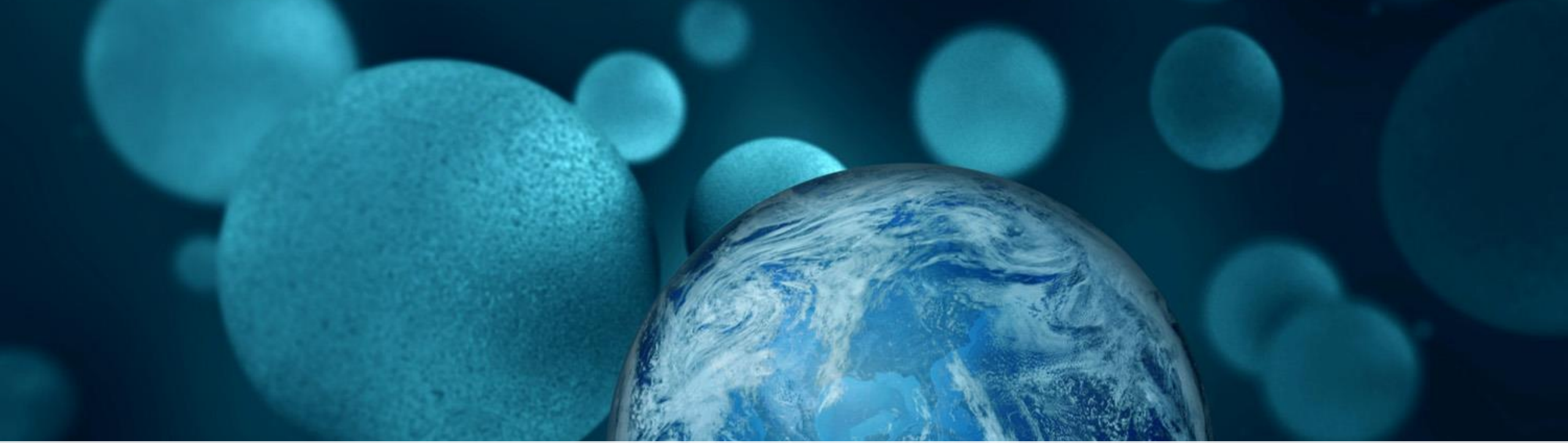


ThermoFisher SCIENTIFIC

Quantitative Analysis of Two Cancer Signaling Pathways Using Multiplex-Immunoprecipitation and Targeted Mass Spectrometry

John C. Rogers, PhD
Senior R&D Manager
Thermo Fisher Scientific

Overview

Background

- Targeted proteomics and mass spectrometry
- Advantages and challenges for targeted mass spectrometry (MS) assays

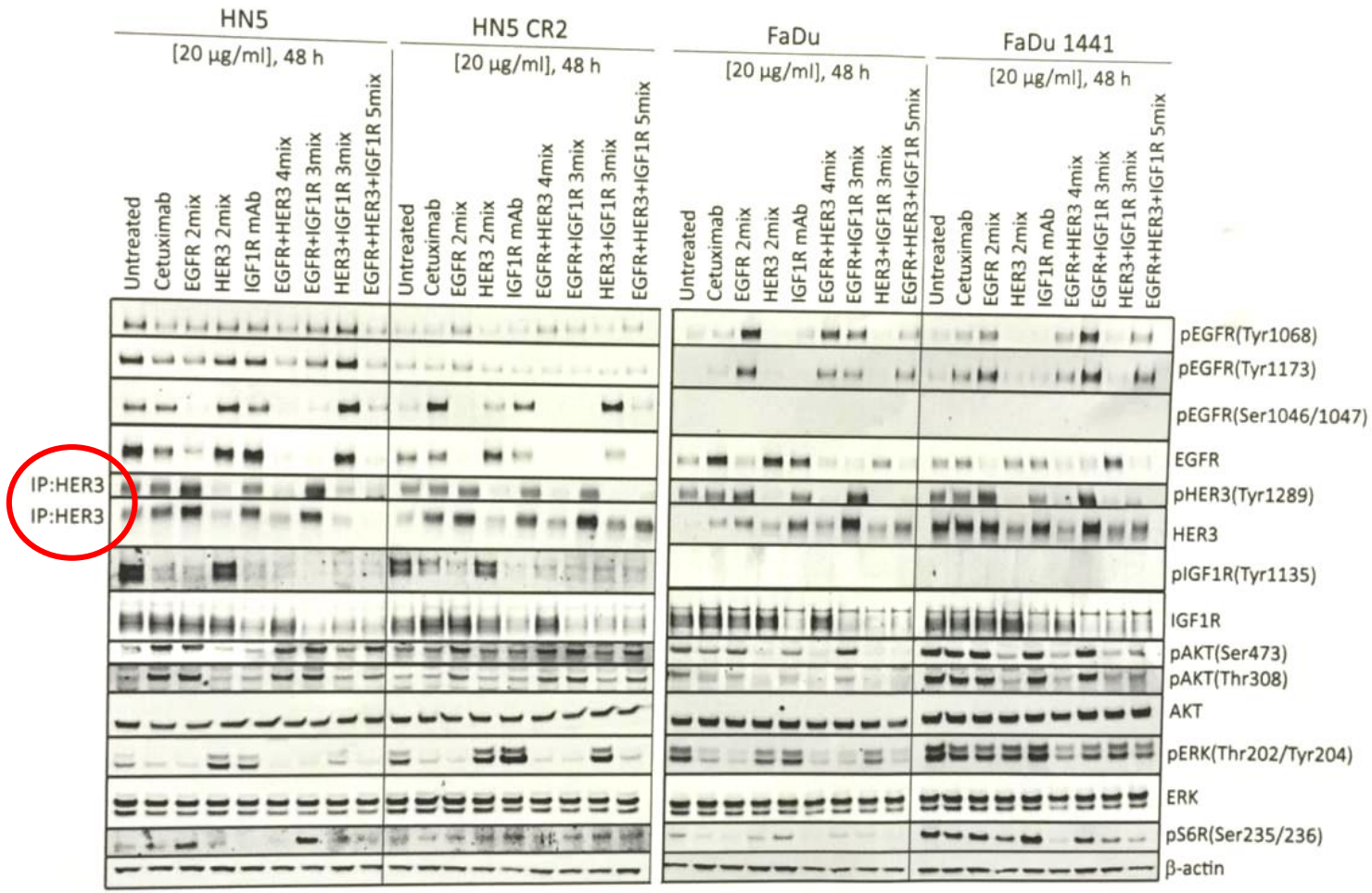
Immunoprecipitation to mass spectrometry (IP-MS)

- Verification of antibody specificity and affinity
- Optimization of target enrichment protocols
- Targeted MS assay development

Quantitation of AKT-mTOR and Ras pathway targets by mIP-tMS assays and benchmarking

How Do Biologists Currently Measure Signaling Proteins?

- **Desire to monitor entire pathways**
- **Low-abundance targets and posttranslational modifications (PTMs)**
- **Many targets**
- **Many conditions**



AACR 2015 Poster Figure

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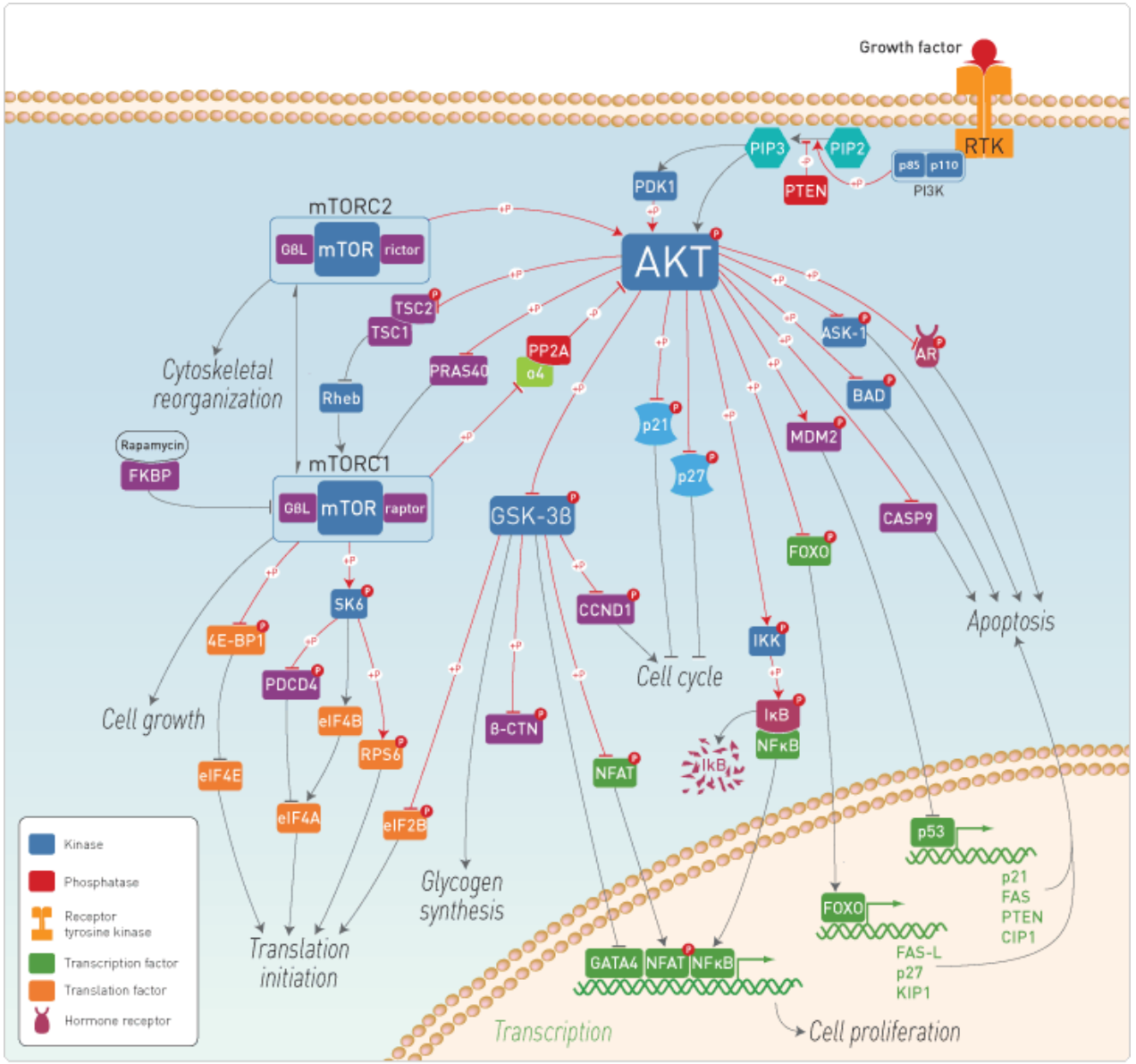
What Does Protein Mass Spectrometry (MS) Offer to Biologists?

- Detect protein isoforms and modifications that cannot be detected by other methods
- Identify and quantitate multiple proteins at a time
- Identify posttranslational modifications and protein interactions
- Can be combined with antibody-dependent methods to improve specificity (immunoprecipitation, flow cytometry)



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What Are the Challenges in MS-based Proteomics?

Challenge:

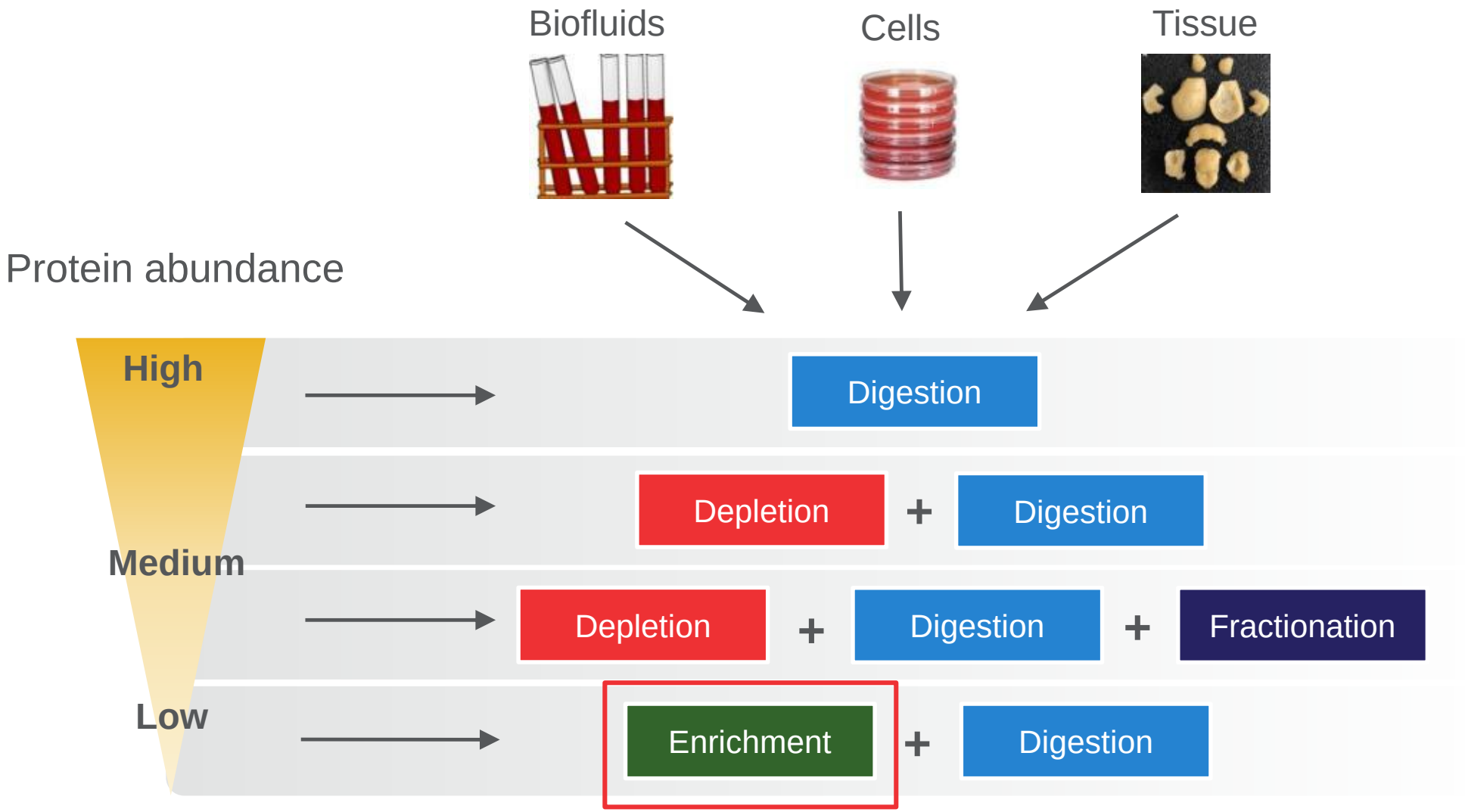
Quantitation of complex samples using MS is challenging due to:

- Wide dynamic range of abundance
- Sensitivity limits of instrumentation
- Ionization suppression
- Missing information for quantitation

Solutions:

- Reduce sample complexity through enrichment (Immunoprecipitation)
- Introduce isotopically labeled internal standards

How Do You Reduce Sample Complexity?



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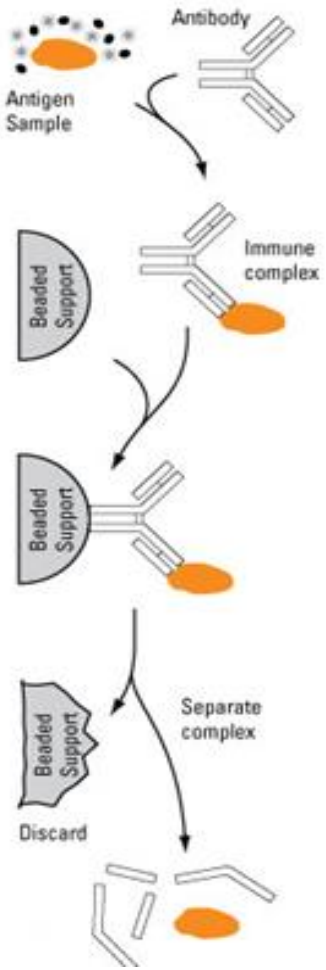
Target-Specific Protein Extraction and Enrichment



Lyse cells

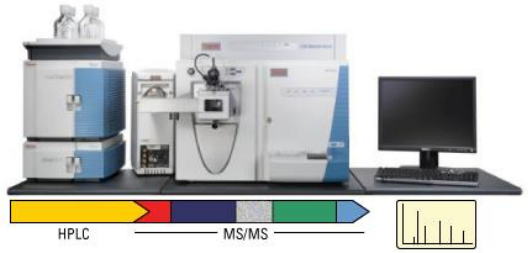
Antigen-Ab complex

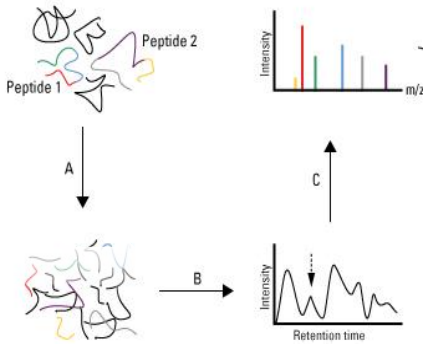
Bind to beads

Wash 3x

Elute

Reduce, alkylate, digest





Target	Cell line	Detected by Q Exactive HF	
		Neat	Enriched-IP
IR	A549	-	+
	HCT116	-	+
IGF1R	A549	+ (4)	+ (22)
	HCT116	-	+
IRS1	A549	-	+
	HCT116	+ (4)	+ (10)
AKT1	A549	-	+
	HCT116	-	+
AKT2	A549	-	+
	HCT116	-	+
PTEN	A549	-	+
	HCT116	-	+
TSC2	A549	-	+
	HCT116	-	+
mTOR	A549	+ (2)	+ (82)
	HCT116	+ (9)	+ (110)
p70S6K	A549	+ (2)	+ (7)
	HCT116	-	+
PRAS40	A549	-	+
	HCT116	+ (2)	+ (8)

Antibody enrichment enhances the detection of targets

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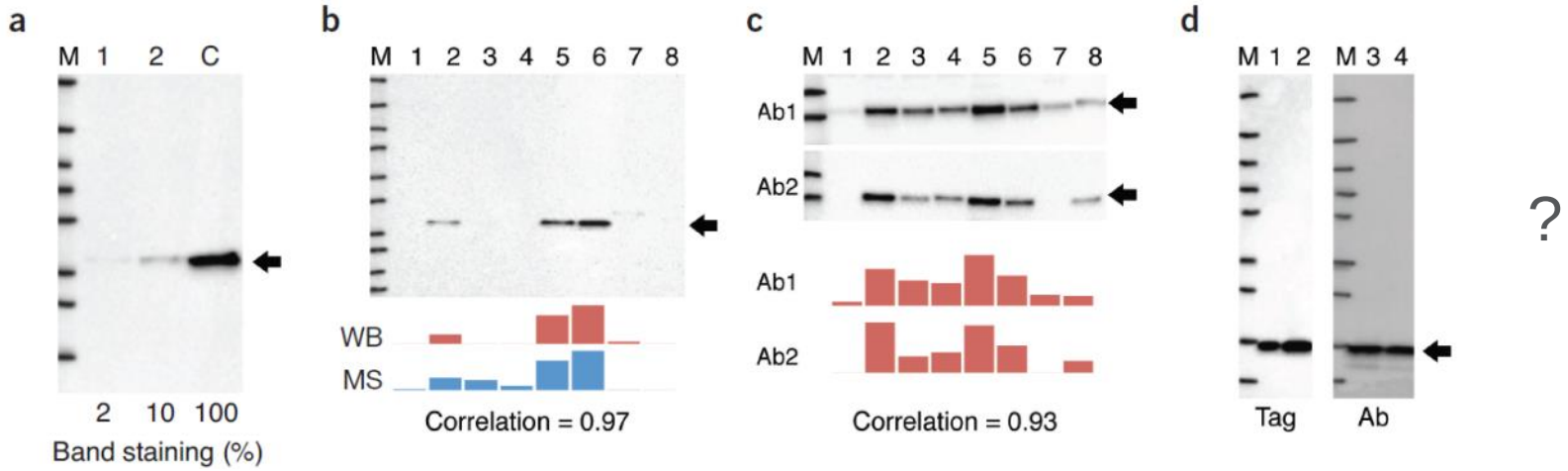
Recommended Antibody Validation* Strategies

A proposal for validation of antibodies

Mathias Uhlen[1], Anita Bandrowski[2], Steven Carr[3], Aled Edwards[4], Jan Ellenberg[5], Emma Lundberg[1], David L Rimm[6], Henry Rodriguez[7], Tara Hiltke[7], Michael Snyder[8] & Tadashi Yamamoto[9]

We convened an *ad hoc* International Working Group for Antibody Validation in order to formulate the best approaches for validating antibodies used in common research applications and to provide guidelines that ensure antibody reproducibility. We recommend five conceptual 'pillars' for antibody validation to be used in an application-specific manner.

Nat Methods. 2016. doi:10.1038/nmeth.3995



?

Methods: Genetic Orthogonality Correlation Tagging IP-MS

*The use or any variation of the word "validation" refers only to antibodies that were subject to functional testing to confirm that a biological target can be appropriately recognized and can be used with the research techniques indicated. The use or any variation of the word "validation" does not ensure that the product was validated to fulfill defined user needs and intended uses.

Only IP-MS can identify off-targets, interacting proteins, and protein modifications

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Overview

Background

- Targeted proteomics and mass spectrometry
- Advantages and challenges for targeted MS assays

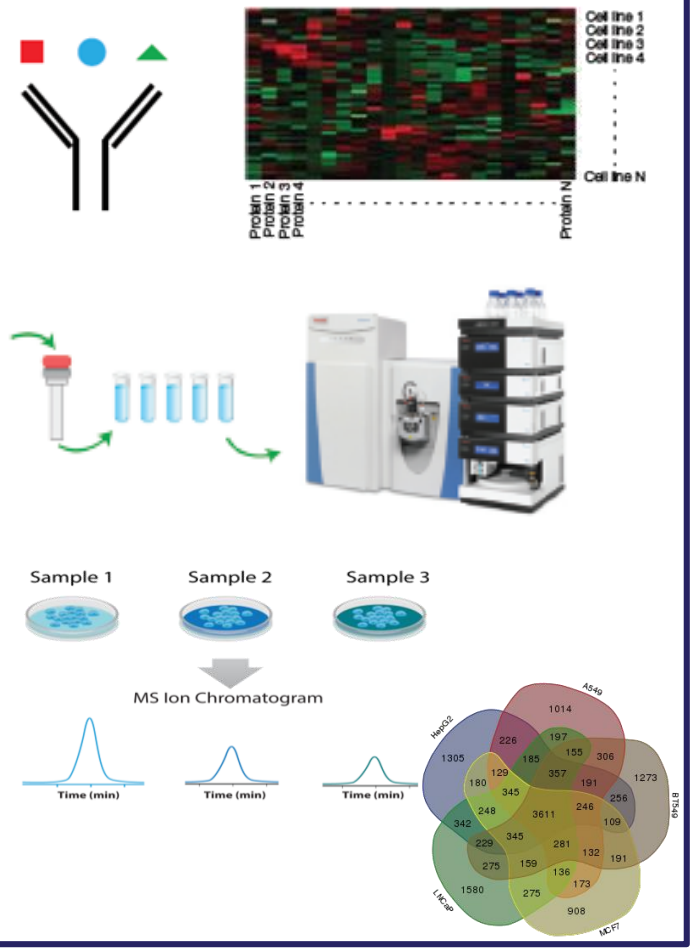
Immunoprecipitation to mass spectrometry (IP-MS)

- Verification of antibody specificity and affinity
- Optimization of target enrichment protocols
- Targeted MS assay development

Quantitation of AKT-mTOR and Ras pathway targets by mIP-tMS assays and benchmarking

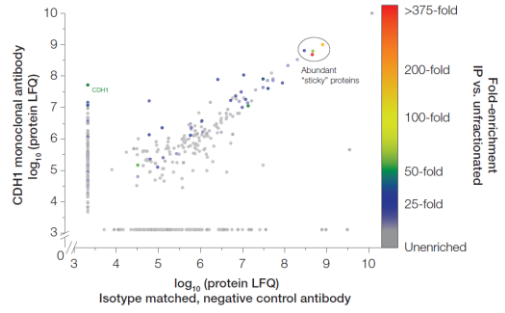
Workflow for Antibody Verification by IP-MS

Select targets, cell lines, and antibodies

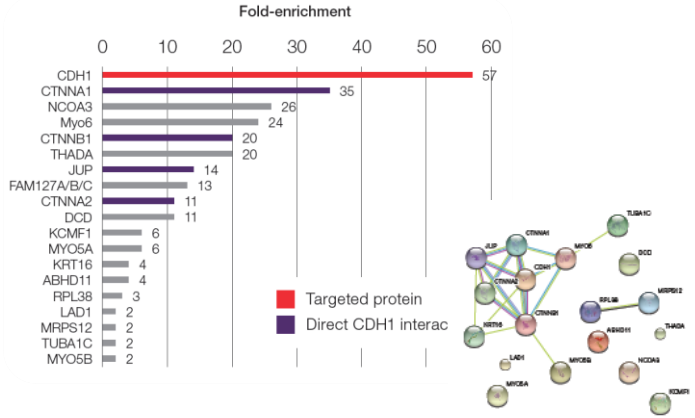


Immuno-capture and LC-MS analysis

Data analysis



$$\text{Fold enrichment} = \frac{\left(\frac{\text{Target protein abundance in IP}}{\text{Total protein abundance in IP}}\right)}{\left(\frac{\text{Target protein abundance in whole lysate}}{\text{Total protein abundance in whole lysate}}\right)}$$



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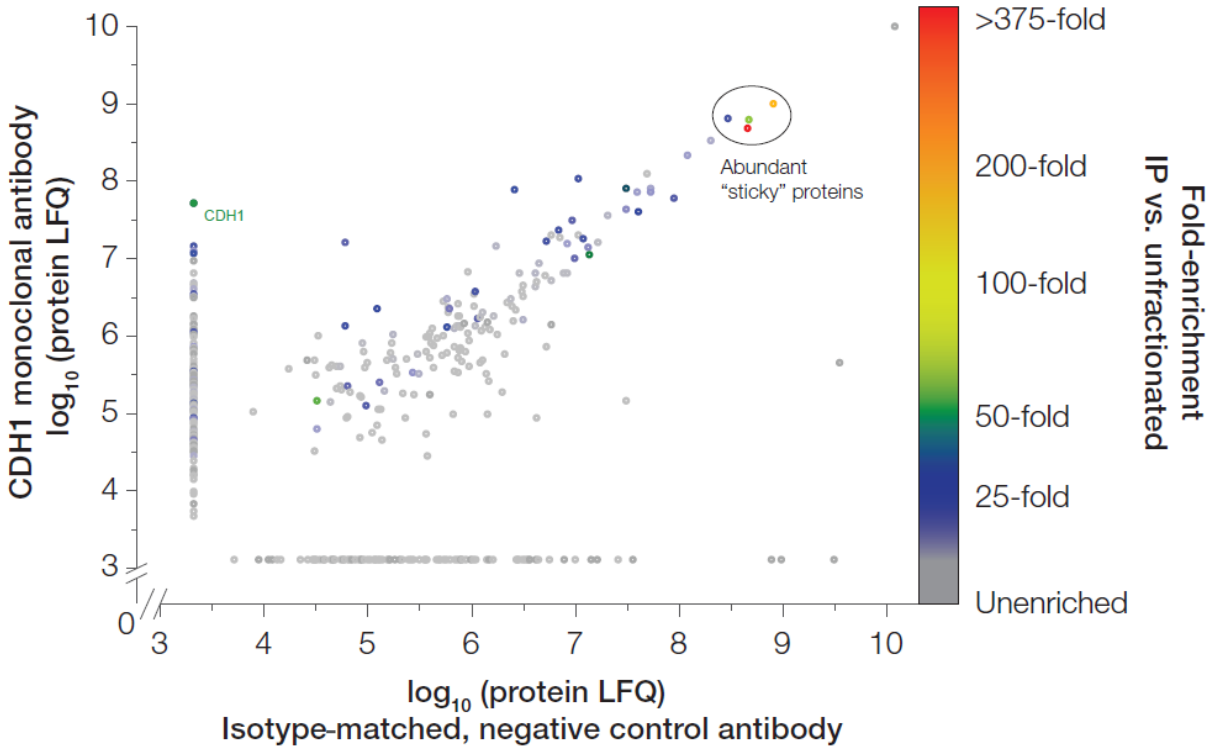
Fold-Enrichment Calculation Ranks Identified Proteins

$$\text{Fold enrichment} = \frac{\left(\frac{\text{Target protein abundance in IP}}{\text{Total protein abundance in IP}}\right)}{\left(\frac{\text{Target protein abundance in whole lysate}}{\text{Total protein abundance in whole lysate}}\right)}$$



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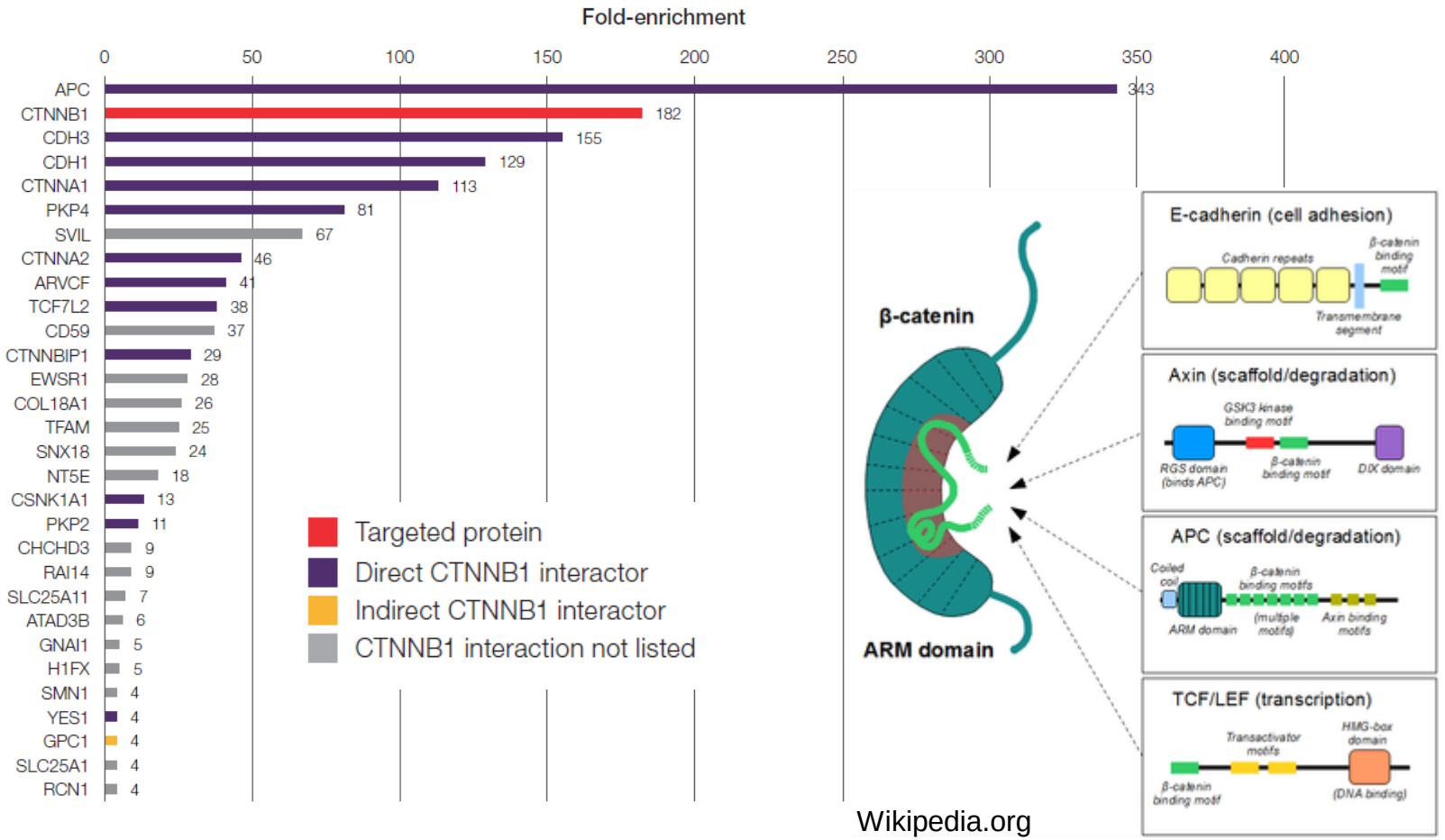
Cross-Verification of IP-MS Results



Wikipedia.org

β-catenin antibody captures cadherins and many native interactors

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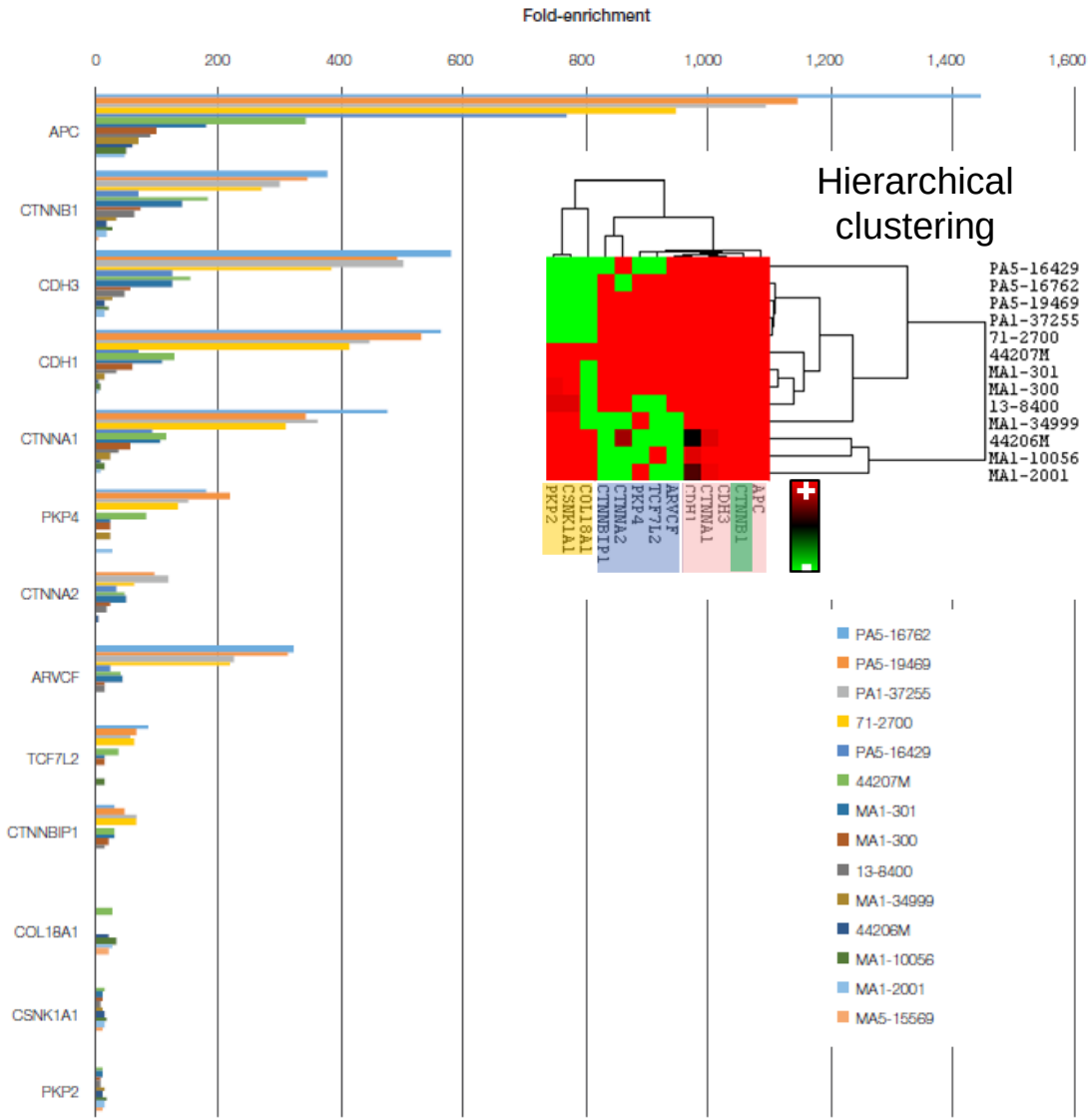
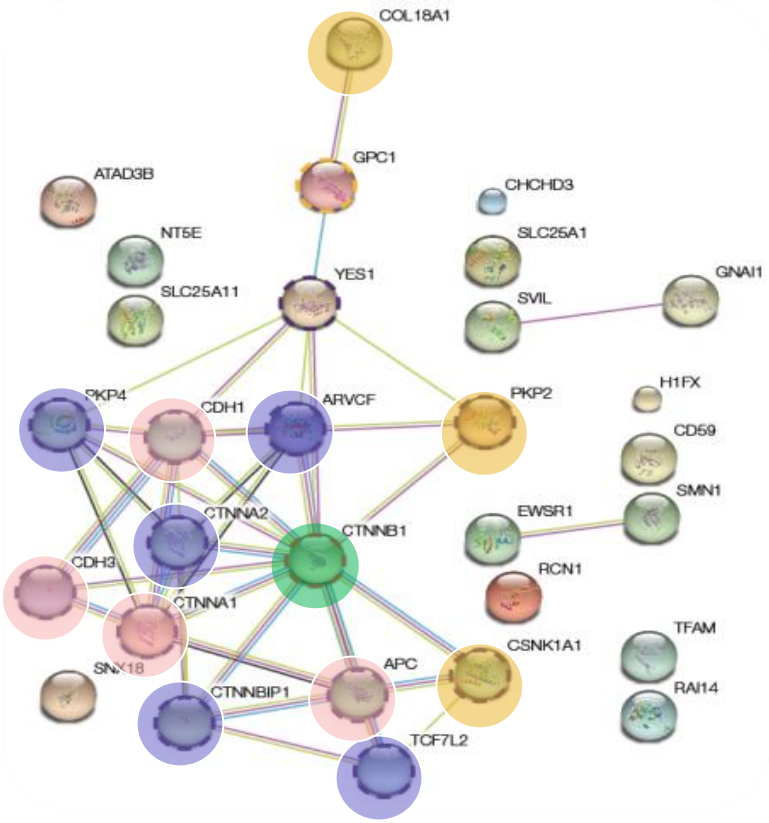
Comparison of β-Catenin Interactors Across Antibodies





- Comparison across antibodies verifies co-IP results
- Clusters of interacting proteins suggest unique complexes or epitopes

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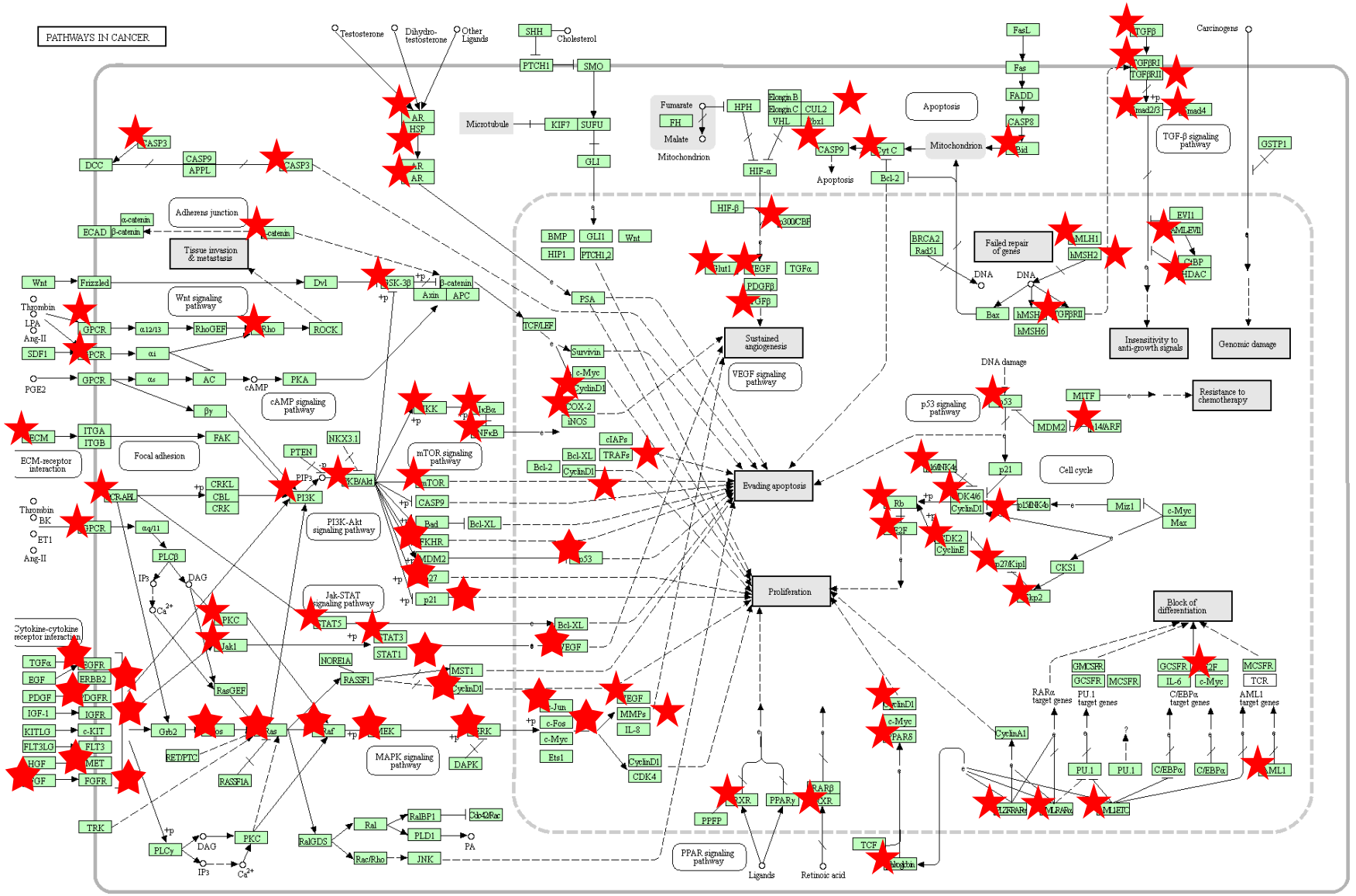
Broad Coverage of Known Cancer Signaling Pathway Proteins



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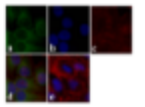
Summary of IP-MS Antibody Verification Efforts

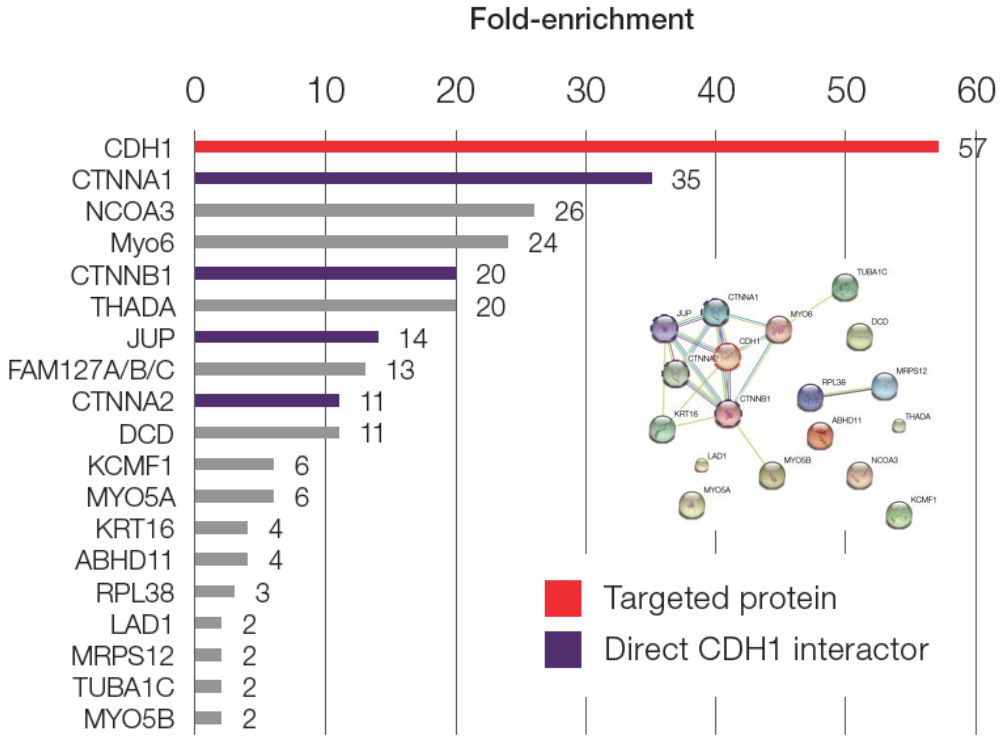
- **Over 1,000 antibodies** screened to >250 signaling pathway targets
- IP-MS verifies the antibody target and provides additional information about antibody performance
- Fold-enrichment provides a simple way to verify antibody performance, assess interactions, and identify off-targets

AKT Pan Polyclonal Antibody | Target Verified by Mass Spectrometry | Cat #: 44-609G, 200 µL

Host	Target Species	Applications	Conjugates	References	Price (USD)
Rabbit	Human, Mouse	Flow, ICC, IF, IHC (P), WB ...	Unconjugated	7	309.00



7 images



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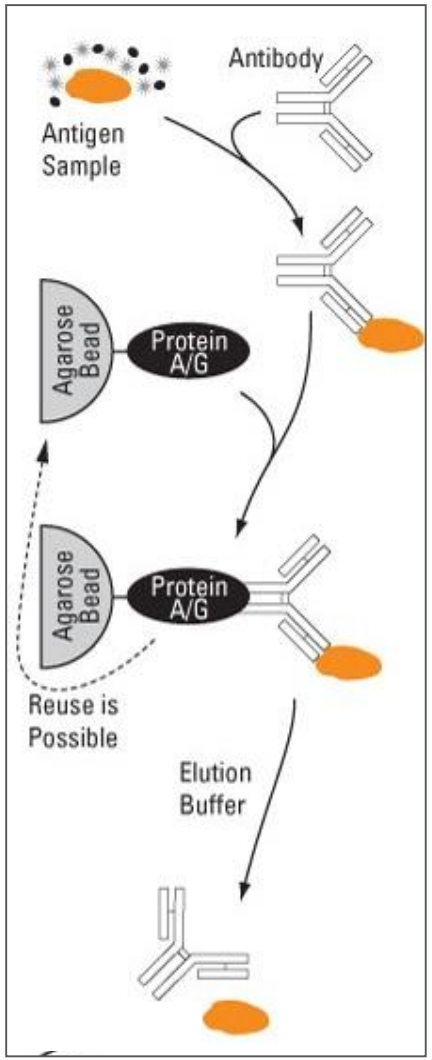
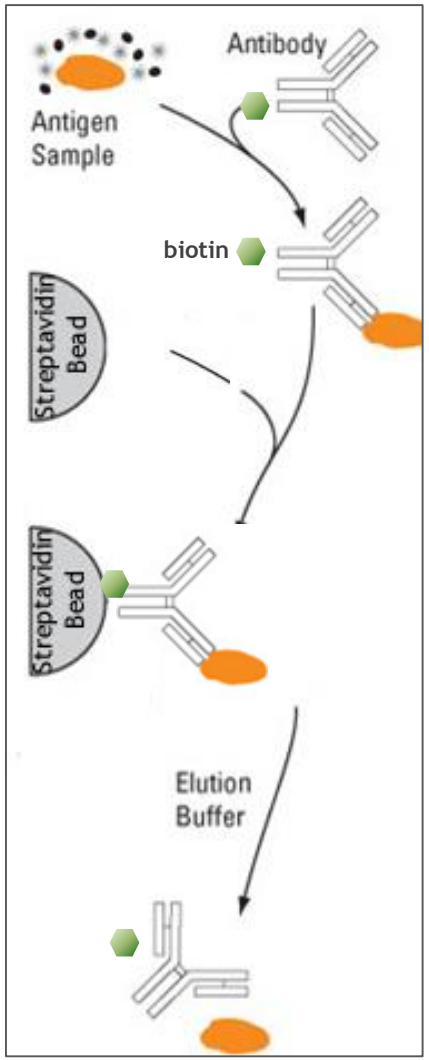
Protein Immunoprecipitation (IP) Methods

Protein A/G IP



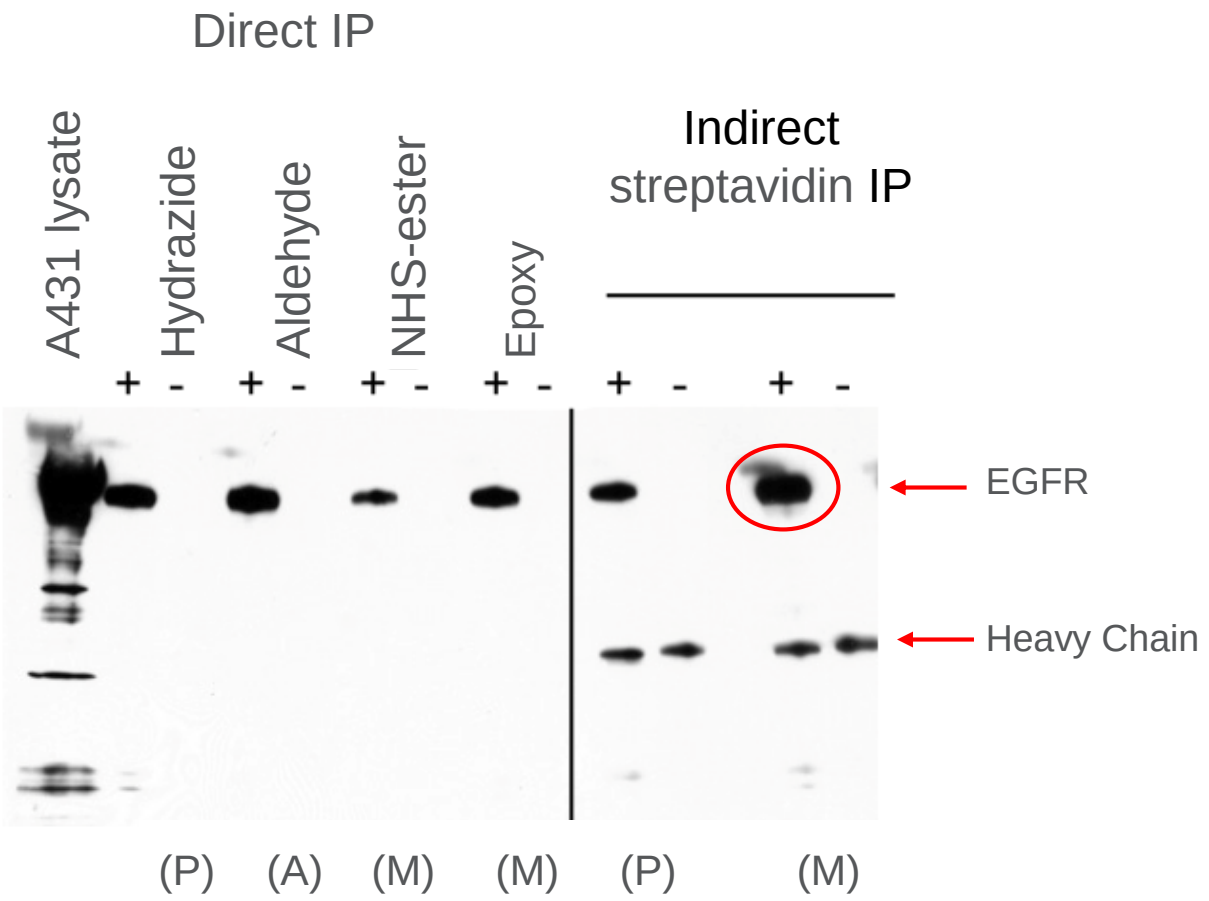
Streptavidin IP



Protein A/G IP	Streptavidin IP
Antibodies with any formulations (BSA)	Requires biotinylated antibody
Best for screening multiple antibodies	Best for protein A/G-verified antibody with no carrier (BSA)
Not suited for biological fluids or vascular tissues	Cells, tissue, and biological fluids

Agarose	Magnetic
Higher capacity	Small-scale enrichment
Low throughput	Easy to handle, automatable

Evaluation of Different Resins for Protein Immunoprecipitation (IP)



P: Polyacrylamide, **A**: Agarose, **M**: Magnetic

+: Anti-EGFR IP, **-** : Rabbit IgG IP

Comparison of Direct and Indirect IP Methods

Success criteria:

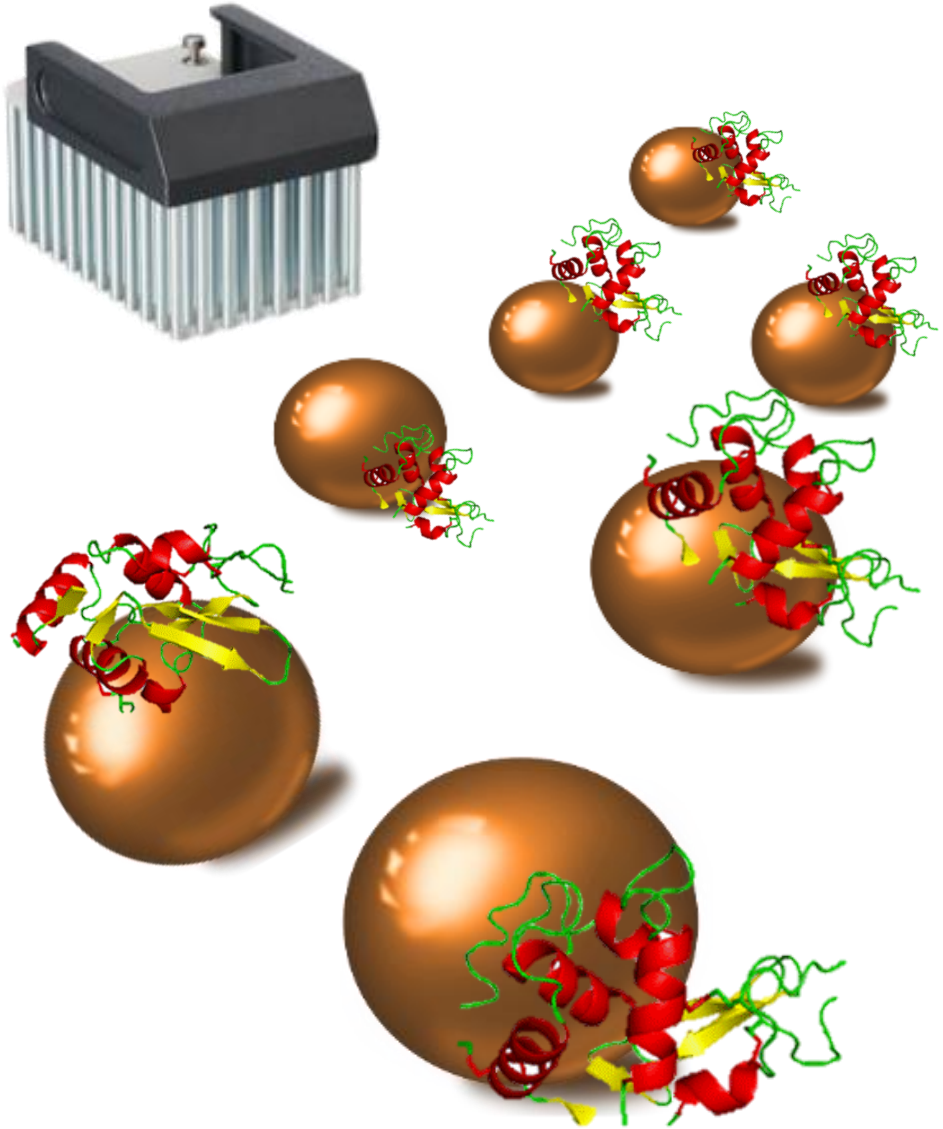
- **# proteins identified <60**
- **EGFR % sequence coverage >60%**

In-solution LC-MS/MS results

	Resin	Anti-EGFR		Rabbit IgG	
		# proteins identified	EGFR % sequence coverage	# proteins identified	EGFR % sequence coverage
Direct IP	Hydrazide (P)	101	79	129	1
	Aldehyde (A)	108	80	157	8
	NHS-Ester (M)	98	57	97	0
	Epoxy (M)	53	71	60	3
Indirect IP	Streptavidin (P)	19	45	14	0
	Streptavidin (M)	38	71	40	2

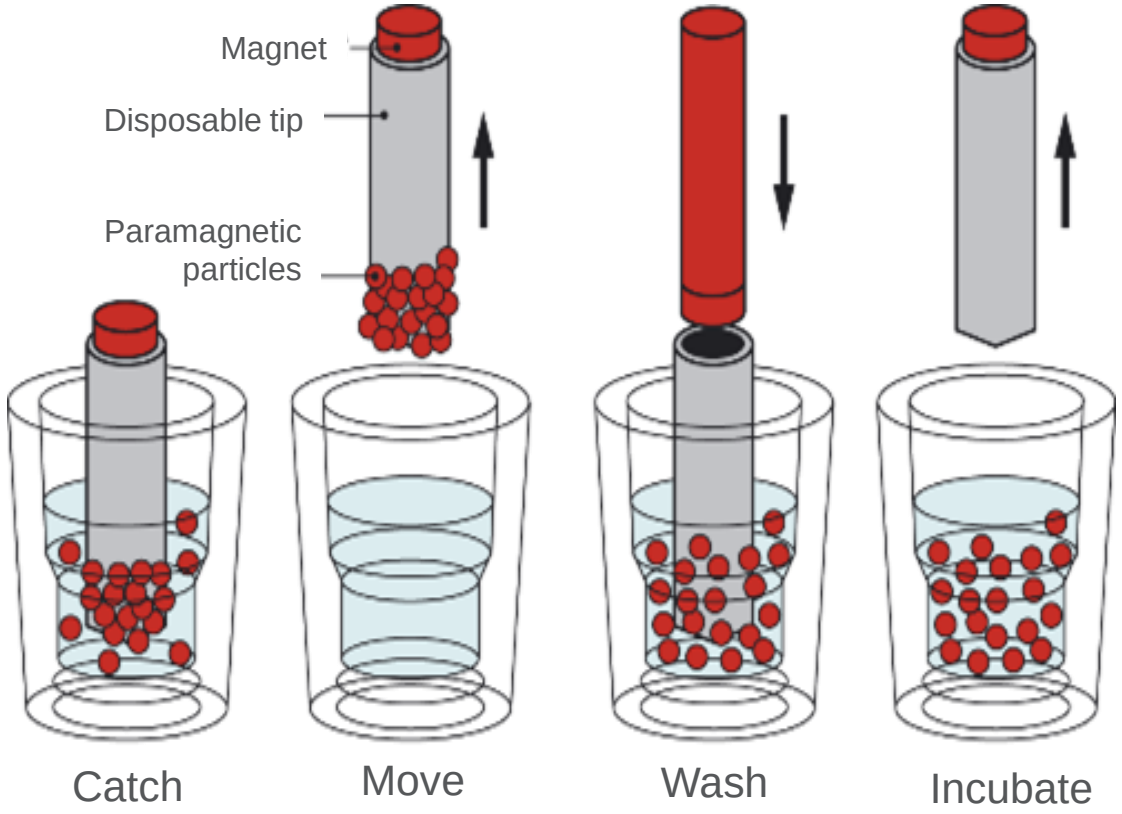
Advantages of Using Magnetic Beads

- Large surface = efficient capture
- Can be used for samples with different viscosities
- Possible to concentrate the sample
- Easy to automate
- Suitable for low to high throughput applications



Overview of the Thermo Scientific™ KingFisher ™ Technology

Magnetic beads are transferred in the process



Thermo Scientific™ Pierce™ magnetic beads and kits

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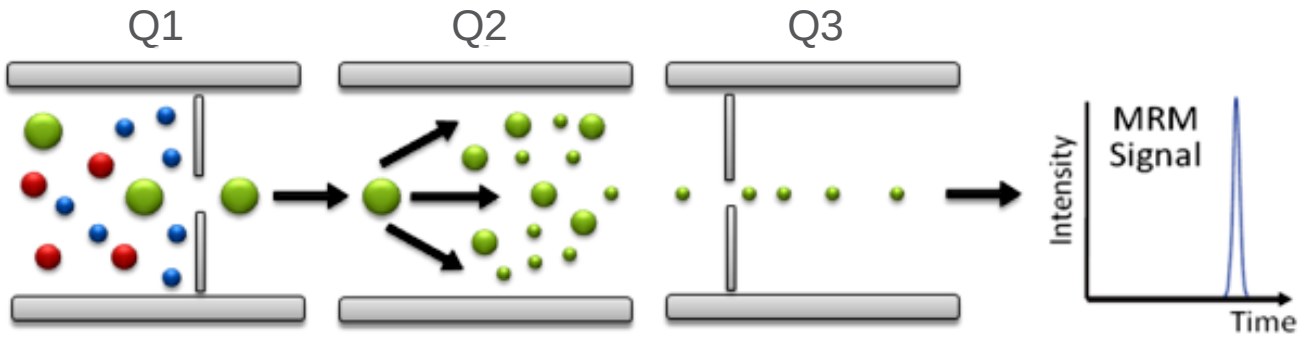
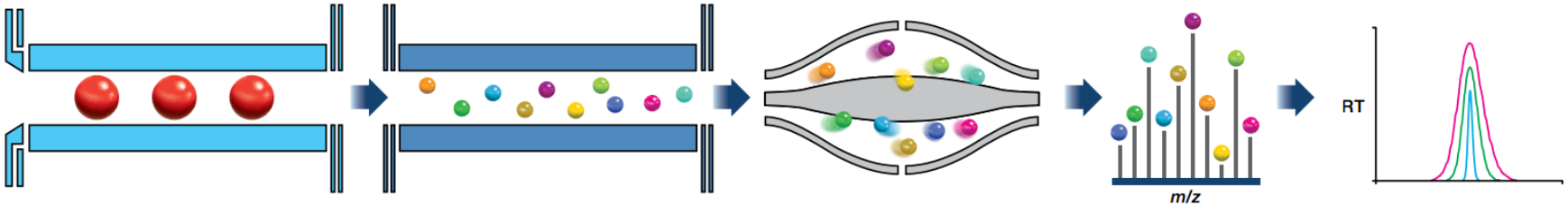
Targeted MS Methods

Selected reaction monitoring (SRM) with a triple quadrupole (QQQ) mass spectrometer



Parallel reaction monitoring (PRM) with the Thermo Scientific™ Q Exactive™ hybrid quadrupole-mass spectrometer



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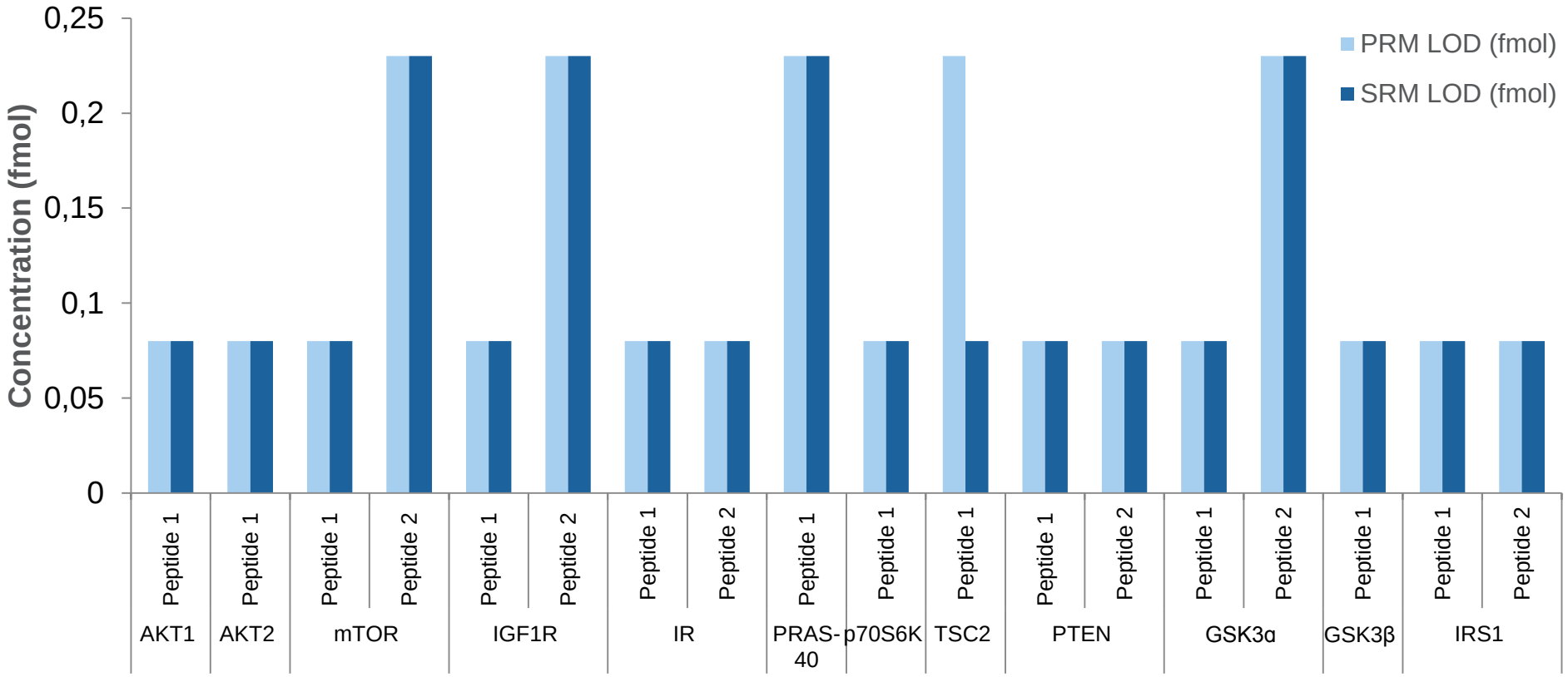
Targeted MS Assays Enable Absolute Quantitation



- **All 12 target peptides were monitored with linear quantitation and 2-3 orders of magnitude**
- **PRM and SRM methods provided essentially the same level of sensitivity**

Overview

Background

- Targeted proteomics and mass spectrometry
- Advantages and challenges for targeted MS assays

Immunoprecipitation to mass spectrometry (IP-MS)

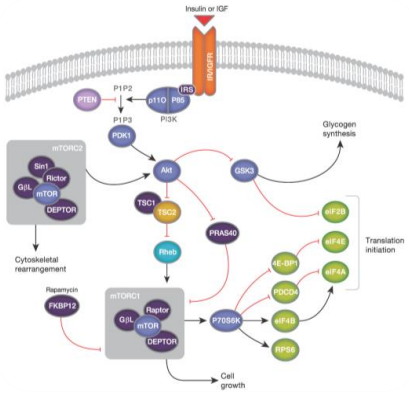
- Verification of antibody specificity and affinity
- Optimization of target enrichment protocols
- Targeted MS assay development

Quantitation of AKT-mTOR and Ras pathway targets by mIP-tMS assays and benchmarking

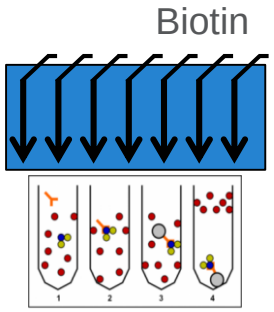
Workflow for Antibody Verification by IP-MS

Total and phosphopeptide pathway profiling

Akt/mTOR & Ras/MAPK pathway targets in cells and tissues

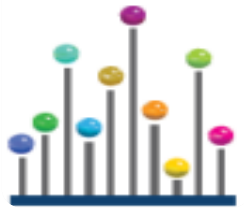


Multiplexed protein immuno-capture

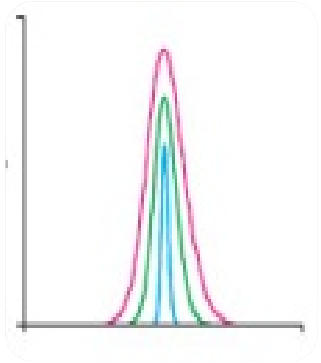


Validated antibodies and peptides in automated magnetic bead workflows

LC-MS analysis



Multiplexed-directed discovery or targeted PRM/SRM quantification



Skyline

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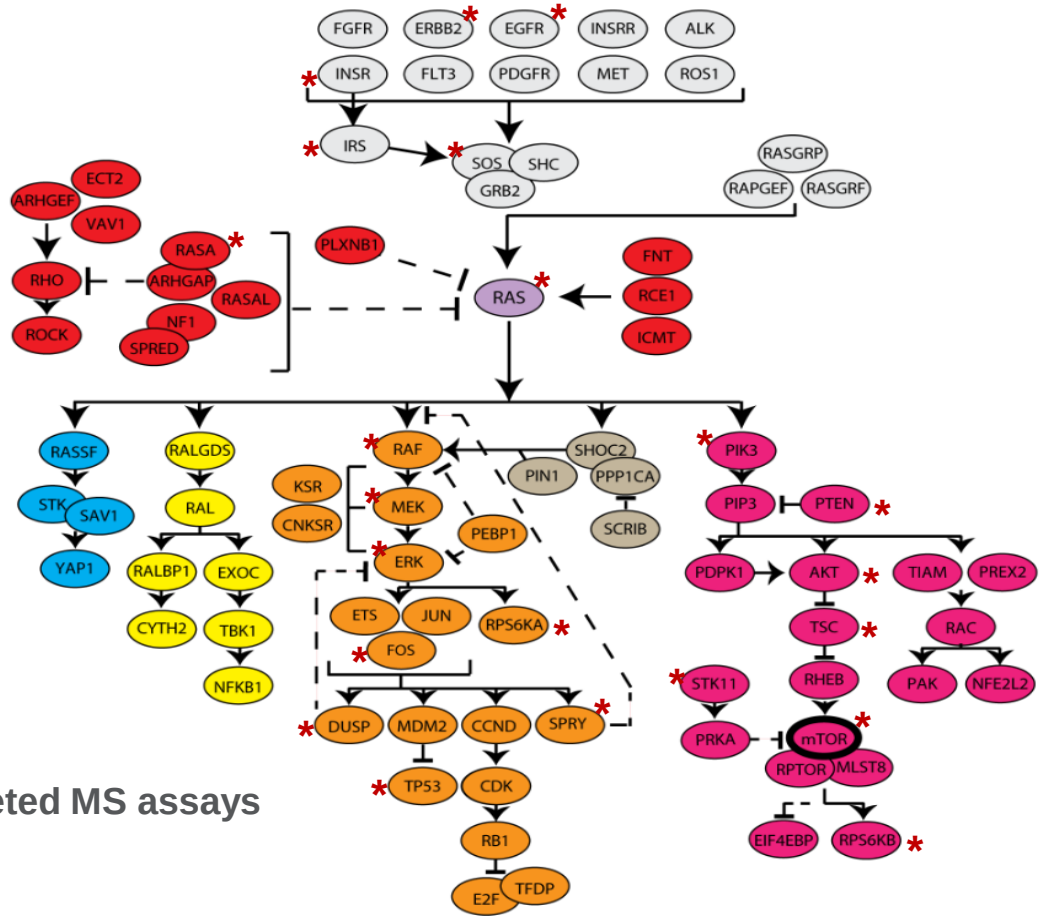
Enrichment of AKT-mTOR and RAS/ERK Pathway Targets

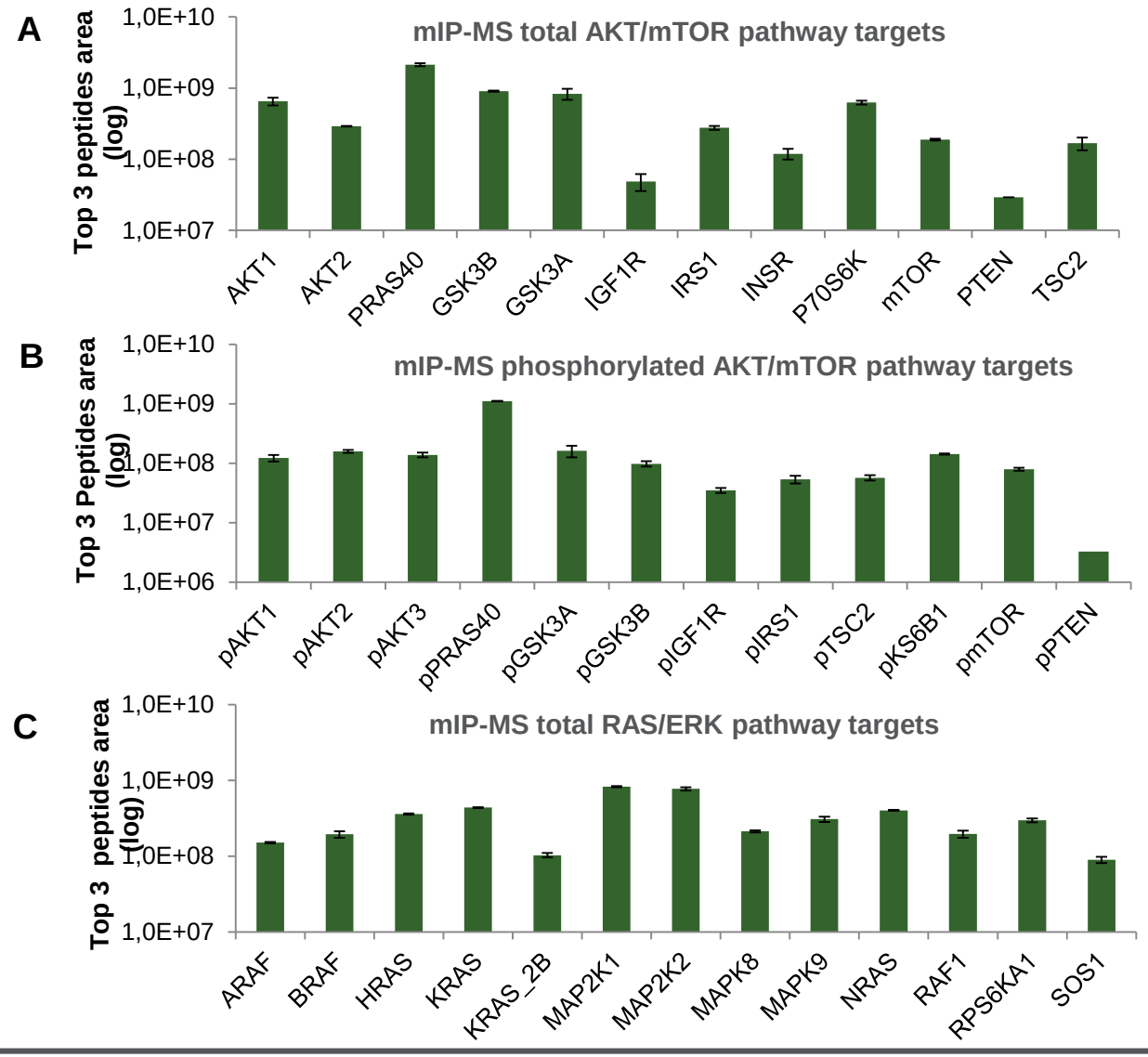


*** Targeted MS assays**

https://www.addgene.org/cancer/ras-pathway/
Dominic Esposito and Frederick National Laboratory for Cancer Research

IP antibody	Target	# of unique peptides	Relevant phospho sites
Phospho AKT	AKT1	20	Ser473
	AKT2	14	Ser474
	AKT3	13	-
AKT1	AKT1	12	-
	AKT2	11	-
PRAS40	PRAS40	8	Thr246
Phospho PRAS40	PRAS40	6	Thr246
Phospho mTOR	mTOR	82	Thr2446, Ser2448
	RICTOR	2	-
	SIN1	3	-
	Gbl	4	-
Pan Ras	HRAS	15	-
	KRAS	13	-
	NRAS	14	-
PIK3R2	PIK3R2	32	Ser262, Ser263
	PIK3CA	29	-
	PIK3CB	30	-
RAF1	RAF1	42	Ser259, Ser621
RSK1	RPS6KA1	60	Ser221, Ser363

Multiplex IP-MS for Total and Phospho-AKT/mTOR and RAS/ERK Pathway Targets



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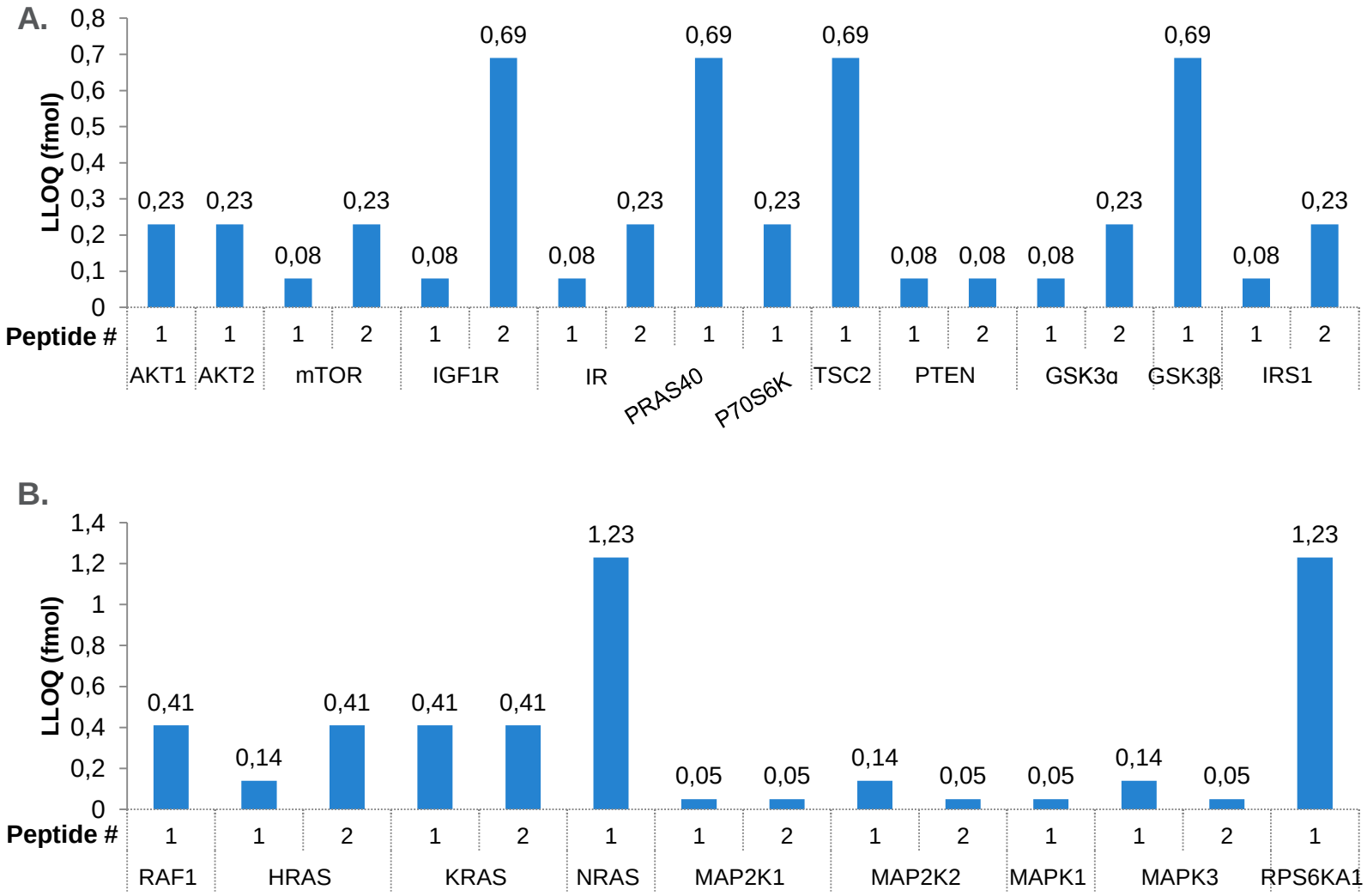
PRM Quantitation Limits of Peptides for AKT-mTOR and RAS/ERK Pathway Proteins



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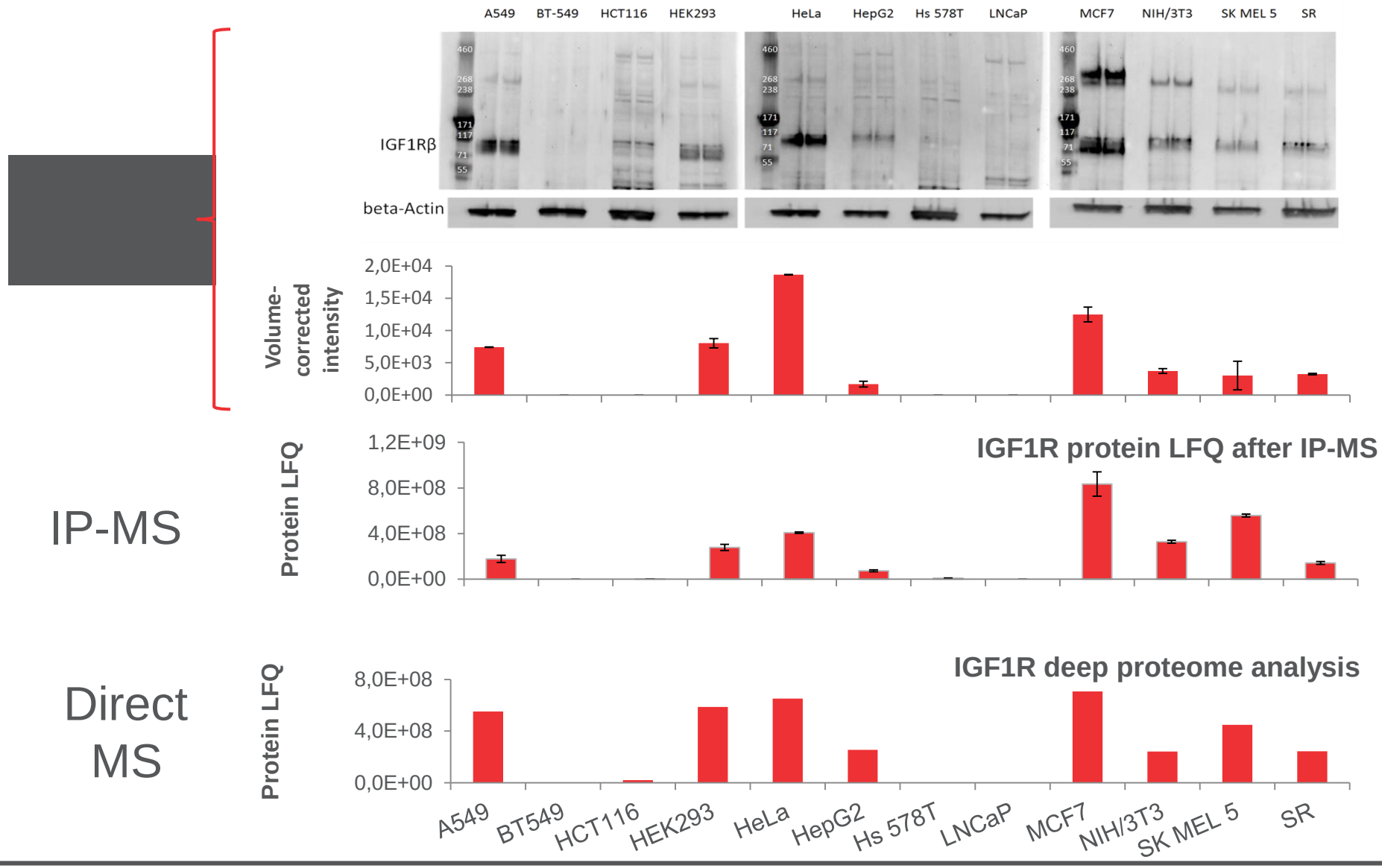
IP-MS Enriches Targets, Interactors, and Modifications

IP antibody	Antibody #	Target	Cell line	IP enriched # of unique peptides		Relevant phosphopeptide ID
				Nest	Enriched-IP	
CTNNB1	4407M	CTNNB1	HCT116	11	33	Ser19, Ser551, Ser552, Ser675
		APC		-	35	
		CDH1		6	13	
		CDH3		6	19	
		CTNNA1		21	64	Ser641, Thr654, Thr658
Pan CDH	PA5-16766	CDH1	A549	-	11	
		CDH2		5	28	
		CDH4		-	7	
		CTNNB1		7	21	
		CTNNA1		27	22	Ser641
CDKN1A	MA1-91243	CDKN1A	HCT116	1	4	
		CDK1		12	7	
		CDK2		5	10	
		CDK4		2	6	
		CCND1		3	7	
PAK1	71-9300	PAK1	HEK293	3	23	Ser174, Thr230
		GIT1		9	19	
		GIT2		3	7	
EGFR	AHR5062	EGFR	A549	18	60	Ser991, Ser1026, Ser1039
TP53	13-4000	TP53	BT549	6	18	Ser9

Orthogonal Detection Methods Show High Correlation

IP-MS

Direct MS



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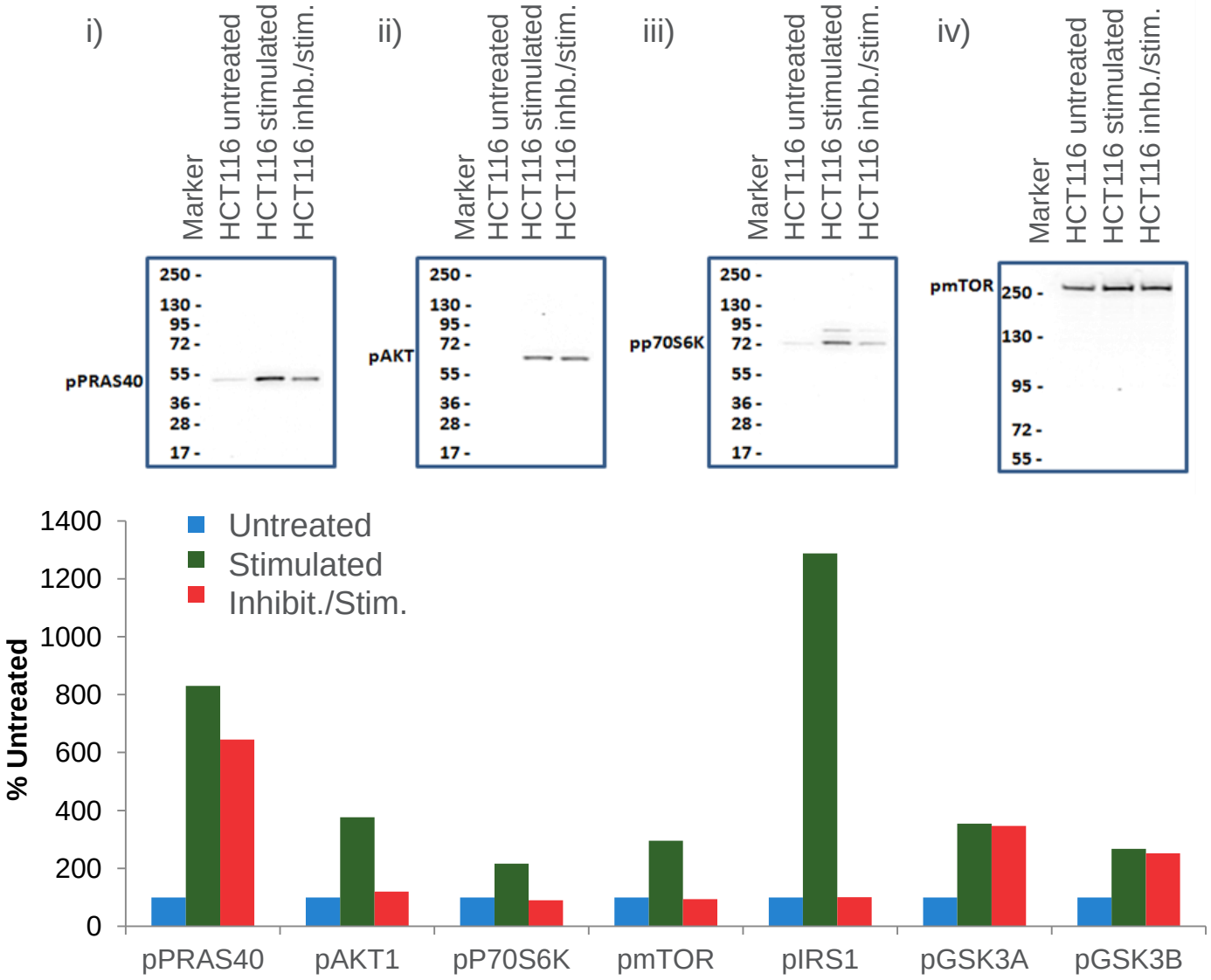
Comparison of Western Blot vs. Targeted IP-MS



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AKT/mTOR and RAS/ERK Pathway Expression Changes

Cell line (condition)	AKT/mTOR pathway		RAS/ERK pathway	
	Total	Phosphorylated	Total	Phosphorylated
HCT116 (stim.)	No change	↑ P	Target-dependent changes	No change
HCT116 (inhib.)		↓ P except GSK3		
A549 (stim.)	No change	↑ P except mTOR	↑ T	↑ P
A549 (inhib.)		↑ P except IRS1		↓ P

Cell line dependent differences in response to PI3K inhibition

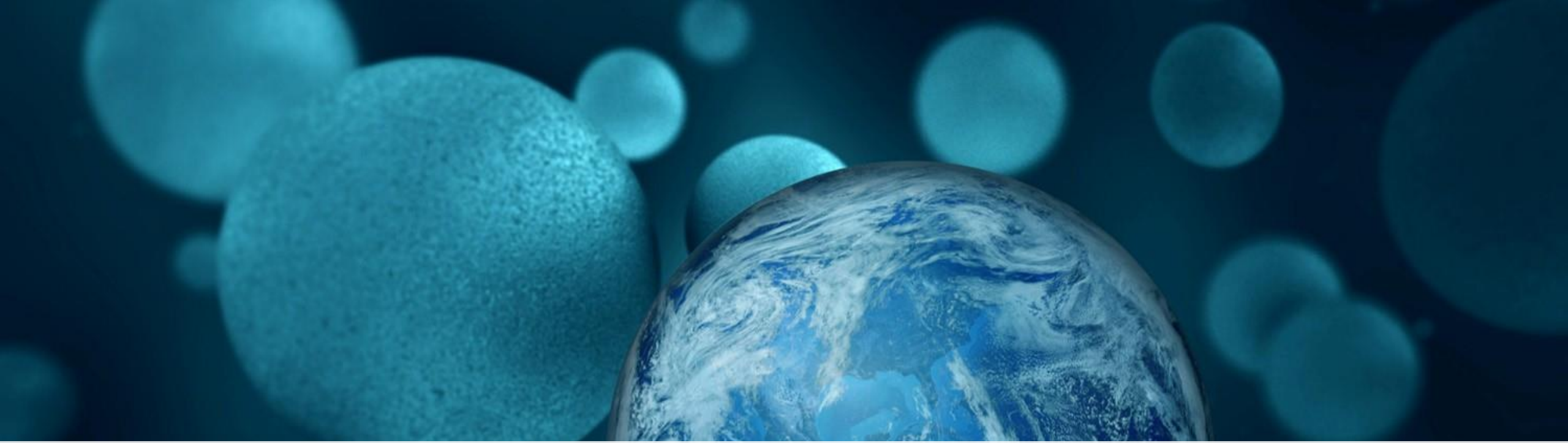
Conclusions

- **Enrichment is necessary for identification and MS quantitation of signaling pathway proteins, interacting partners, and PTMs**
- **Thermo Scientific™ Pierce™ MS-Compatible Magnetic Immunoprecipitation IP Kits (Protein A/G and Streptavidin) resulted in a higher yield of AKT/mTOR and RAS/ERK pathway proteins and fewer nonspecific binding proteins than with other beads/resins**
- **Total and phosphorylated target mIP-tMS assays allowed simultaneous quantitation of multiple AKT/mTOR and RAS/ERK pathway proteins and modifications in treated cell lines**
- **Elucidating specific pathway differences between A549 and HCT116 cells will lead to better understanding and treatment of lung and colon cancer.**
- **mIP-tMS assays were benchmarked against western blot:**
  - Overall good correlation observed for AKT/mTOR and RAS/ERK pathway proteins
  - Variability between techniques for some targets could be due to antibody specificity

Acknowledgements

- Bhavin Patel
- Penny Jensen
- Greg Potts
- Leigh Foster
- Abid Hasseb
- Kay Opperman
- Matt Baker
- Brian Johnson
- Wayne Considine
- Carrie Clothier
- Kevin Harvey

Q&A



Thank you

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