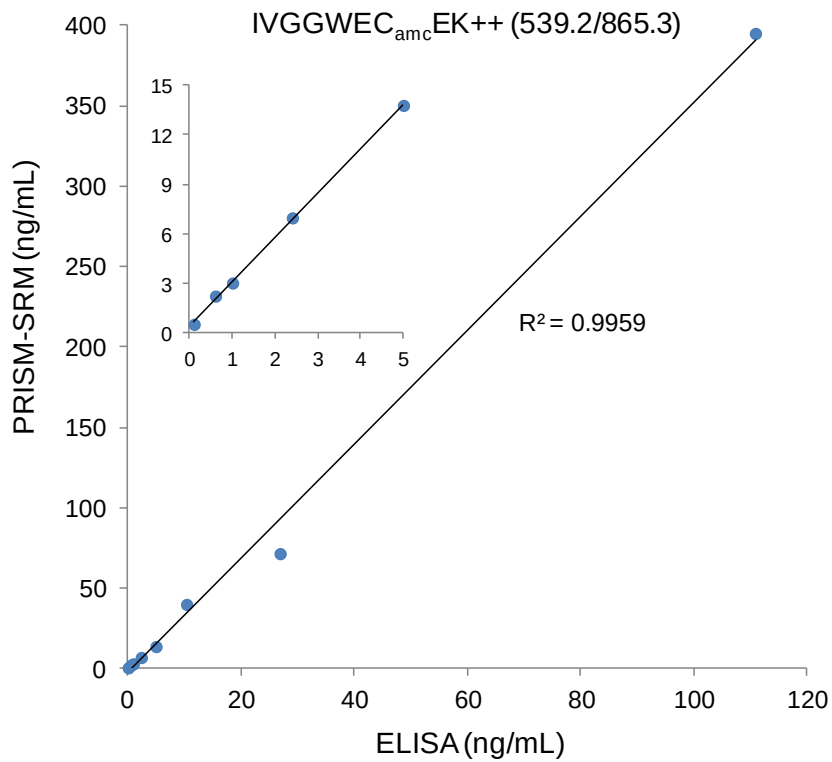
Heavy standards



All protein concentrations were reported in the range of 1 to 300 ng/mL

Correlation with ELISA values





High reproducibility and high peptide recovery using RP capillary column
PRISM-SRM (total PSA concentration); ELISA (specific PSA form)

Limit of Quantification (LOQ) for standard proteins spiked into female plasma

Proteins	Surrogate peptides	Method	LOQ (ng/ml) Non-depleted	LOQ (ng/ml) IgY14-depleted
Bovine carbonic anhydrase	DGPLTGTYR	Direct SRM	500	10
		PRISM-SRM	5	0.5
	DFPIANGER	Direct SRM	1000	10
		PRISM-SRM	1	0.05
Bovine beta-lactoglobulin	VLVLDTDYKK	Direct SRM	250	5
		PRISM-SRM	5	0.05
	VYVEELKPTPEGDLEILLQK	Direct SRM	2000	10
		PRISM-SRM	5	0.05
Prostate-specific Antigen (PSA)	IVGGWEC$_{amc}$EK	Direct SRM	1000	100
		PRISM-SRM	1	0.1
	LSEPAELTDAVK	Direct SRM	2000	10
		PRISM-SRM	0.5	0.05

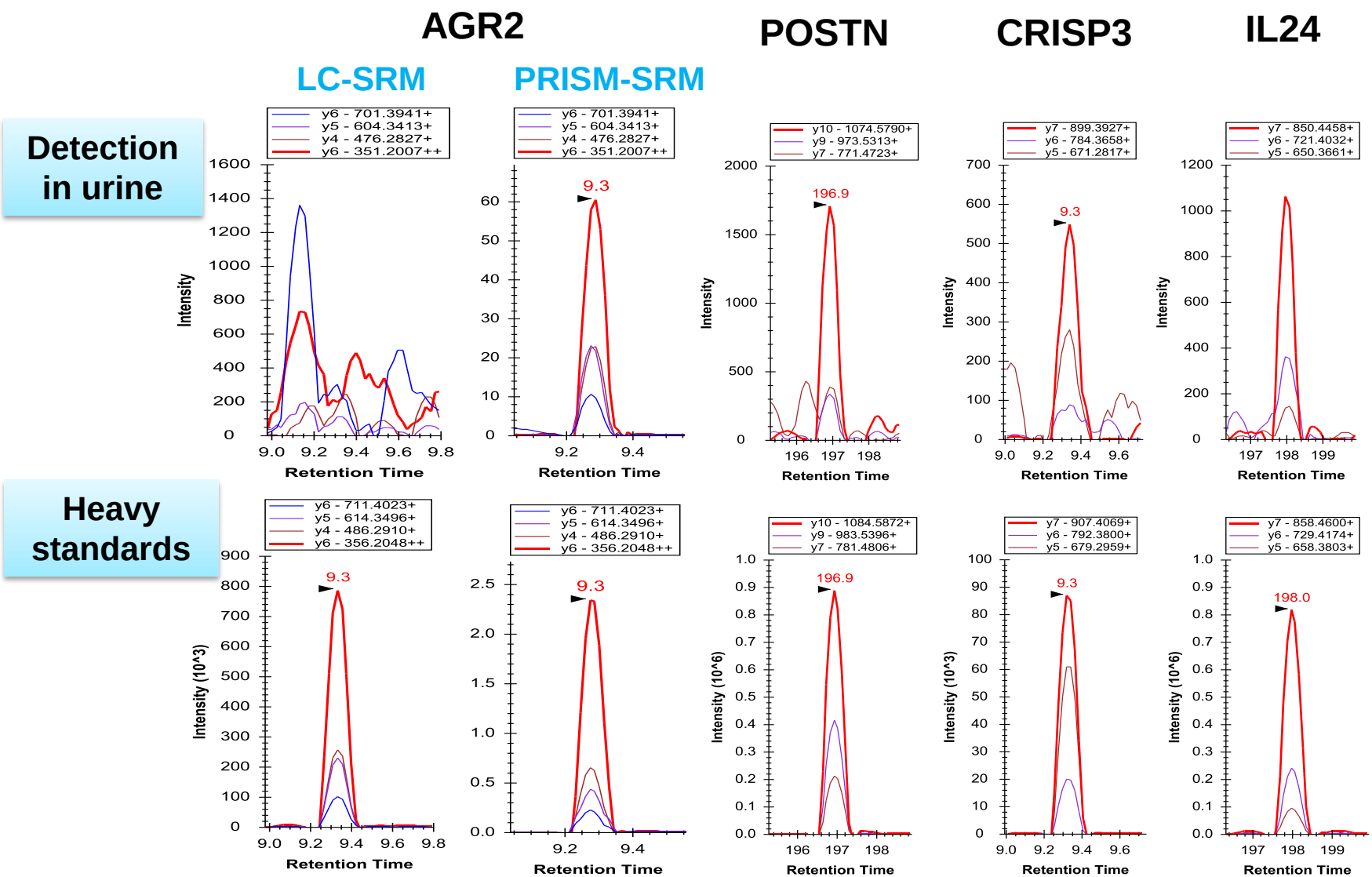
~200-fold SRM sensitivity enhancement

PRISM *vs* SISCAPA-SRM

	PRISM	SISCAPA
Sensitivity	50-100 pg/mL (10-50 µL) 0.5-5 ng/mL (2 µL)	~2 ng/mL (10-50 µL) ~ 50 pg/mL (1 mL)
Sample recovery	≥ 90%	10%-70%
Reproducibility *(median CV)*	~10%	~10%
Sample throughput	Moderate	Relatively high
Reagent	No antibody	Antibody
Success rate	High	Moderate
Lead time	A few weeks for synthetic peptides	up to 24 weeks

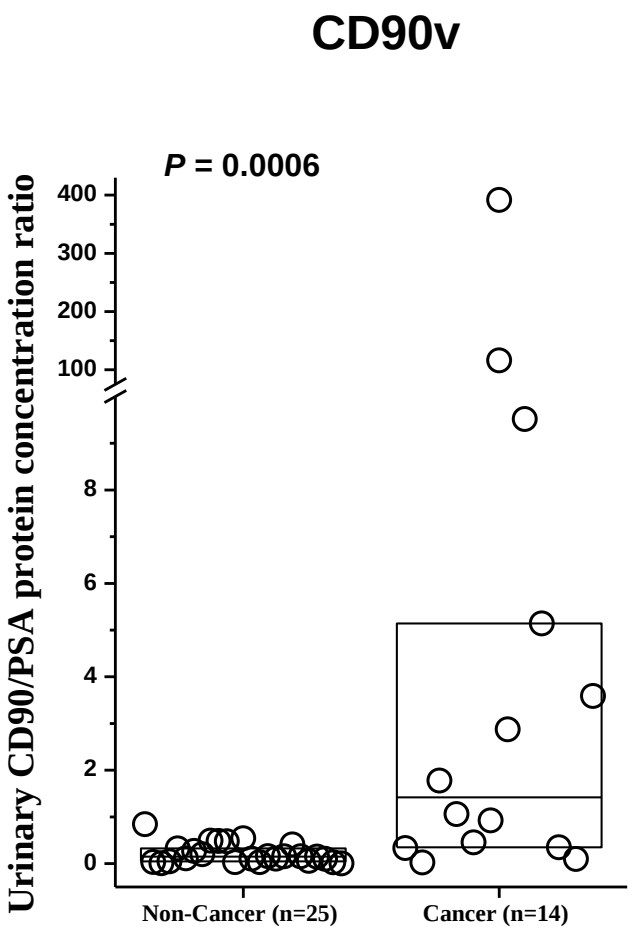
Shi et al. ***Proc Natl Acad Sci*** 2012, 109, 15395-15400.
Shi et al. ***J Proteome Res*** 2013, 12, 3353-3361.
Whiteaker et al. ***Mol Cell Proteomics*** 2010, 9, 184-196.
Whiteaker et al. ***Mol Cell Proteomics*** 2012, 11(6):M111.015347.
Kuhn et al. ***Mol Cell Proteomics*** 2012, 11(6):M111.013854.

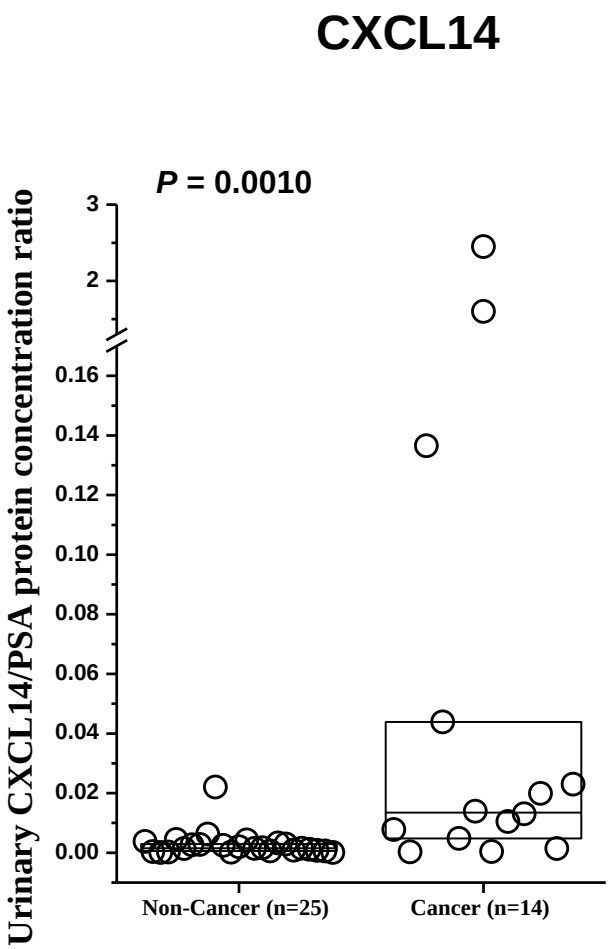
Detection of prostate cancer urinary markers by PRISM-SRM



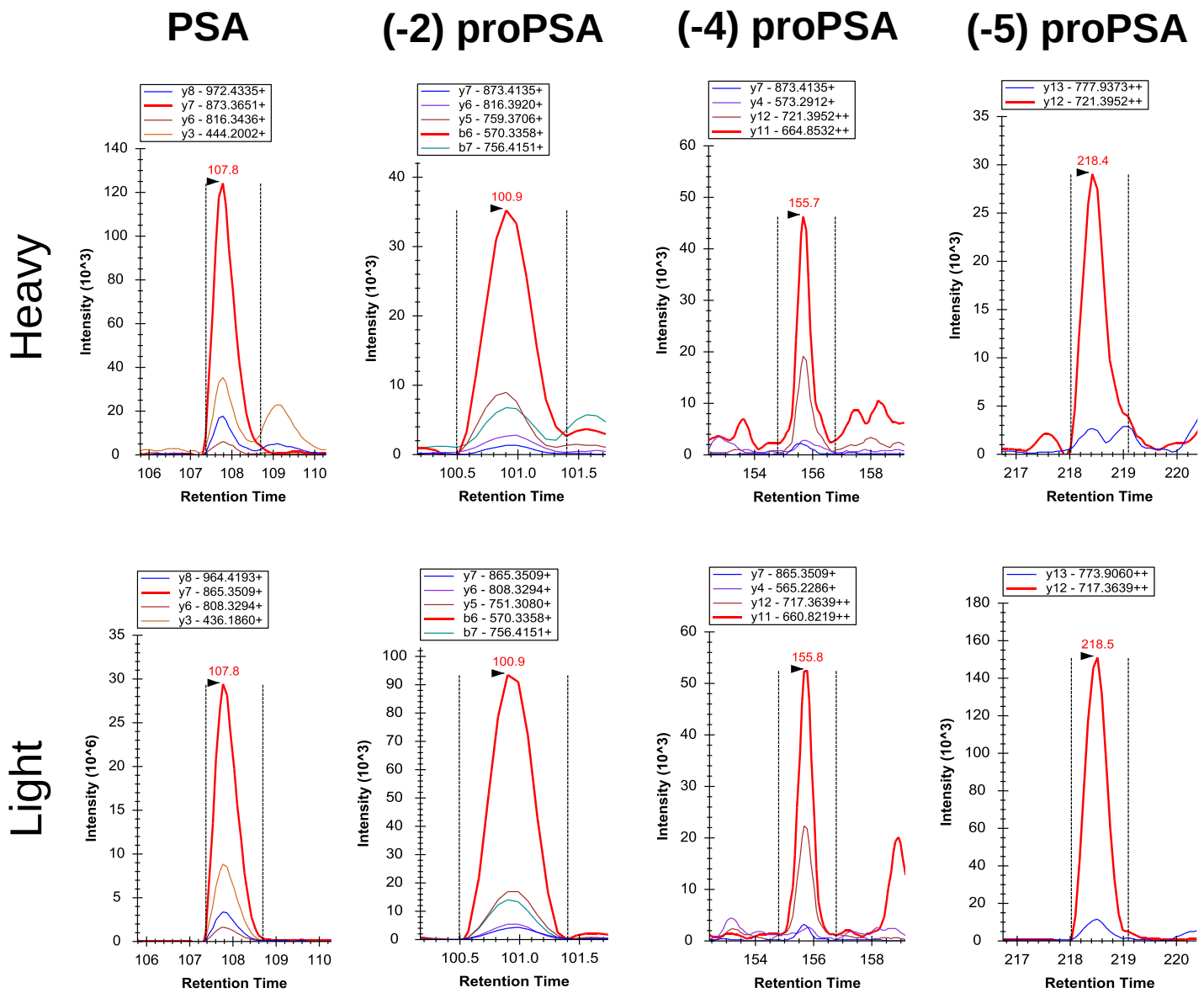
✓ 12 out of 14 candidate proteins were quantified in 48 subjects

Promising urinary markers for prostate cancer





proPSA isoforms in prostate cancer patient urine



Shi et al., unpublished results

PRM for isoforms with similar masses (Osteopontin, a lung cancer marker in serum)

QNLLAP**Q**TLPS**K**

m/z=655.377

QNLLAP**E**TLPS**K**

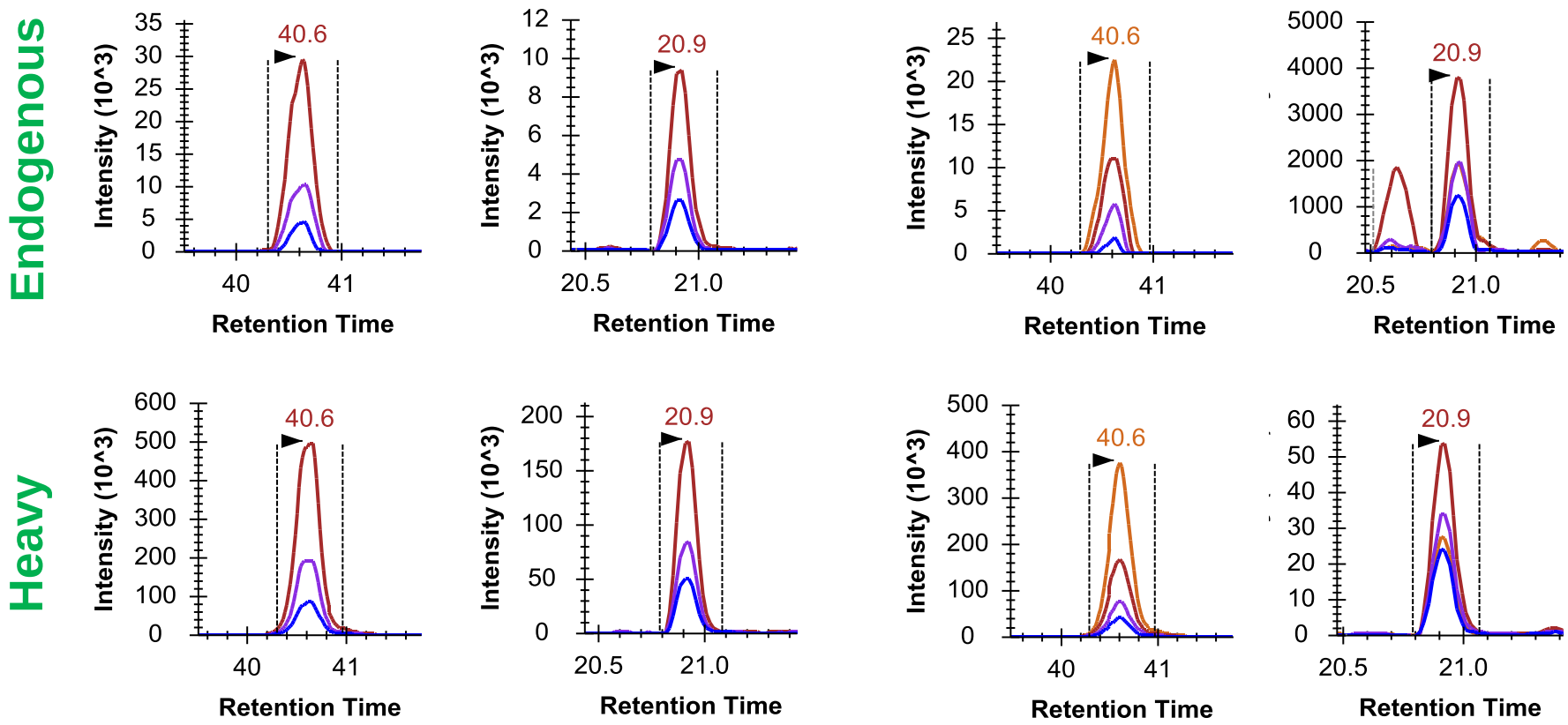
m/z=655.870

PRISM-PRM L/H=0.0547

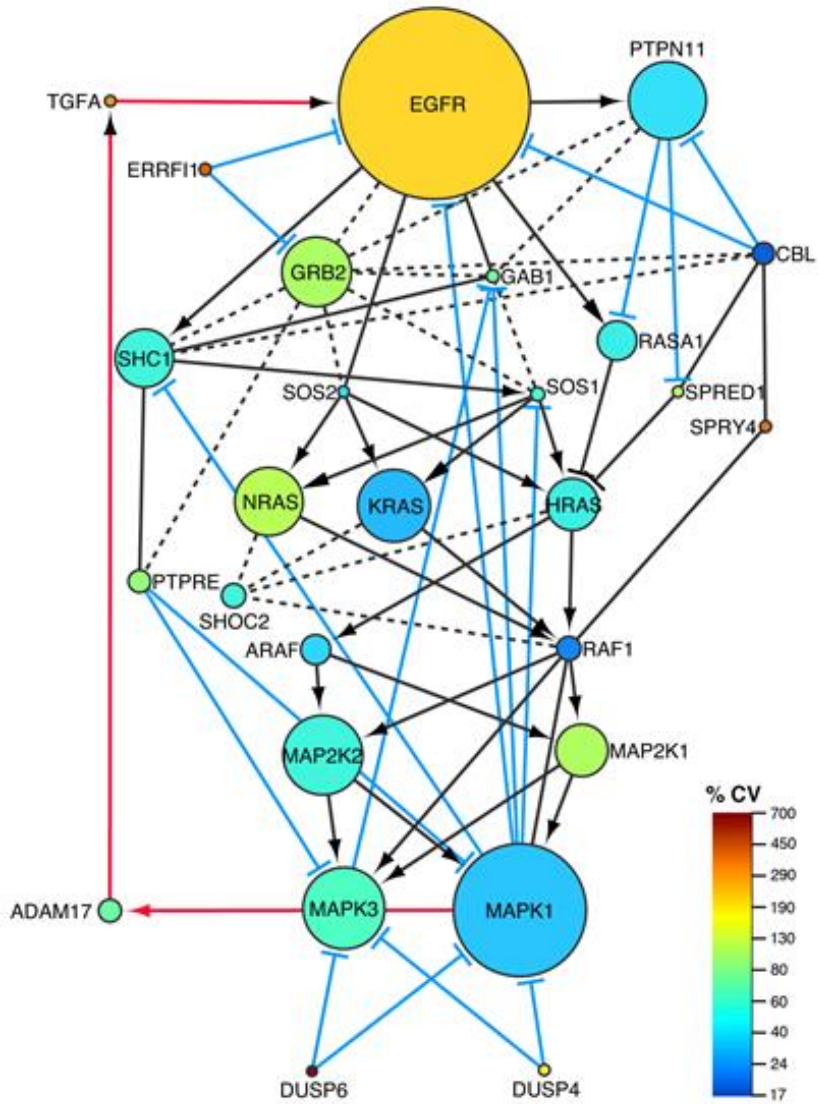
PRISM-SRM L/H=0.0539

PRISM-PRM L/H=0.0572

PRISM-SRM L/H=0.0612



Absolute quantification of EGFR signaling network



Gene	WB	2D-LC/MS global analysis (pooled sample)	Gene	WB	2D-LC/MS global analysis (pooled sample)
EGFR	Y	√	MAPK1	Y	√
SHC1	Y	√	AKT1	N	
GRB2	Y	√	PDPK1	N	
GAB1	-		PIK3R1	Y	
PTPN11	Y	√	PIK3R2	-	
SOS1	N		PEBP1	-	√
SOS2	-		TGFα	-	
HRAS	Y	√	CBL	Y	
KRAS	-		ERRFI1	-	
NRAS	-	√	DUSP4	Y	
RASA1	Y	√	DUSP6	-	
ARAF	Y		SPRED1	-	
RAF1	-		SPRY4	-	
MAP2K1	Y	√	PTPRE	-	
MAP2K2	Y	√	SHOC2	-	
MAPK3	Y		ADAM17	-	

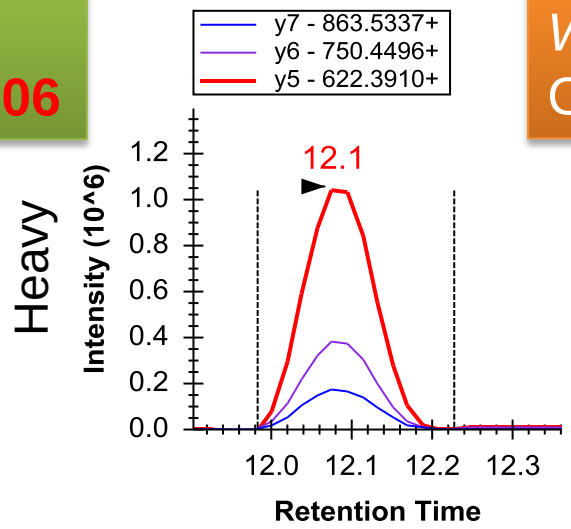
WB: 14/32
Global MS/MS: 11/32

Shi, et al., Science Signaling, 2016

Low-abundance signaling proteins

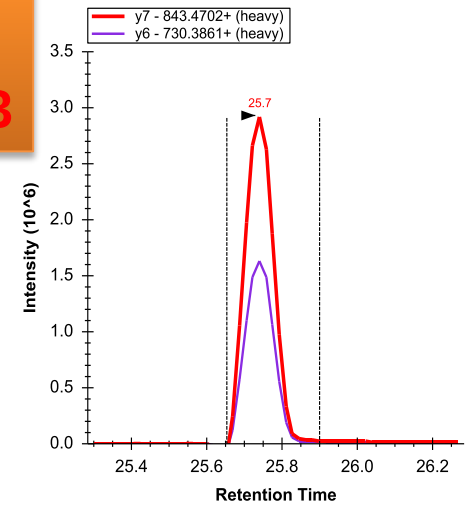
SOS1
WB (×)
Copies/cell: **1306**

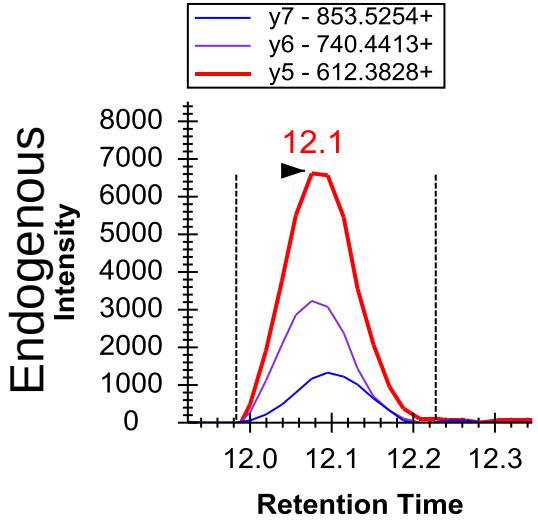
EYIQPVQLR

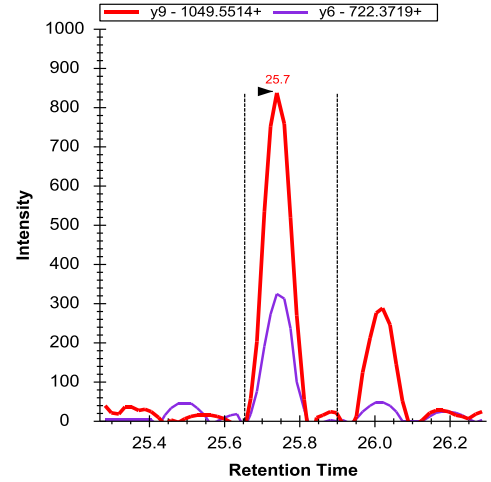


DUSP6
WB (×)
Copies/cell: **103**

DSTNLDVLEEFGIK

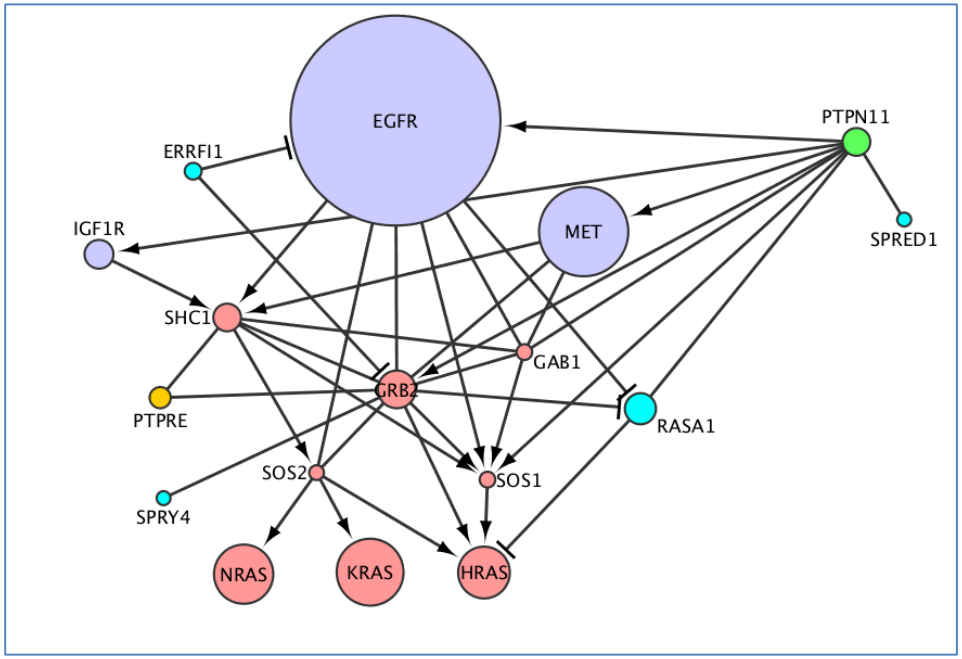




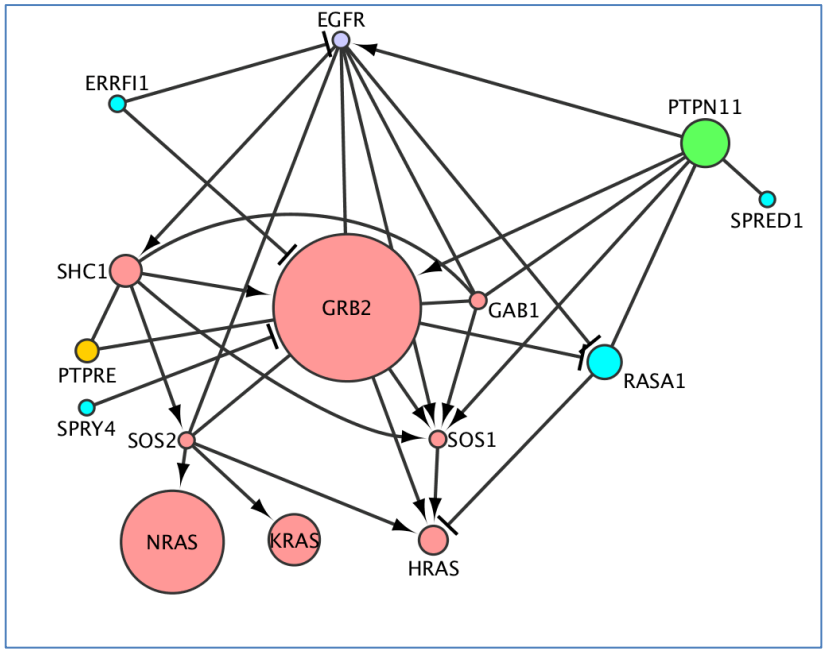


EGFR adaptor layer

HMEC

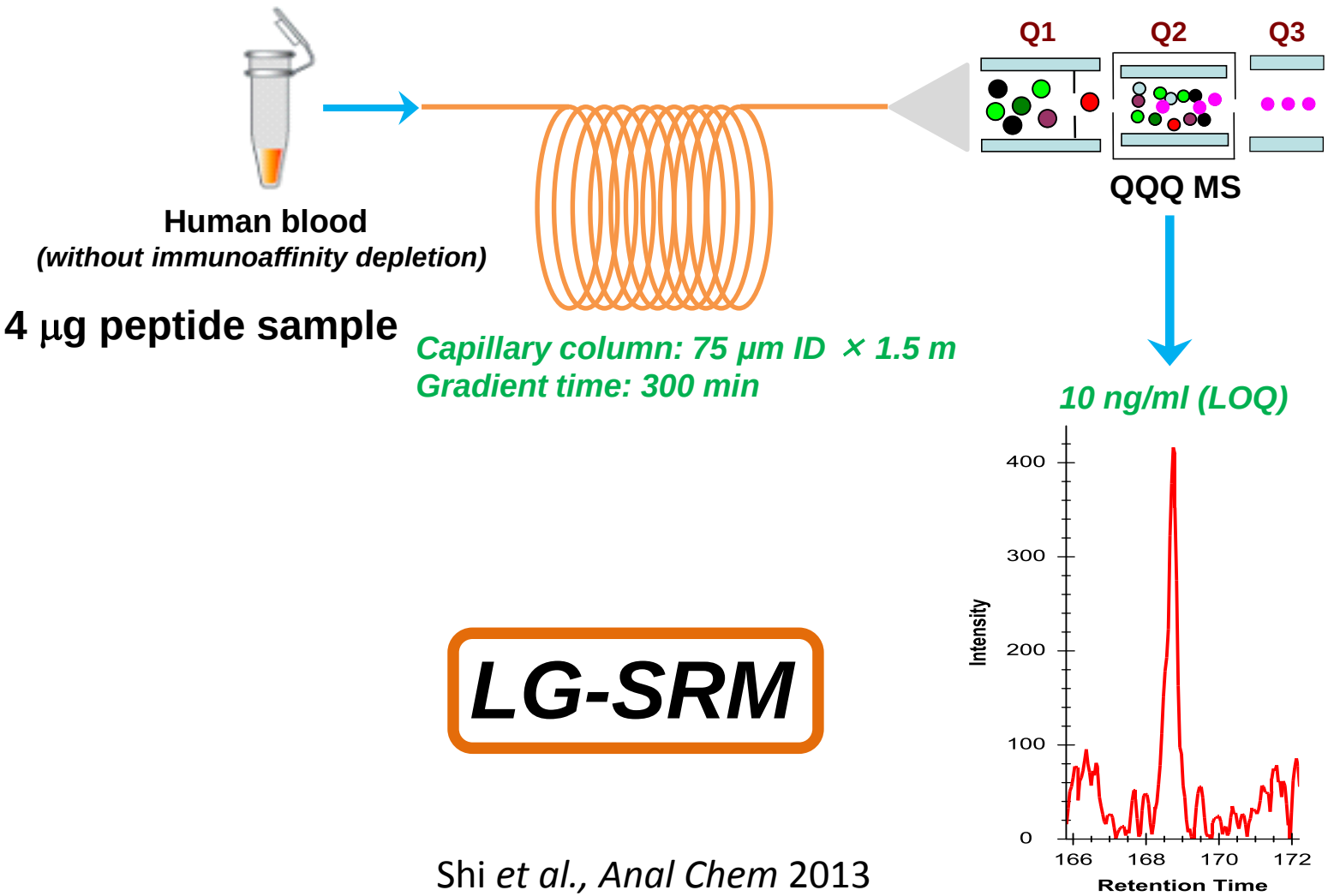


MCF-7



The difference in ratios of receptor and adaptors across different cell types explains vastly different responses of Erk or Akt activation in response to EGF

Long-Gradient (LG)
--- a complementary technique for small clinical samples

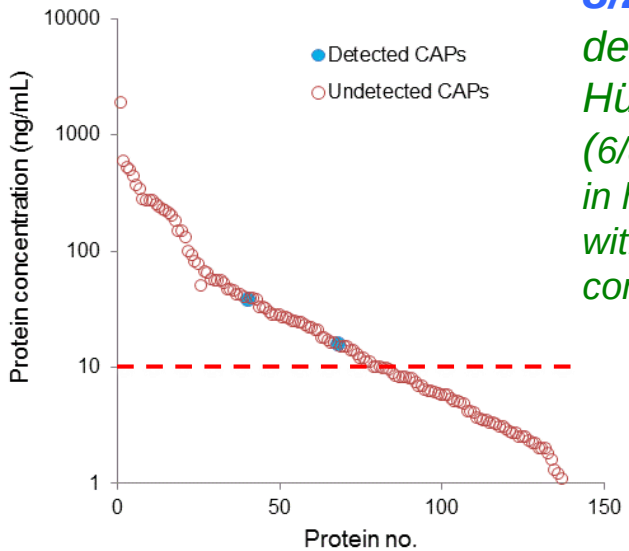


LG-SRM

Shi *et al., Anal Chem* 2013

Quantification of 220 cancer-associated proteins in human serum/urine

LC-SRM

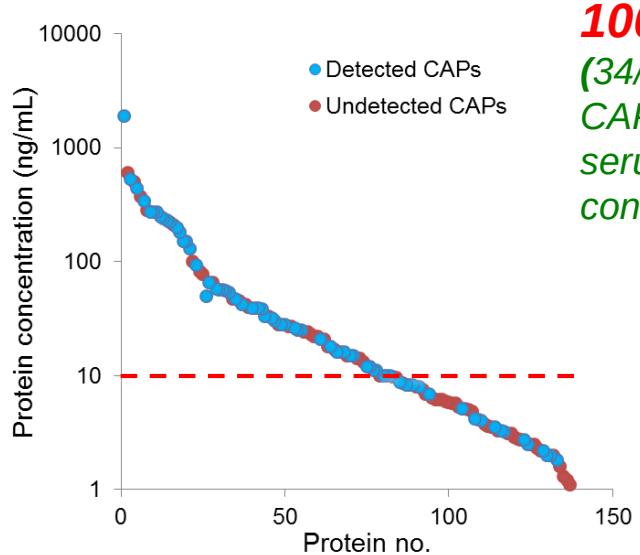


8/220

detected by Hüttenhain et al.

(6/8 detected CAPs in human plasma with unknown concentrations)

LG-SRM



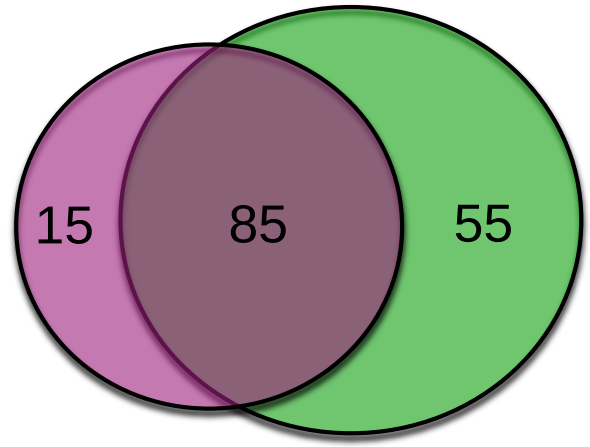
100/220

(34/100 detected CAPs in human serum with unknown concentrations)

Selection criteria for 220 CAPs:

- ❑ Most CAPs (212/220) were not detected by LC-SRM in depleted human plasma
- ❑ Most CAPs (138/220) were reported to have concentrations below 200 ng/mL levels in human plasma/serum

Hüttenhain et al. ***Sci Transl Med*** 2012, 4, 142ra94.



Detected CAPs in human serum

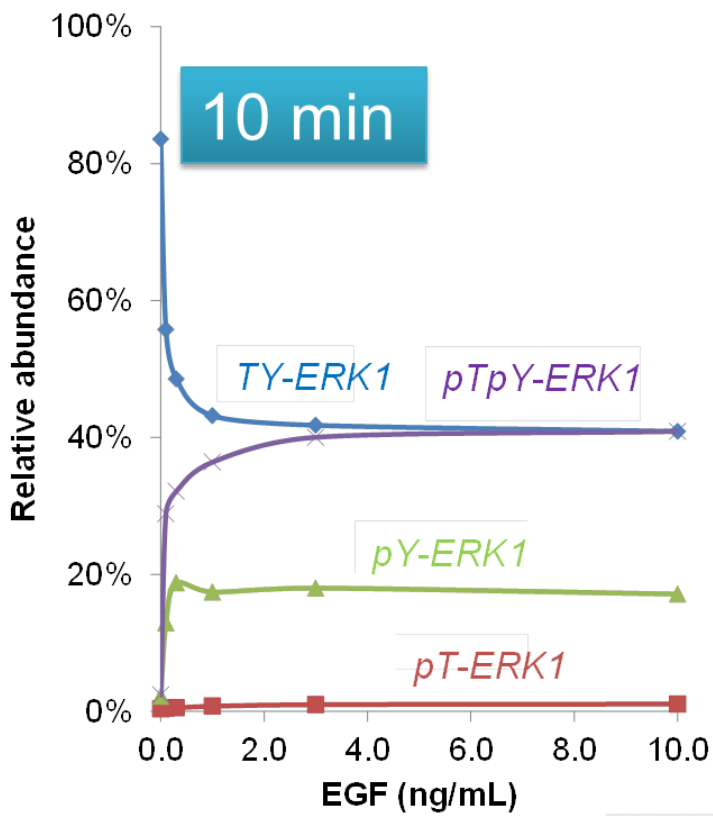
Detected CAPs in human urine

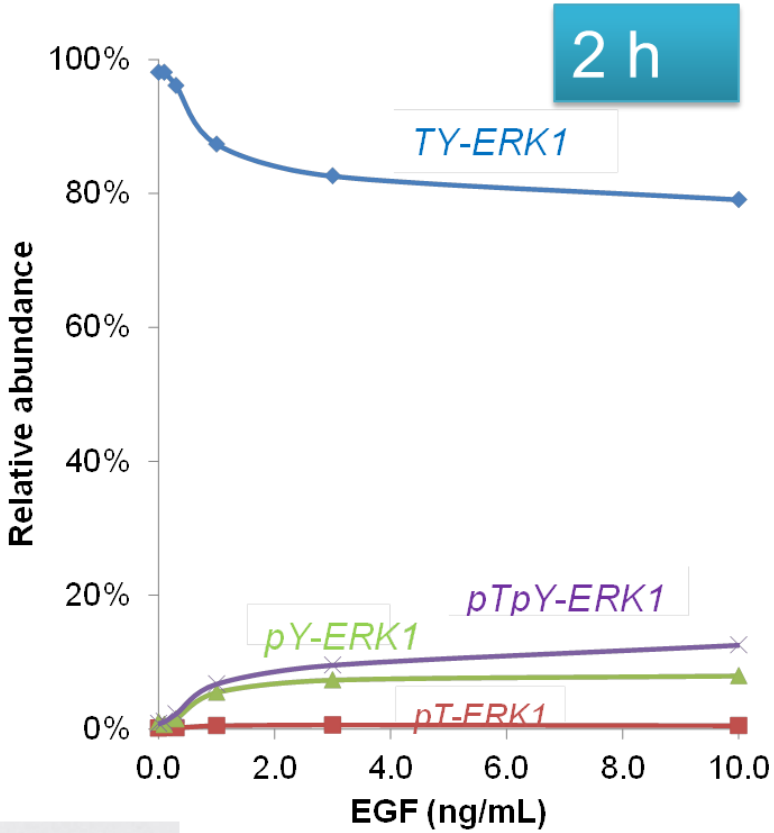
Direct quantification of low-abundance phosphorylation signatures

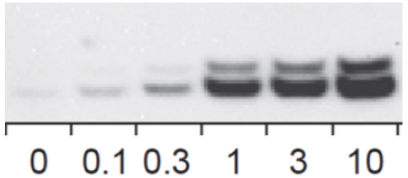
Surrogate peptides for ERK1/2 phosphorylation analysis

Isoform	Standard sequence
ERK1	LADPEHDHTGFLTEYVATR (TY-ERK1)
	LADPEHDHTGFL**pT**EYVATR (pT-ERK1)
	LADPEHDHTGFLTE**pY**VATR (pY-ERK1)
	LADPEHDHTGFL**pT**E**pY**VATR (pTpY-ERK1)
ERK2	VADPDHDHTGFLTEYVATR (TY-ERK2)
	VADPDHDHTGFL**pT**EYVATR (pT-ERK2)
	VADPDHDHTGFLTE**pY**VATR (pY-ERK2)
	VADPDHDHTGFL**pT**E**pY**VATR (pTpY-ERK2)

Direct quantification of ERK phosphorylation stoichiometries

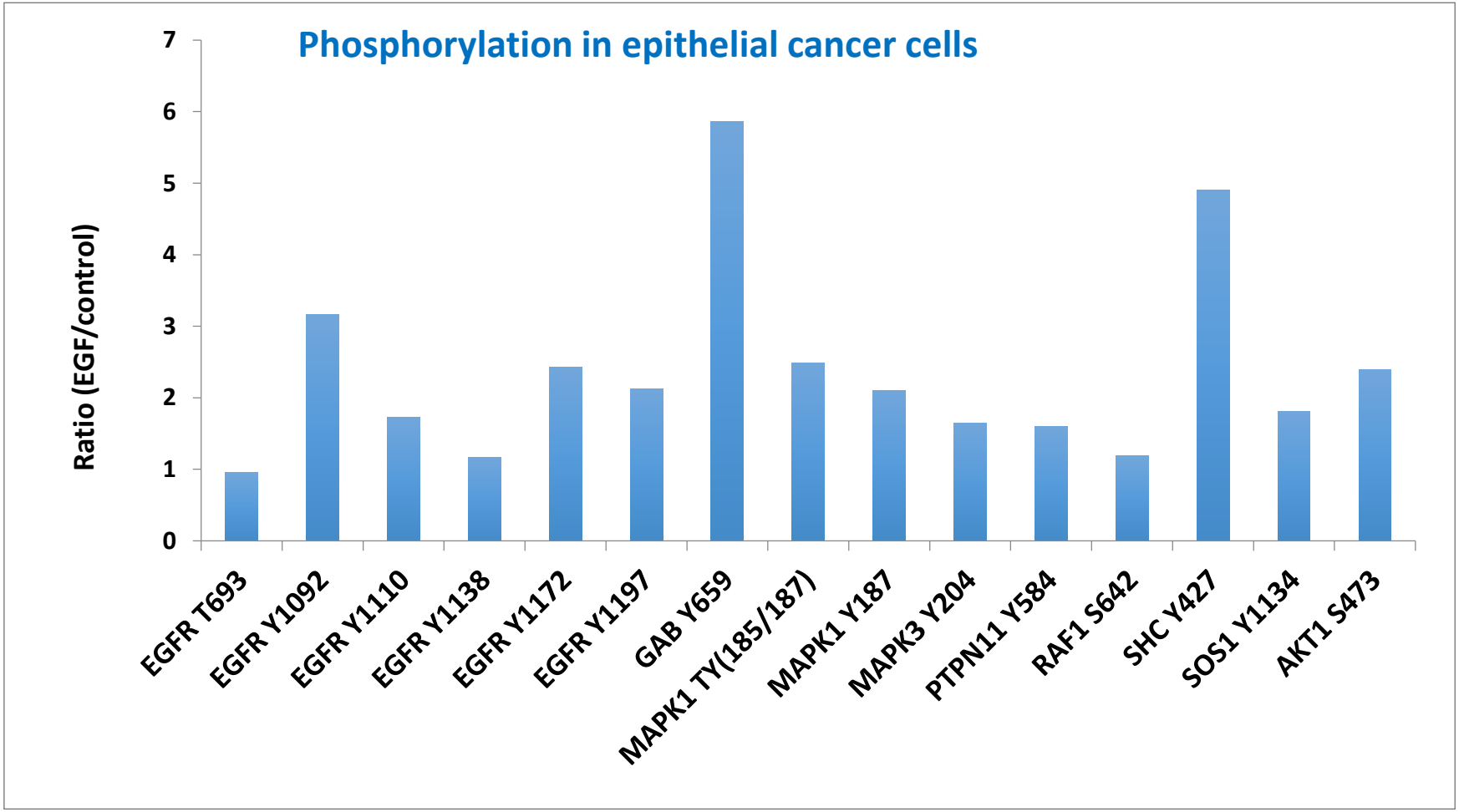






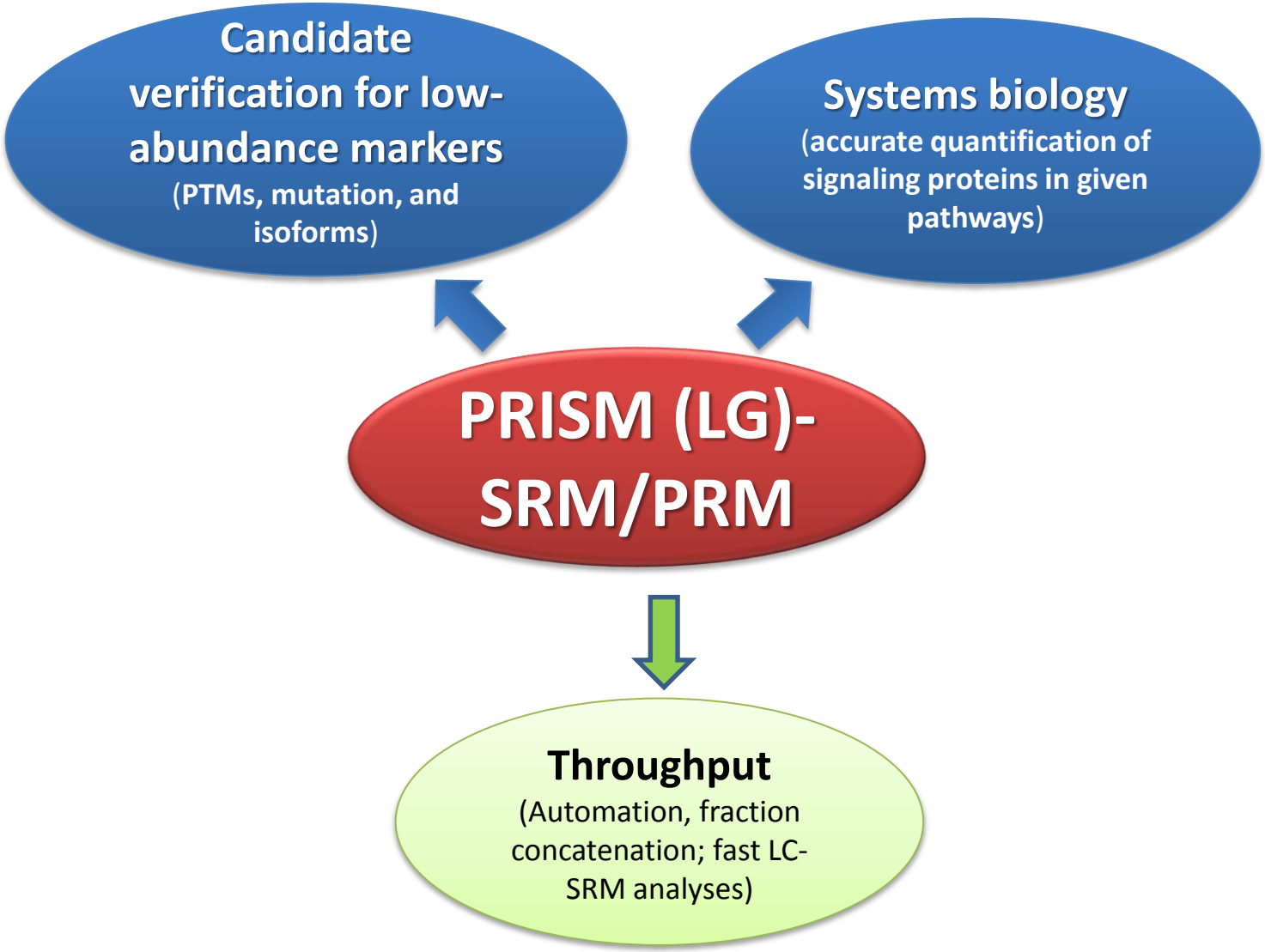
- Maximal ERK activation: **0.3-1 ng/mL** EGF at peak activation (10-min); **3 ng/mL** EGF at steady state (2-h)
- EGF dose at the peak activation close to physiological levels in humans (~**0.5 ng/mL**)

Targeted phosphorylation measurements



Summary

Targeted MS platform	LOQs (starting material)	
	Human plasma/serum	Mammalian cells
Conventional LC-SRM	~1 µg/mL (0.5 µg)	
LG-SRM	~10 ng/mL (4 µg)	
IgY14-LG-SRM	~1 ng/mL (10 µL)	
PRISM-SRM	~1 ng/mL (2 µL)	~100 copies/cell (~25 µg)
		~1000 copies/cell (ERK phosphorylated forms)
IgY14-PRISM-SRM	~50 pg/mL (10 µL)	

- **Front-end separation:** PRISM (LG)-S/PRM or DIA-MS
- **High-throughput PRISM-MS:** effective PRISM fraction multiplexing and sample multiplexing
- **Large-scale targeted quantification:** cellular signaling networks and biomarker pre-verification

Potentials and limitations