Targeted MS and its Application in Biology and Medicine

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Discovery defines a reduced set of "sentinel" marks that need to be repeatedly measured in a range perturbations

Perturbations:

- Disease
- Development
- Drug
- KO/KI

Analyte Valley of Death

Past: Westerns; Immunoassays

Not all proteins and PTMs of interest observed in all experiments

Desired assay properties:

- Highly specific
- Sensitive
- Highly precise
- Multiplexed
- Interference-free

Precisely measure selected analytes in all experiments – no missing data!

Conventional protein measurement methods have major limitations

Properties of the Measurements	Western blotting	Immunoassay
Specificity of method	Highly variable	Good
Standards (proteins) available for analytical validation	No	Few
Internal standards used for high inter-laboratory reproducibility	No	No
Validated assays for high % of proteome	No	No
Quantitative	No to semi	Yes
Moderate-to-high throughput	No	Yes
Can be highly (e.g., >20) multiplexed	No	No
Interferences detectable and avoidable	No	No
New assays easy to generate (time, reagents, cost, expertise)?	No	No

Targeted proteomics: hypothesis-driven technology for biology and medicine

Classic "Discovery" MS

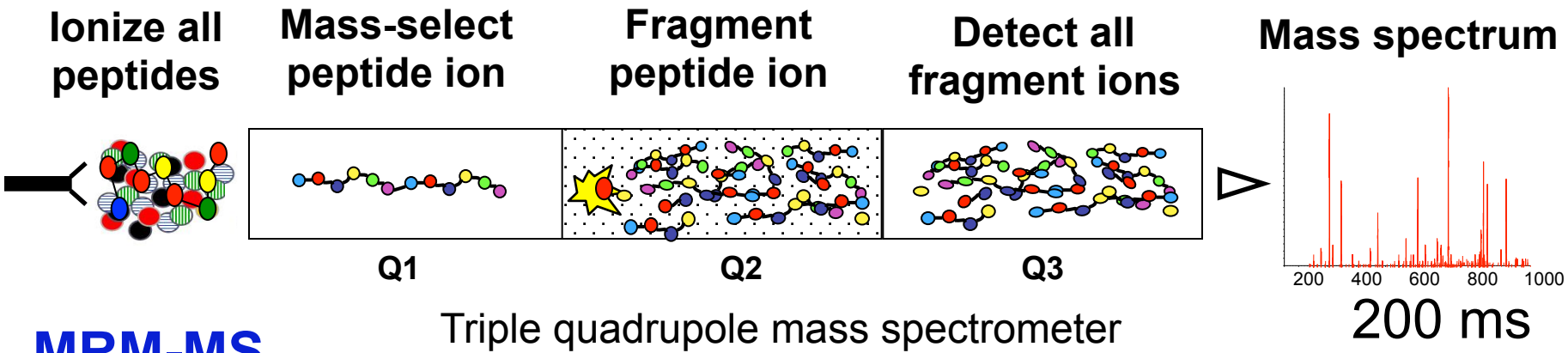
- Lots of "arrows" shot
- Many, many peptides hit
- What you hit is not up to you
- Peptides not repeatedly detected across experiments
- Good relative quantification if labeling is used

Targeted MS Methods

- Fewer arrows shot
- Fewer peptides hit
- What you hit is defined by user
- All selected peptides hit all the time
- Quantification is highly precise and can be accurate using internal standards.
- 50-1000x more sensitive than Discovery

How targeted MS (MRM-MS) differs from conventional MS/MS

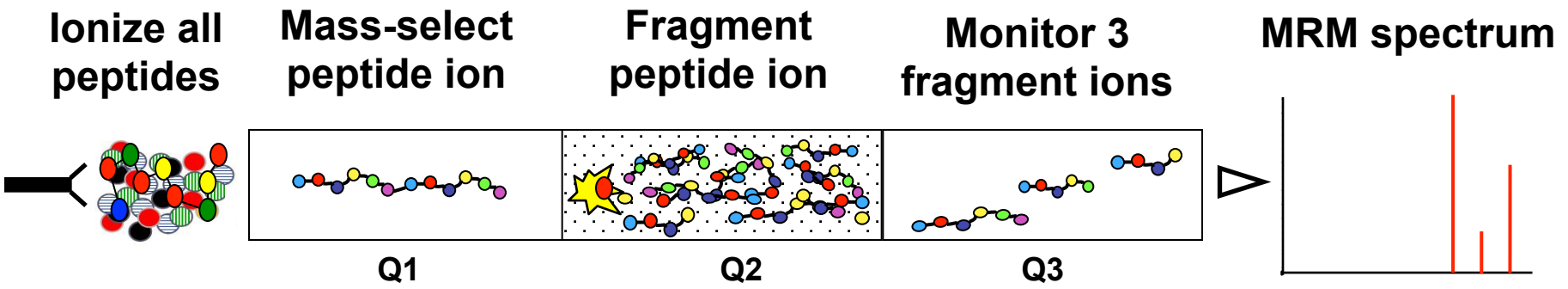
MS/MS Operating Mode



Triple quadrupole mass spectrometer

200 ms

MRM-MS



Triple quadrupole mass spectrometer

10 ms

Terminology:

SRM – Selected Reaction Monitoring
MRM – Multiple Reaction Monitoring

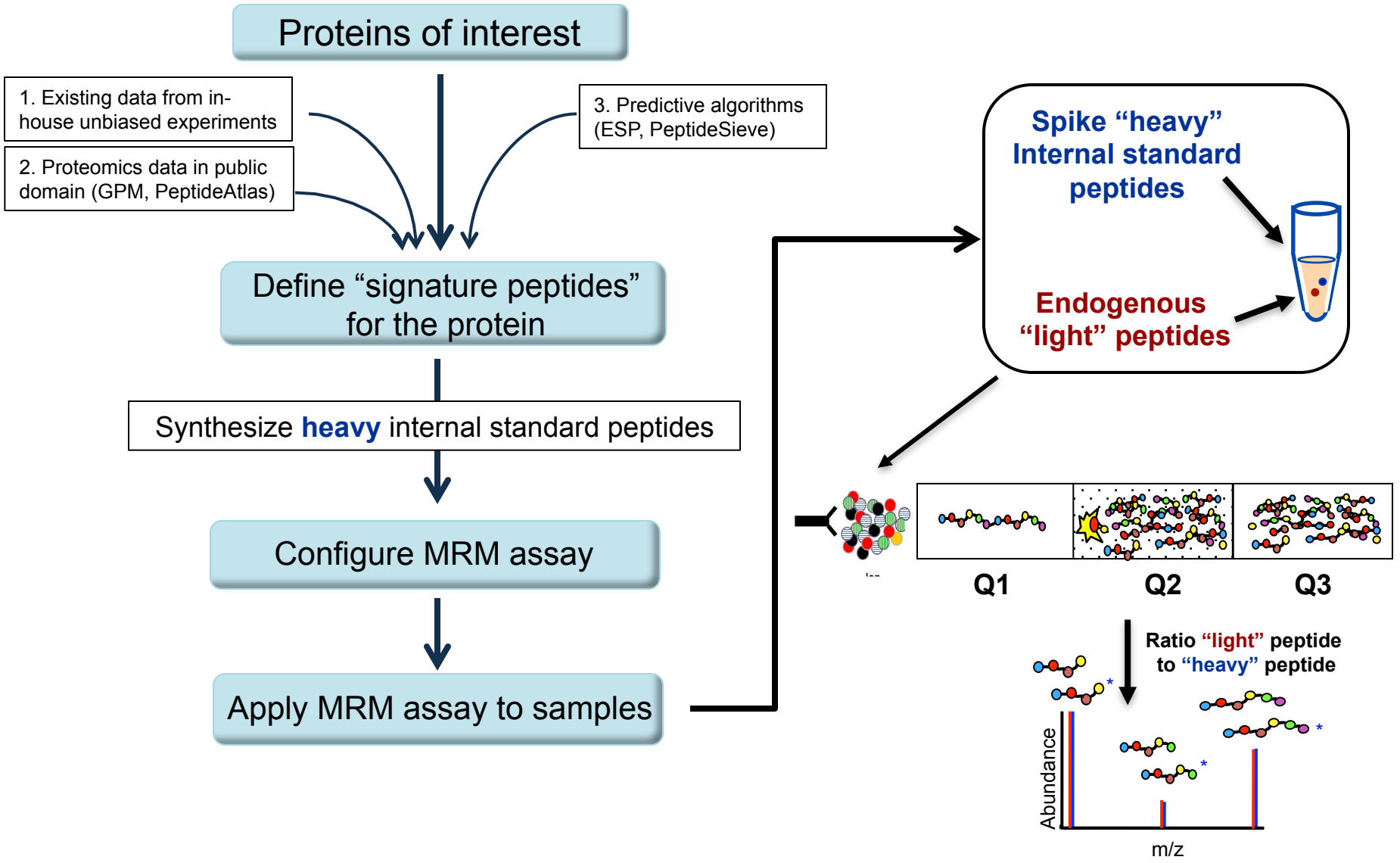
} Refer to the same targeted MS method on a triple quadrupole Mass spectrometer

PRM – Parallel Reaction Monitoring → Refers to a targeted MS method on high resolution MS instrument

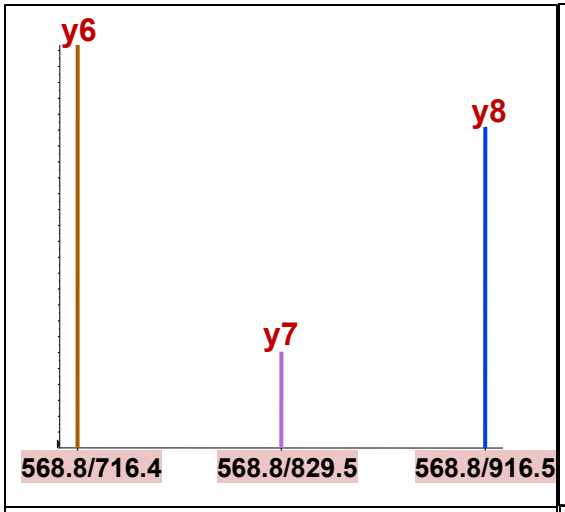
Transition – Precursor coupled to a specific product ion (Q1/Q3 pair)

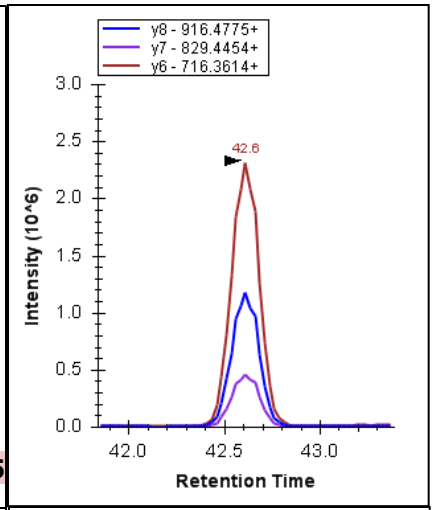
Peak Area Ratio (PAR) – $\frac{\text{Peak Area (light)}}{\text{Peak Area (heavy)}}$

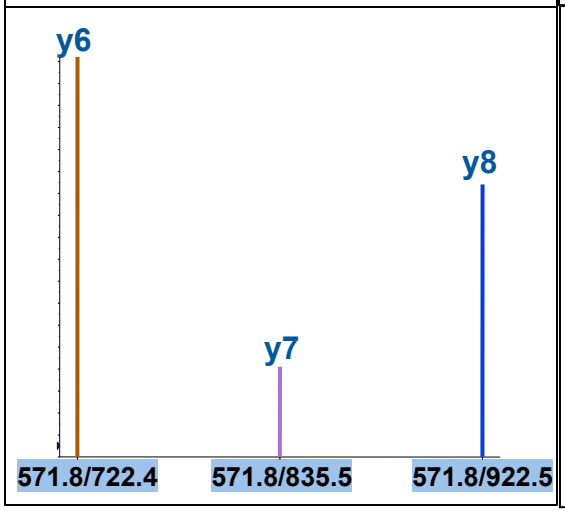
MRM assay construction

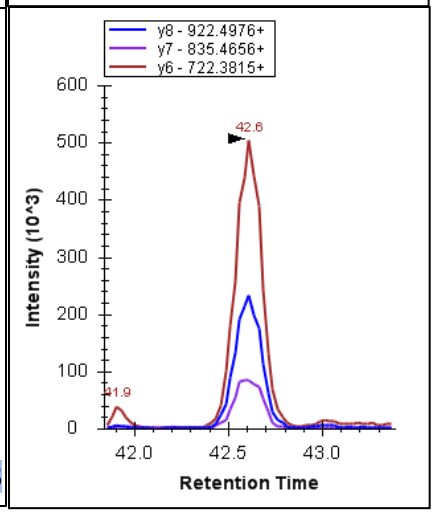


Monitoring a pair of $^{12}C/^{13}C$ peptides by MRM-MS









G Y S I F S Y A T $[^{12}C_6]$K

y8 y7 y6

MH^+ = 1136.6

$[M+2H]^{2+}$ = 568.8

G Y S I F S Y A T $[^{13}C_6]$K

y8 y7 y6

MH^+ = 1142.6

$[M+2H]^{2+}$ = 571.8

$$PAR = \frac{[^{12}C_6] \text{ Peak Area}}{[^{13}C_6] \text{ Peak Area}}$$

Key Steps in Developing Targeted Quant. Assays

- Selection of Targets
  - Empirical MS data or known targets of interest?
  - Synthesis of heavy and light peptide targets
- Characterization of Targets
  - Selection of transitions and assay optimization
- Determination of Assay Figures of Merit
  - LOD/LOQ
  - Assay Precision
- Testing on typical samples
  - Demonstrates robustness of assay
  - Surveys assay for utility in samples of interest
- Application to real samples

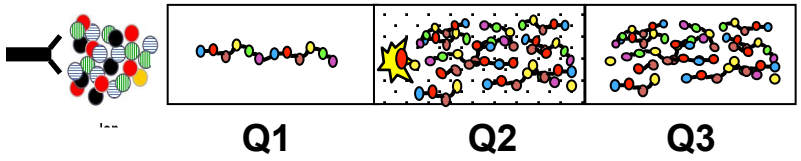
Response Curves and Figures of Merit

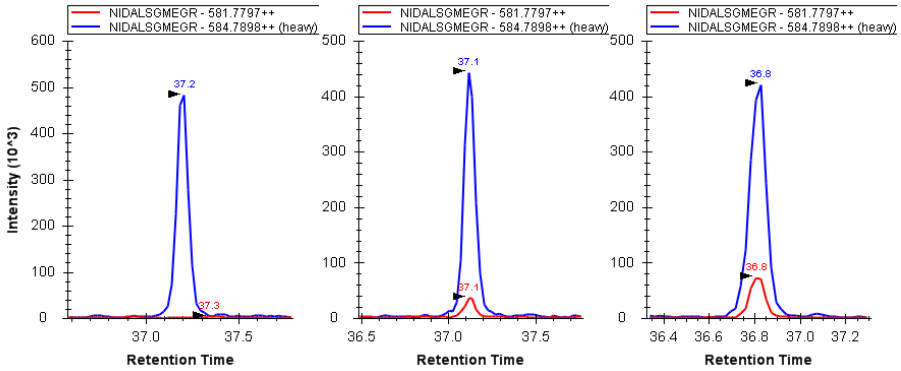
Heavy peptide at constant conc.

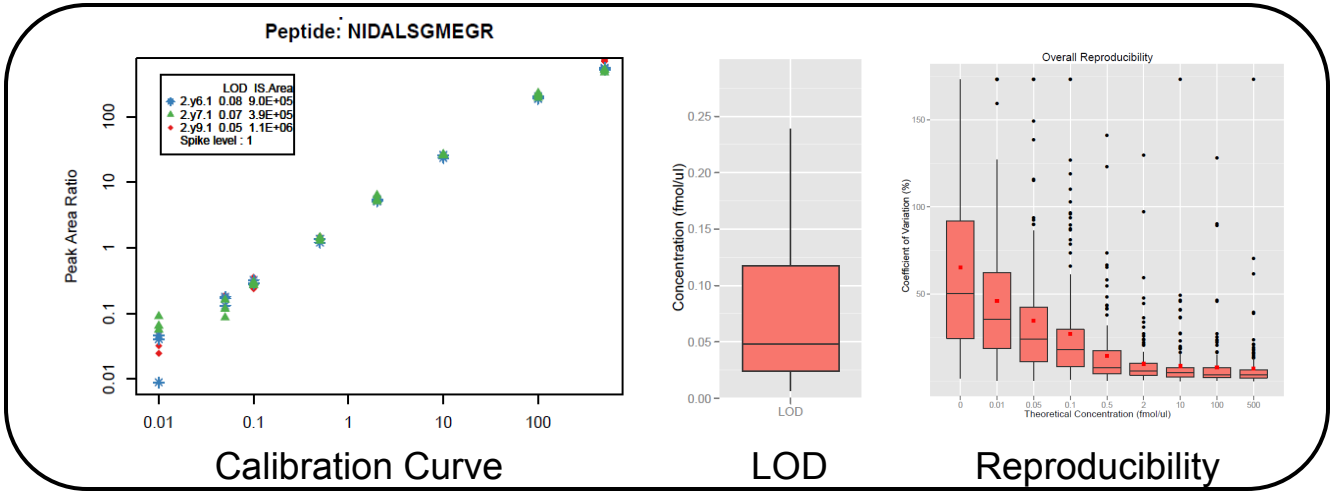
Light peptide at varying conc.

Relevant Background

Light	Heavy
0	0.5
0.05	0.5
0.1	0.5
0.5	0.5
1	0.5

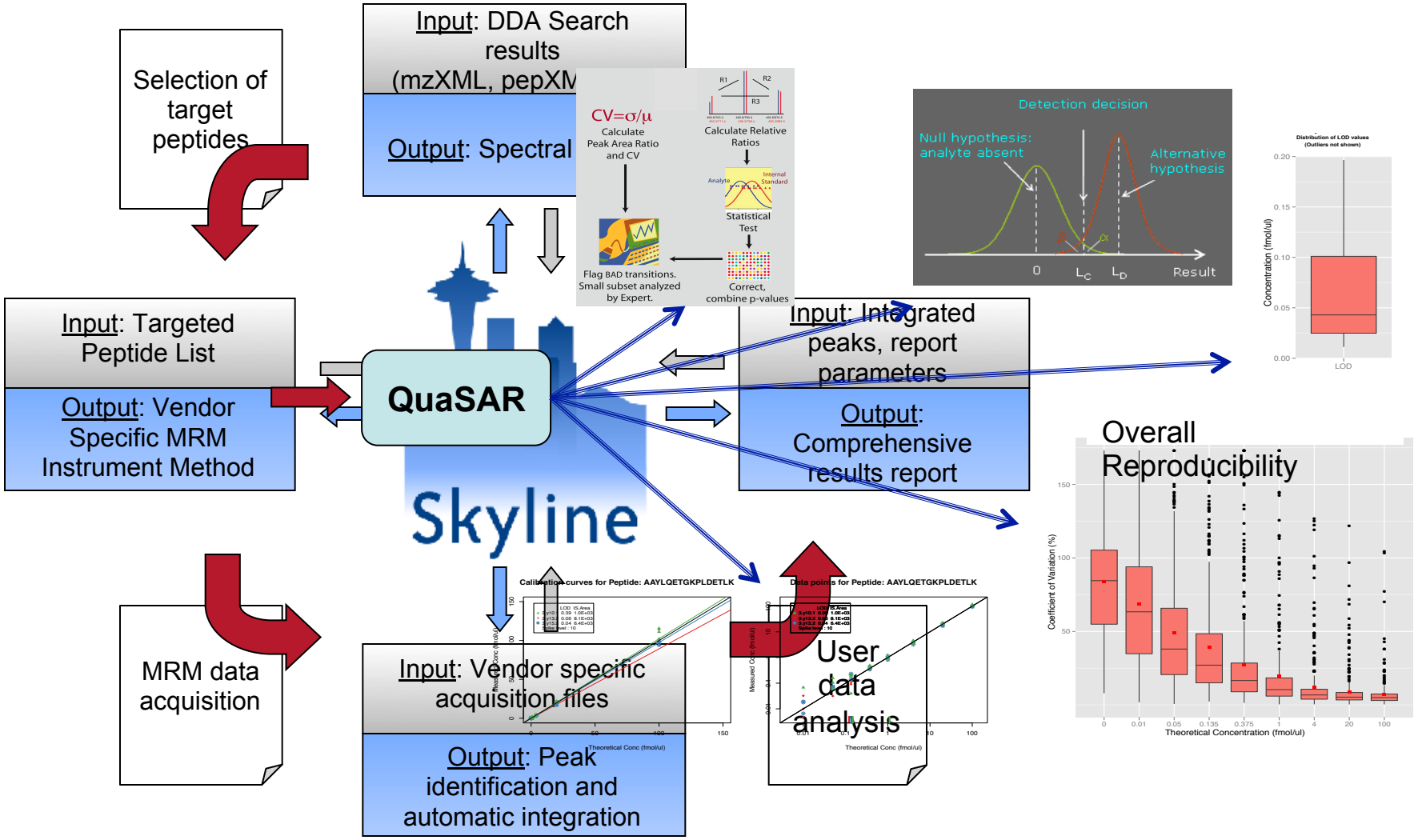






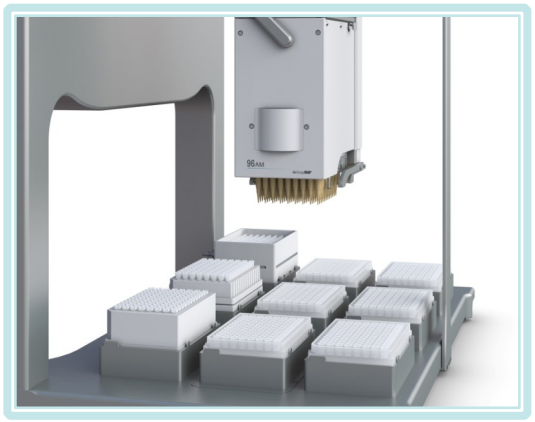
Skyline: an essential free tool for targeted assays
QuaSAR: fully integrated into Skyline

skyline.gs.washington.edu

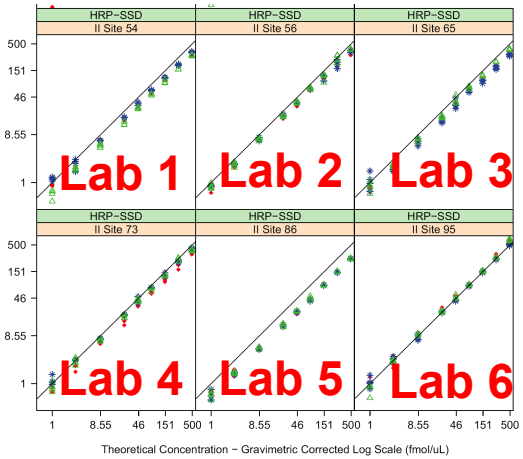


MRM-MS is precise, reproducible, robust, and can be highly multiplexed

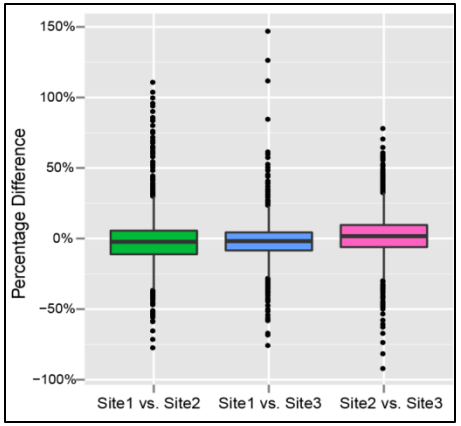
Automated Sample Processing



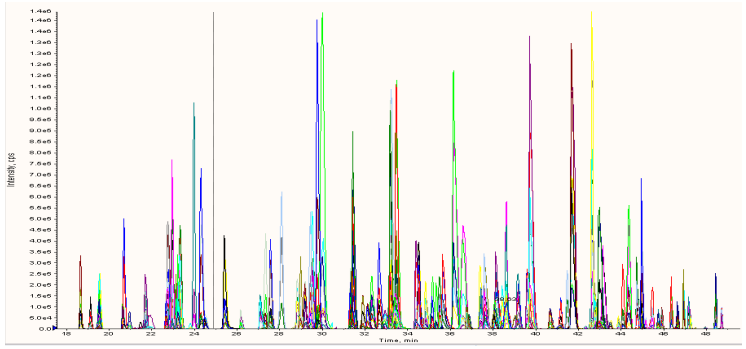
Precise and Reproducible



Robust



High Multiplex and Information Content

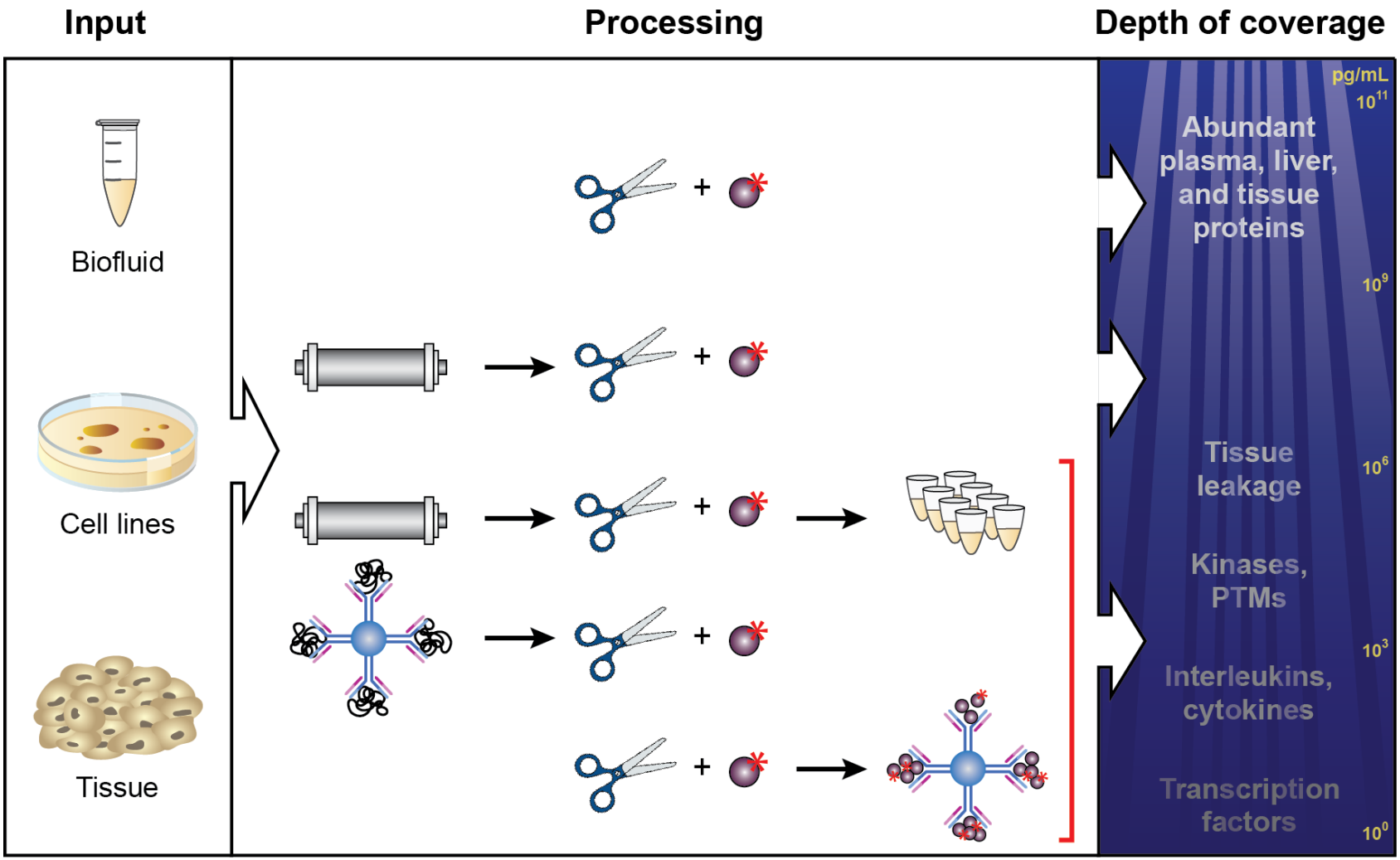


800 MRM assays; 2400 transitions

Numerous, well documented Studies

- Addona (2009) *Nature Biotech*
- Whiteaker (2011) *Mol Cell Proteomics*
- Addona (2011) *Nature Biotech*
- Kuhn (2011) *Mol Cell Proteomics*
- Hüttenhain (2012) *Sci Transl Med*
- Kennedy (2013) *Nature Methods*
- *Keshishian (2014) Mol Cell Proteomics*

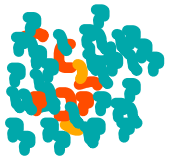
Enrichment methods increase limits of detection



Gillette and Carr
Nature Methods, 2013

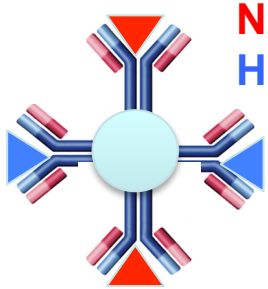
MRM-MS with Ab-capture of peptides decreases assay complexity and increases robustness (iMRM or SISCAPA[1])

Peptides in digested sample



Add ^{13}C-labeled signature peptide

Capture on Ab-coated magnetic beads



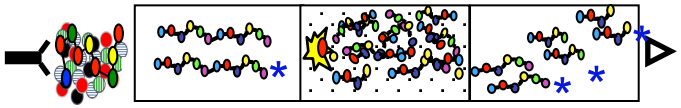
Native (^{12}C) peptide

Heavy Stnd (^{13}C) peptide

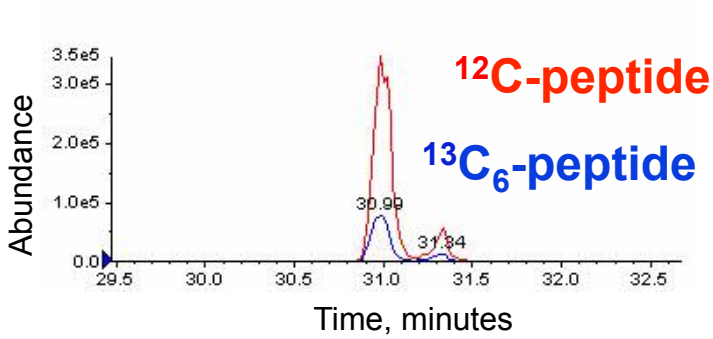
1000's - >10,000 fold enrichment

Wash; elute peptides

MRM-MS



Ratio ^{13}C-peptide to ^{12}C-peptide by MRM-MS



^{12}C-peptide

$^{13}C_6$-peptide

Advantages of SISCAPA

- Simpler sample handling prior to LC-MS/MS to reach ng/mL
- Only requires 1 Ab
- Easy to obtain useful anti-peptide Abs (>75% success)

1. Anderson et al. (2004) J. Prot. Res.

When Would You Use Targeted Quantification?

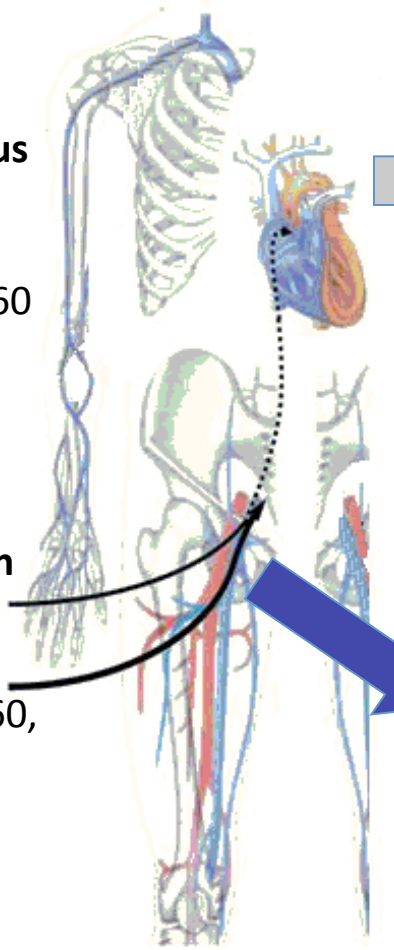
- You have a candidate list (10's - >100) of proteins and/or phosphopeptides you want to repeatedly measure under varying conditions, cell types, etc.
  - Precise, relative quantification across samples
  - Control vs stimulated cell lines
  - Normal vs disease-state tissue or plasma

- Wherever change of abundance is needed
  - Exact peptide sequence is monitored and quantified
  - Small changes in abundance can be determined (<<2-fold)

- Case Studies

Discovery and Verification proteomics in a human model of myocardial injury: planned myocardial infarction (PMI)

Coronary Sinus Samples
Time (min):
Baseline, 10, 60



Femoral Vein Samples
Time (min):
Baseline, 10, 60, 240, 1440

Discovery

Goal: Generate list of candidate protein biomarkers and specific peptides to target

Technique: LC-MS/MS with extensive fractionation

Sample: Proximal fluid (coronary sinus)

Quantitative Verification

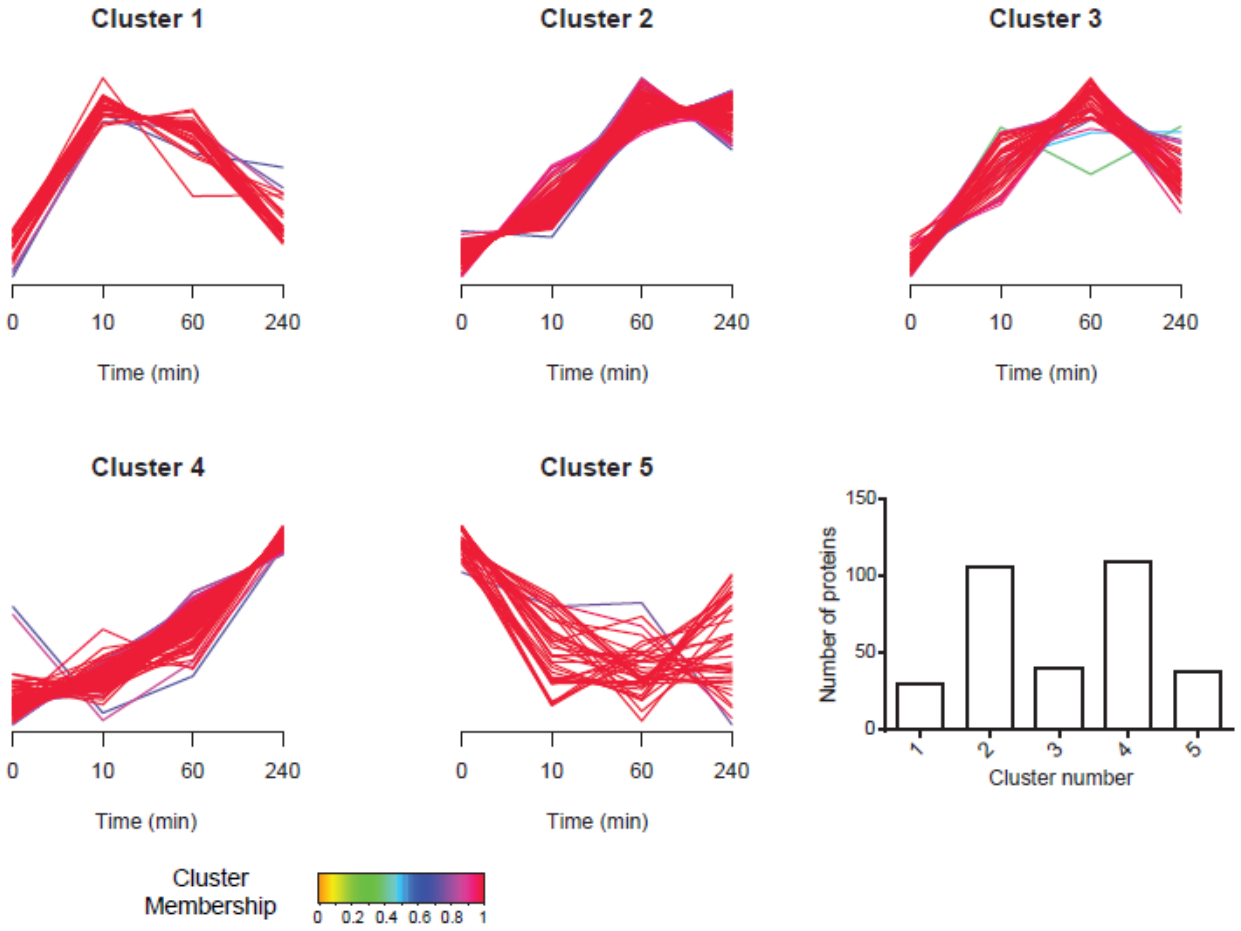
Goal: Quantify protein abundance in cases, controls

Technique: fraction-MRM-MS and immunoassay

Sample: Peripheral plasma of cases (PMI, SMI, ischemia) and controls (routine catheterization)

Addona et al. *Nature Biotechnology* (2011) 29: 635

Fuzzy C-means clustering identified 333 regulated proteins in 5 clusters: pick peptides from proteins of interest from each cluster

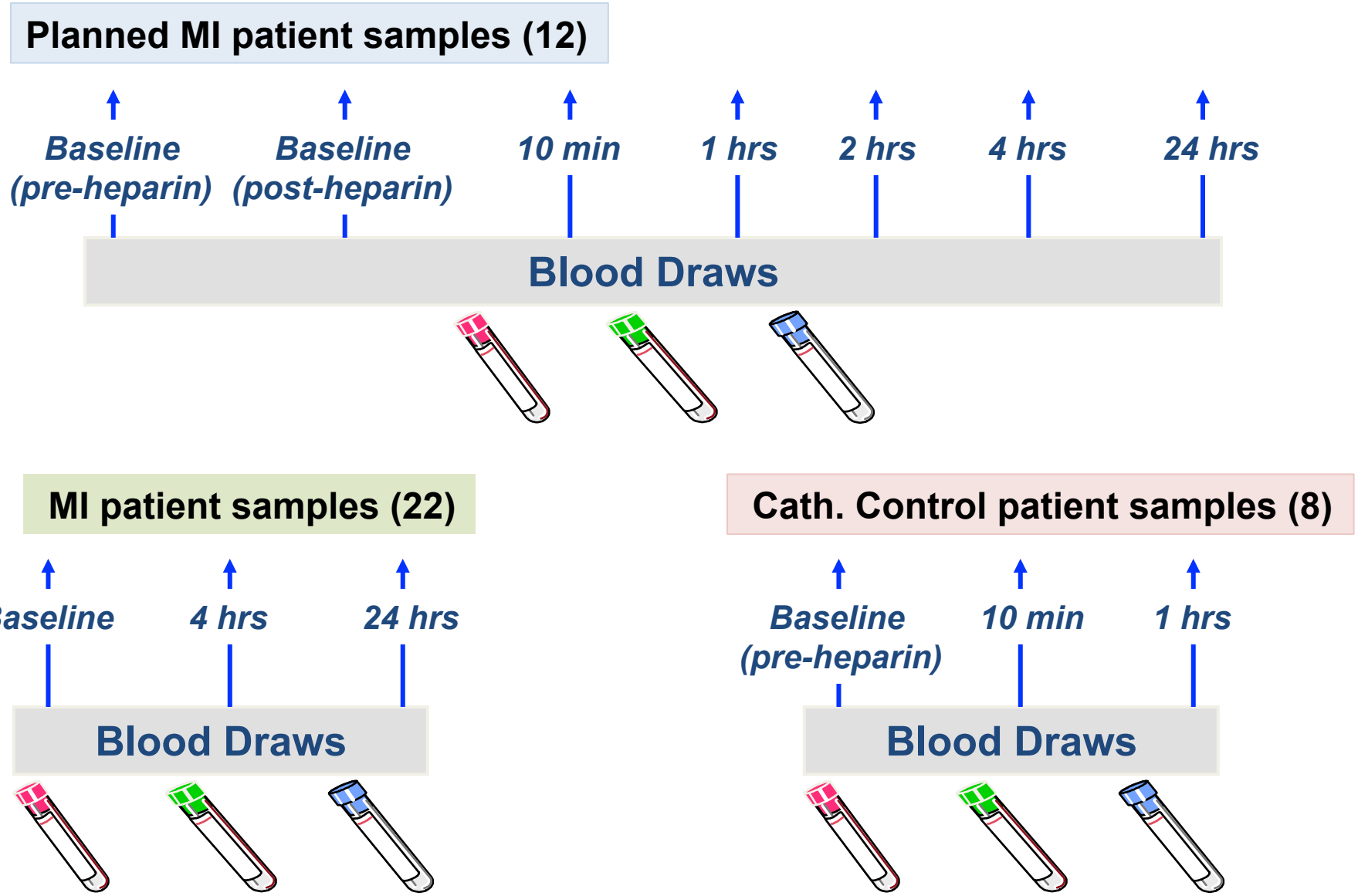


Keshishian et al. Mol Cell Proteomics, 2015

23 Antibodies for 23 Peptides/ 13 Protein targets

Protein	Peptide	Ug Ab in the mix
Troponin I	NITEIADLTQK	2
IL 33	TDPGVFIGVK	2
	VLLSYYESQHPSNESGDGVDGK	2
ACLP Aortic carboxypeptidase-like protein 1	ILNPGEYR	2
	DTPVLSELPEPVVAR	2
FHL1 four and a half LIM domains 1 isoform 5	AIVAGDQNVEYK	2
	NPITGFGK	2
MYL3 Myosin light chain 3	AAPAPAPPPEPERPK	2
	ALGQNPTQAEVLR	2
	HVLATLGER	2
TPM1 Isoform 4 of Tropomyosin alpha-1 chain	LVIIESDLER	2
	SIDDLEDELYAQK	**1**
	HIAEDADR	2
ITGB1 Isoform Beta-1C of Integrin beta-1	GEVFNELVGK	**1**
TAGLN2 Transgelin-2	ENFQNWLK	2
TAGLN1 Transgelin-1	AAEDYGVIK	2
FGL2 Fibroleukin	ELESEVNK	**1**
	EEINVLHGR	2
SCUBE2 Signal peptide	GSVACECRPGFELAK	2
FSTL1 Follistatin-related protein 1	IQVDYDGHCK	2
	LDSSEFLK	2
SPON1 Spondin-1	VEGDPDFYKPGTSYR	2
	AQWPAWQPLNVR	2

Patient samples for immunoMRM (iMRM)



Overview of Broad's Automated iMRM Workflow

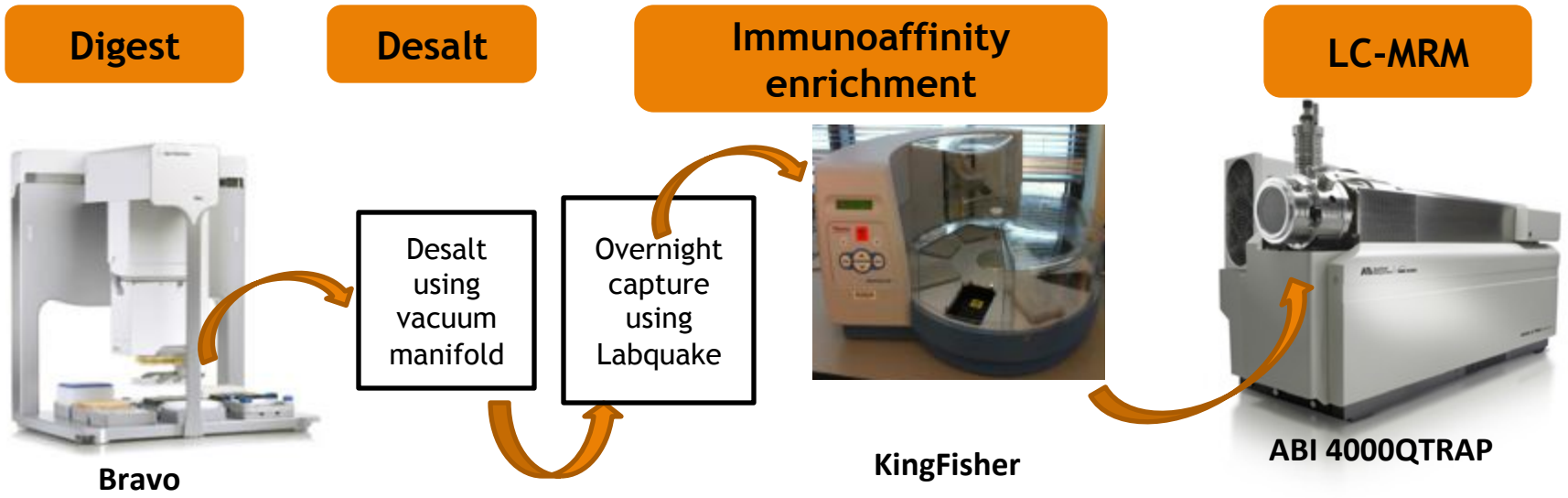


Plate of 30ul Digested Plasma (not depleted)

- Add heavy peptide mix over after digestion, prior desalting

Capture Target Peptides

- 23 antibody Mastermix
- 2ug beads per ug Ab
- Crosslinked Ab to bead

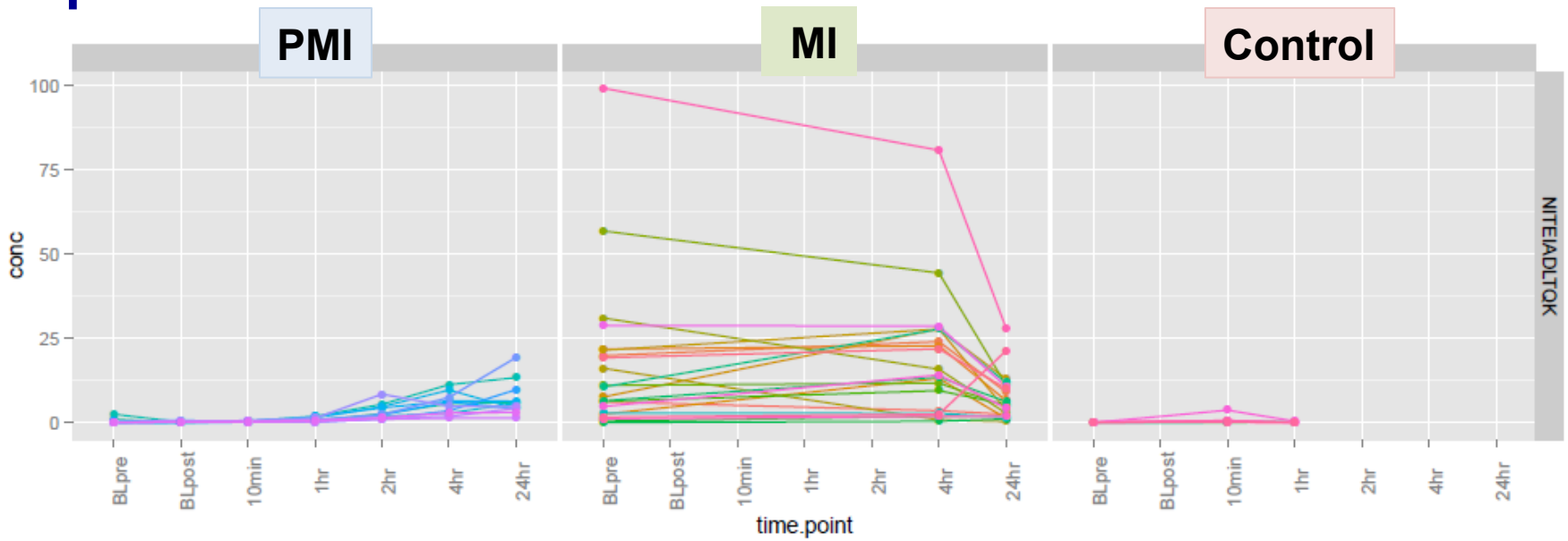
Wash and Elute Target Peptides

- Wash 2x in 1x PBS, 0.03% CHAPS
- Wash 1x in 0.1x PBS, 0.03% CHAPS
- Elute in 3%ACN, 5% HOAc

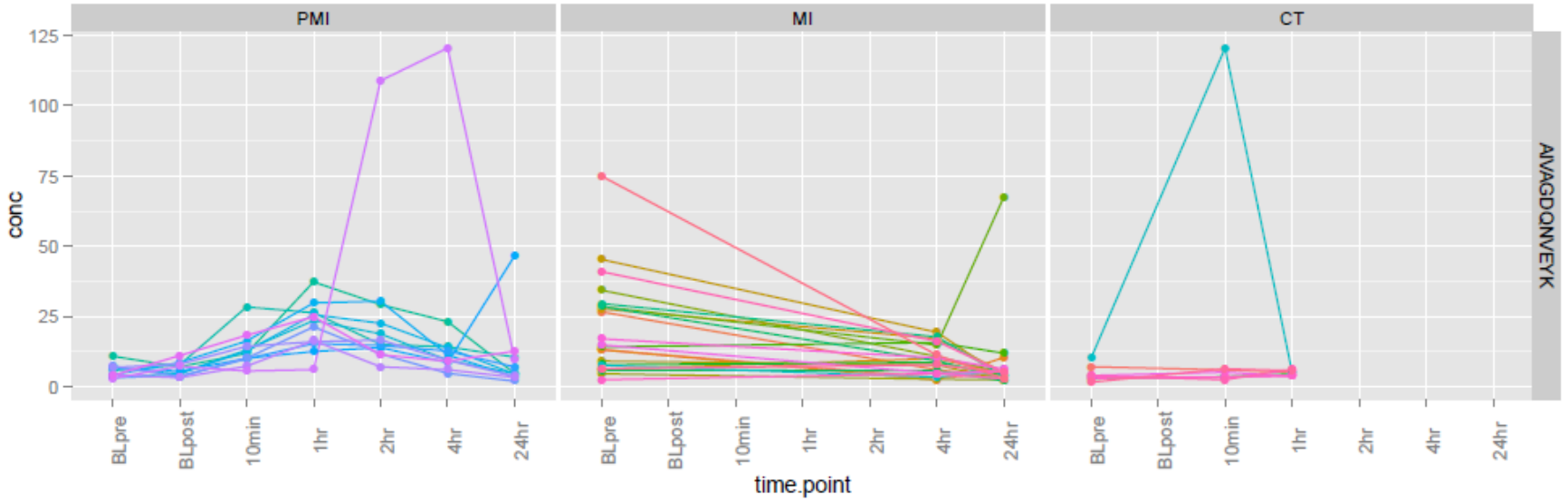
Analyze

- LC-MRM-MS
- Skyline
- QuaSAR

Troponin I

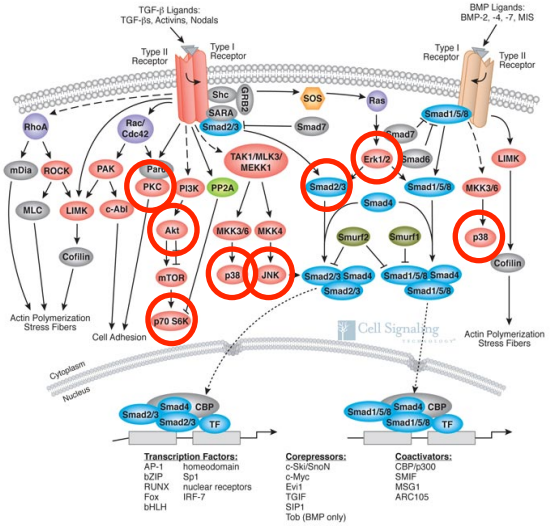


FHL1

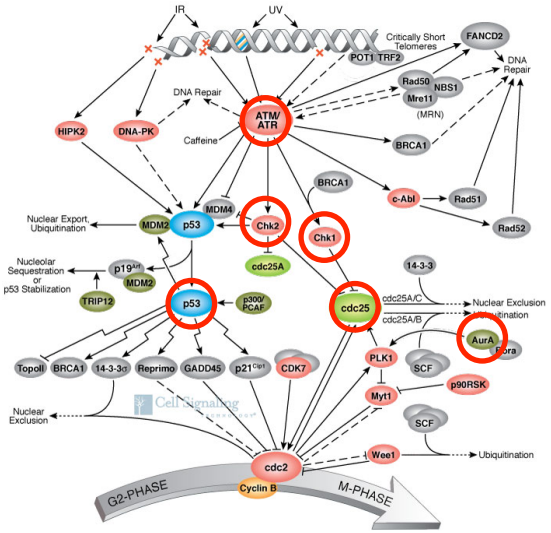


Case Study 2: Quantification of Phosphopeptides

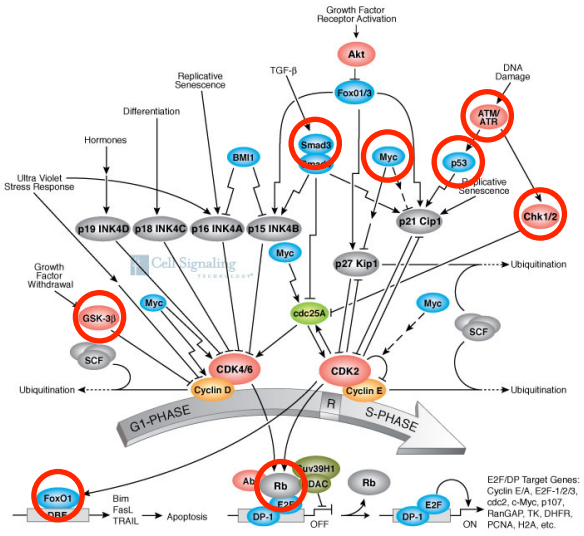
TGFB Signaling



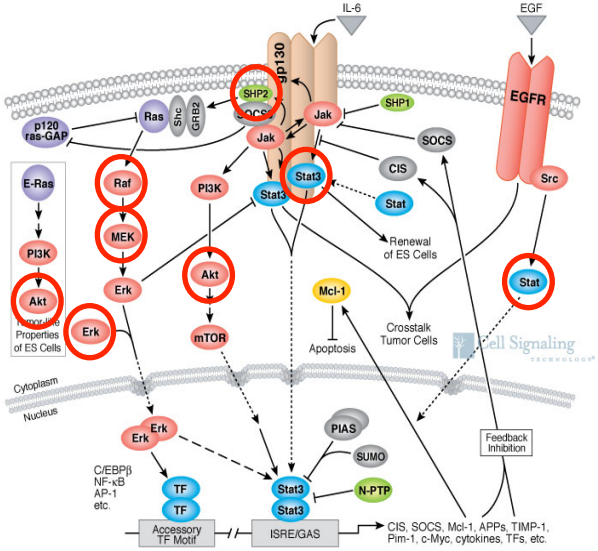
Cell Cycle Control: G2/M DNA Damage Checkpoint



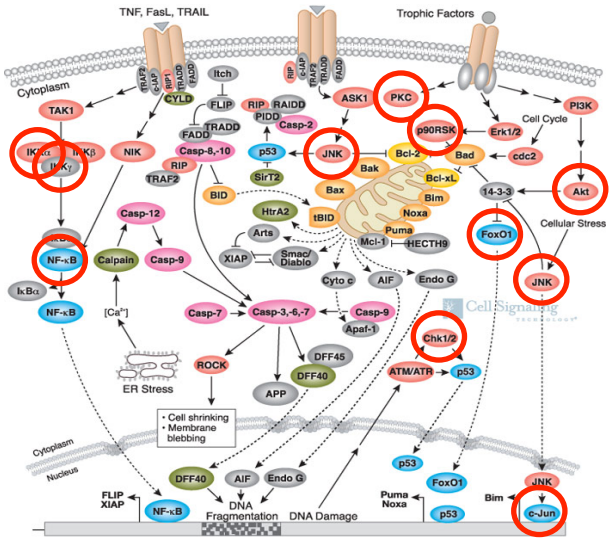
Cell Cycle Control: G1/S Checkpoint



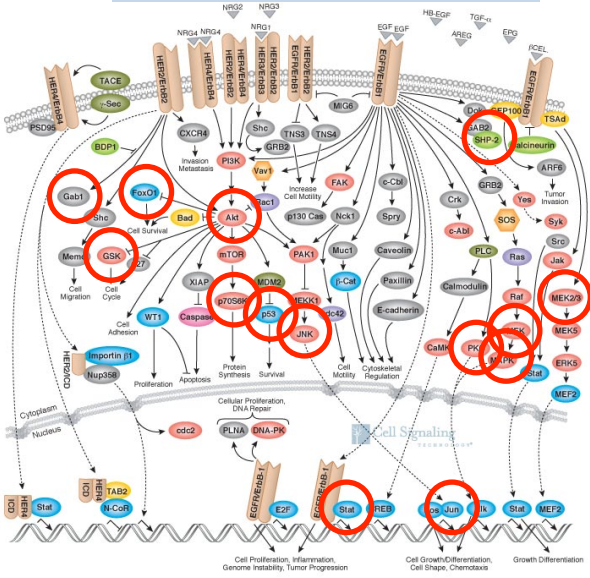
JAK / STAT Signaling



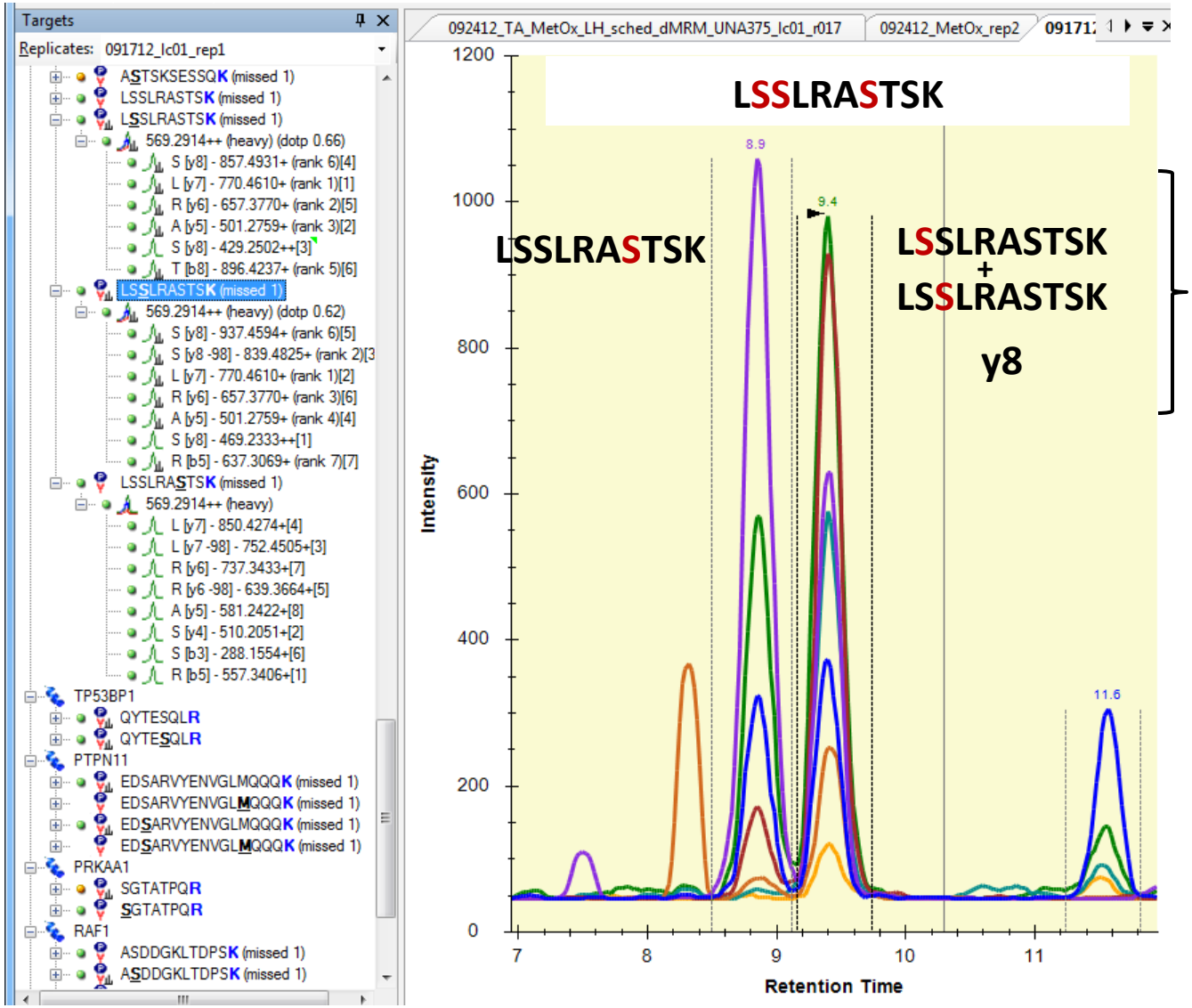
Regulation of Apoptosis



Erb B / HER Signaling



Challenges with Phosphopeptide Isoforms

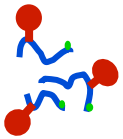


- Co-elution AND same molecular mass:
  Limits number of transitions for quantification
- Even if peptides don't co-elute, identifying which is which is challenging without standards

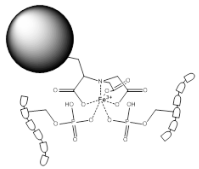
Develop targeted-MS peptide and phosphopeptide marker panels

Tissue homogenate, cell lysate

Lys-C; Trypsin

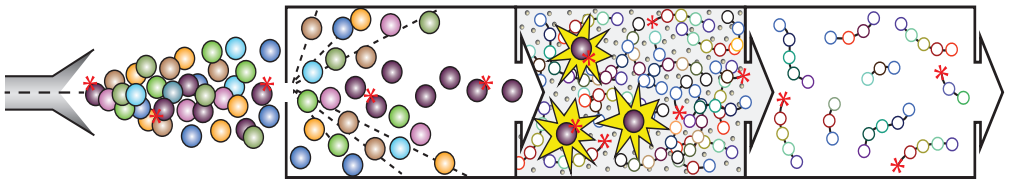


Add labeled phosphopeptides

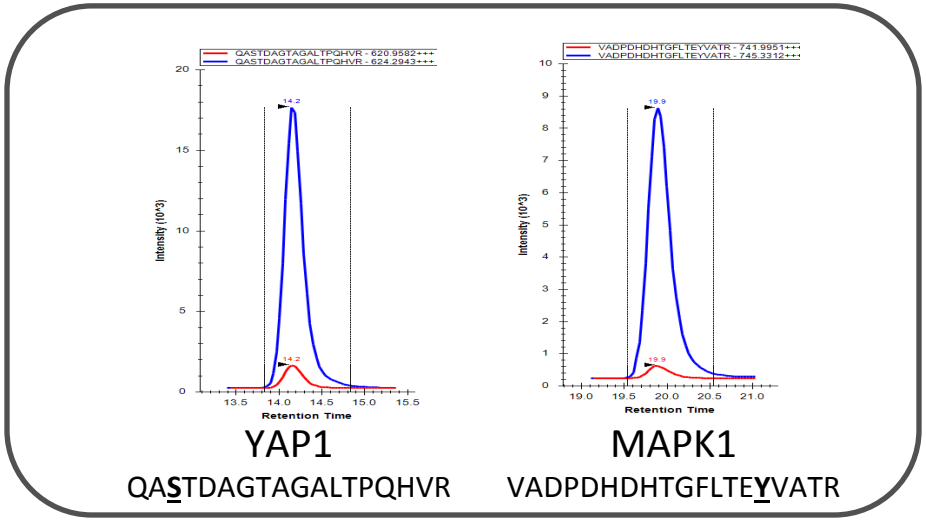


IMAC

Enriched Phosphopeptides



LC-MRM-MS



YAP1 QA**S**TDAGTAGALTPQHVR

MAPK1 VADPDHDHTGFLTE**Y**VATR

Selectively detect and quantify proteins and phosphopeptides, including ischemia-associated markers identified

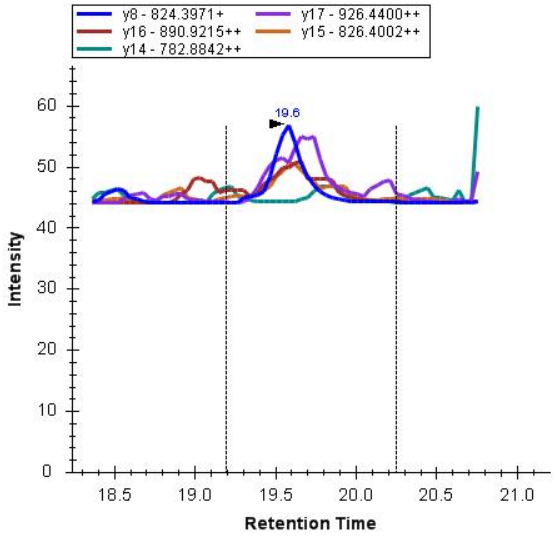
Enrichment is necessary for phosphopeptide measurements

- There is no significant endogenous signal for phosphorylated targets in unenriched samples
- Enrichment increases detection of some of the endogenous peptides in cell lysate
- After enrichment, percent of SM hits that are phosphorylated is 98-99%

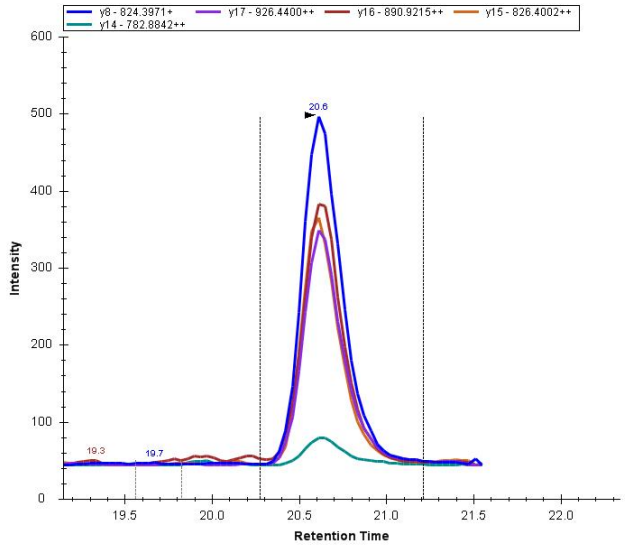
GSK3B (S9)

TT**S**FAES**C**KPVQQPSAFGSMK

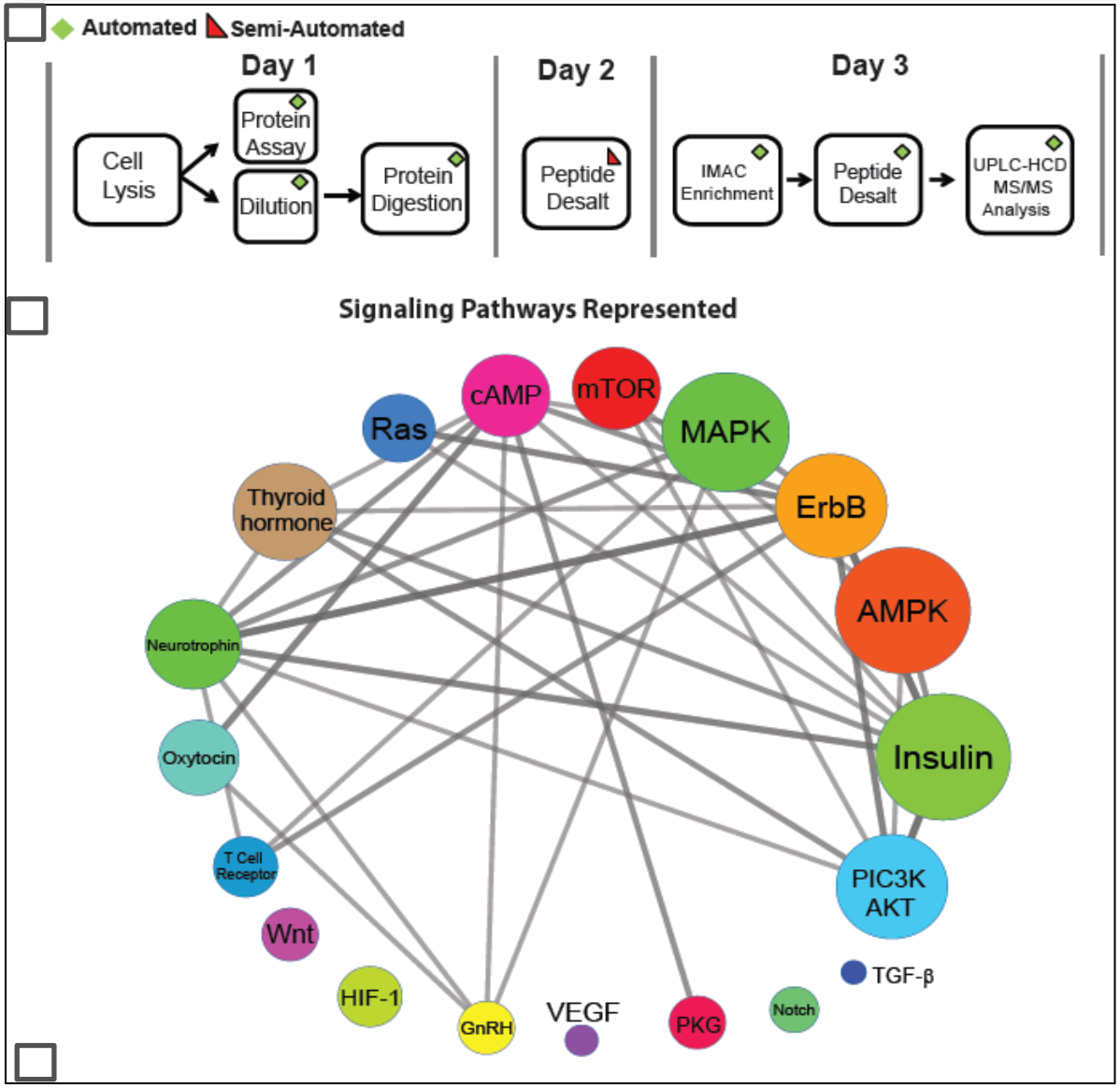
Unenriched

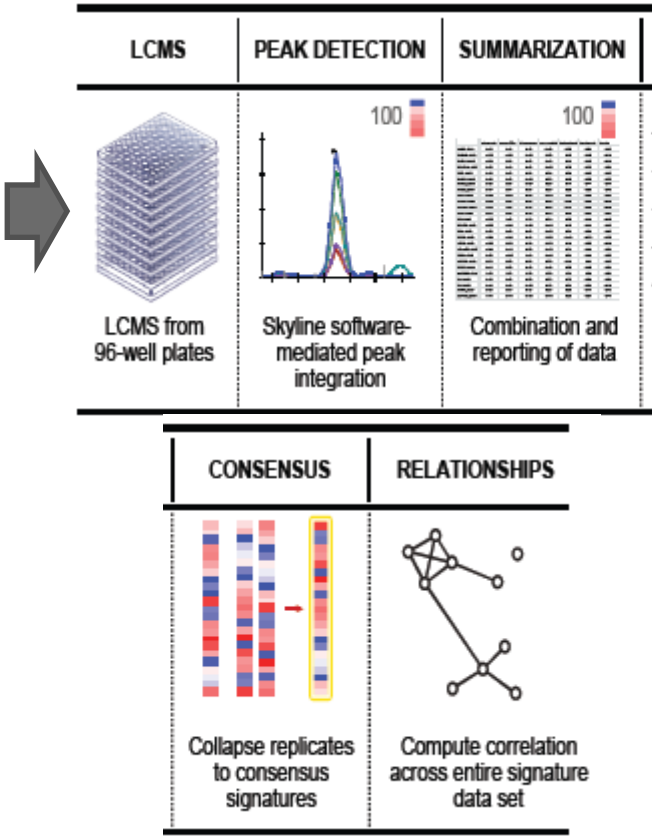


Enriched (IMAC)



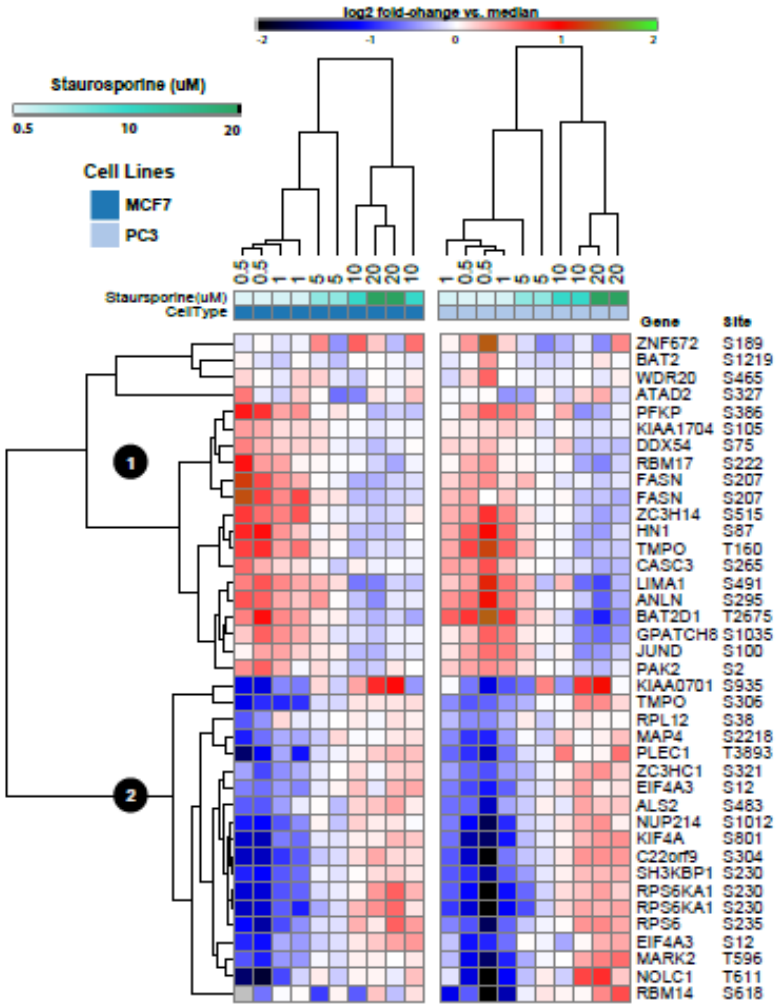
Measuring phosphosites to discover similarities in cell signaling among phenotypes elicited by drug treatment ("P100 assay")

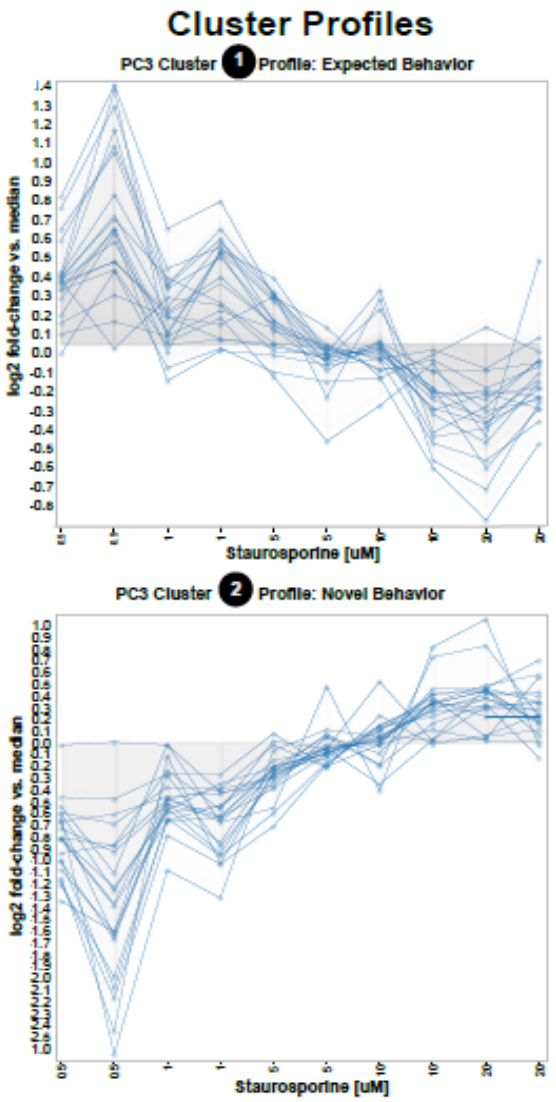




Abelin, Jaffe et al. Proteomics Platform

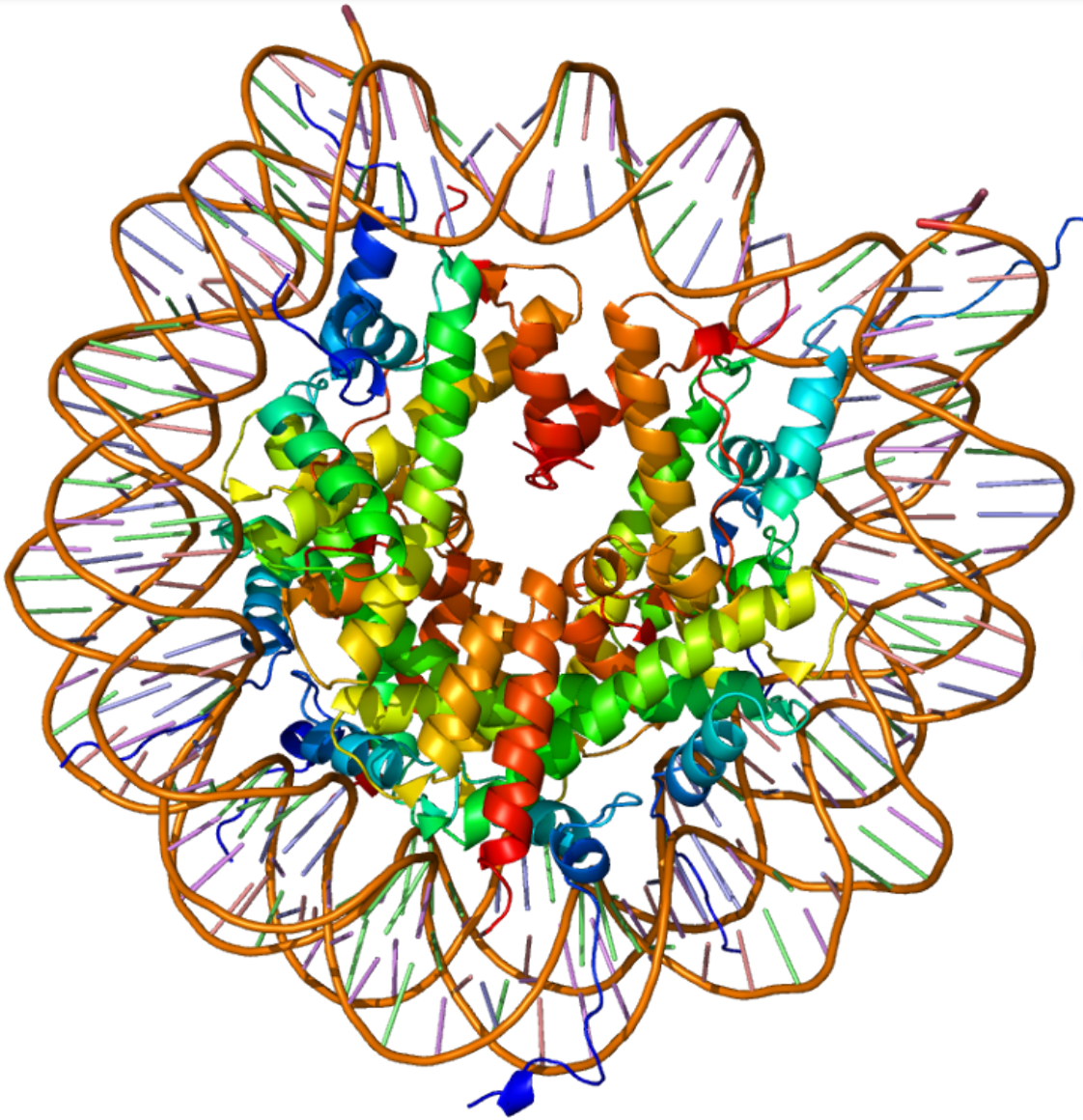
Example application of P100 Assay: dose response to kinase inhibitor

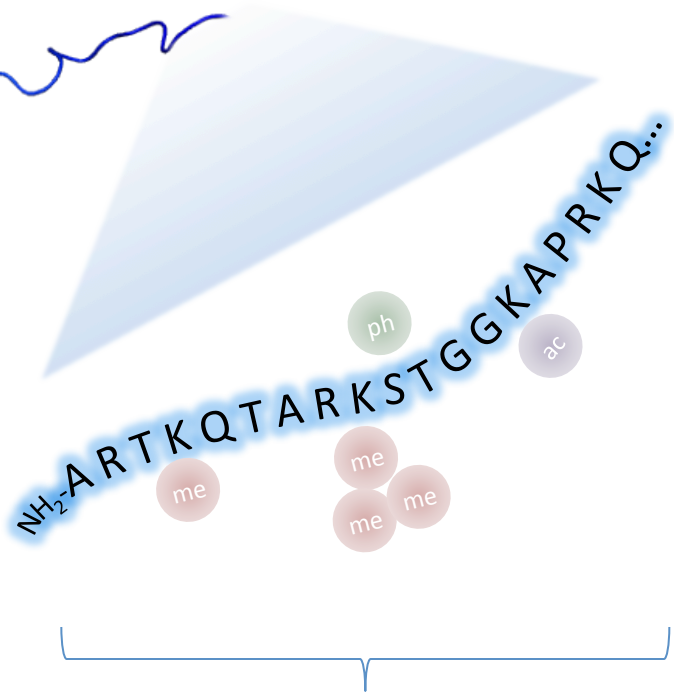




Abelin et al. submitted

Case Study 3: Histones and their post-translational modifications





Associated with transcriptional regulatory states of genomic loci

Source: PDB 1AOI, Luger et al. *Nature* (1997)

Histones are not amenable to traditional proteomics approaches

Histone H3:

```
          10        20        30        40        50        60
ARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHRYRPGTVALREIRRYQKSTEL

          70        80        90       100       110       120
LIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEACEAYLVGLFEDTNLCAIHAKRVTIM

          130
PKDIQLARRIRGERA
```

Red: Trypsin cleavage sites

- Trypsin digestion would create many short peptides and would do so inconsistently due to presence of numerous inhibiting modifications

Histones are not amenable to traditional proteomics approaches

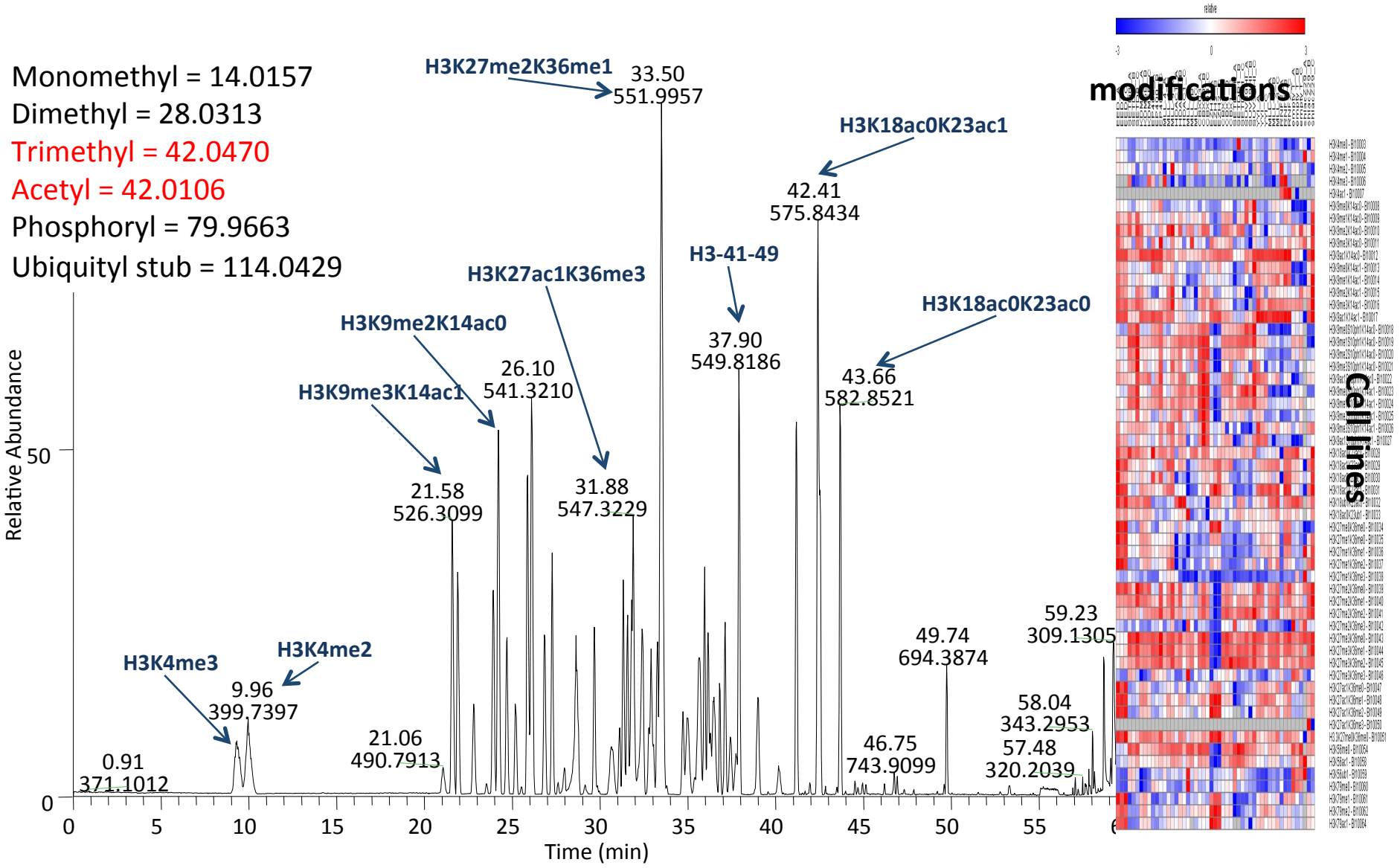
Histone H3:

```
          10        20        30        40        50        60
ARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHRYRPGTVALREIRRYQKSTEL

          70        80        90       100       110       120
LIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEACEAYLVGLFEDTNLCAIHAKRVTIM

          130
PKDIQLARRIRGERA
```

- Propionylate lysine
  - Consistent peptides
  - Helps with retention since histone peptides are very hydrophilic

Source: Garcia et al. *Nature Protoc.* (2007)

Highly multiplexed MRM-MS is an equally valuable tool in biology: assays for modified peptides



Benefits of Targeted MS for peptide/protein assays

- High molecular specificity
- Quantitative
- Works in any matrix (cells, tissue, plasma, urine, etc.)
- Works equally well for posttranslationally modified peptides
- ‘Sensitivity and assay performance already sufficient to assay many proteins of interest (mid-pg/mL to high ug/mL)
- Interferences can detected and avoided (unlike Westerns, IHC, immunoassays, aptamers)
- Does not require immunoassay-grade antibodies (2/protein)
- Assays can be highly multiplexed (>100 plex now routine)
  - Not possible with antibodies; can be done with aptamers
- Assays are transferable with high interlab reproducibility
- Multiple MS platforms now capable of targeted MS experiments (not just triple quads any longer)

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