Highly Selective Phosphopeptide Enrichment Workflow

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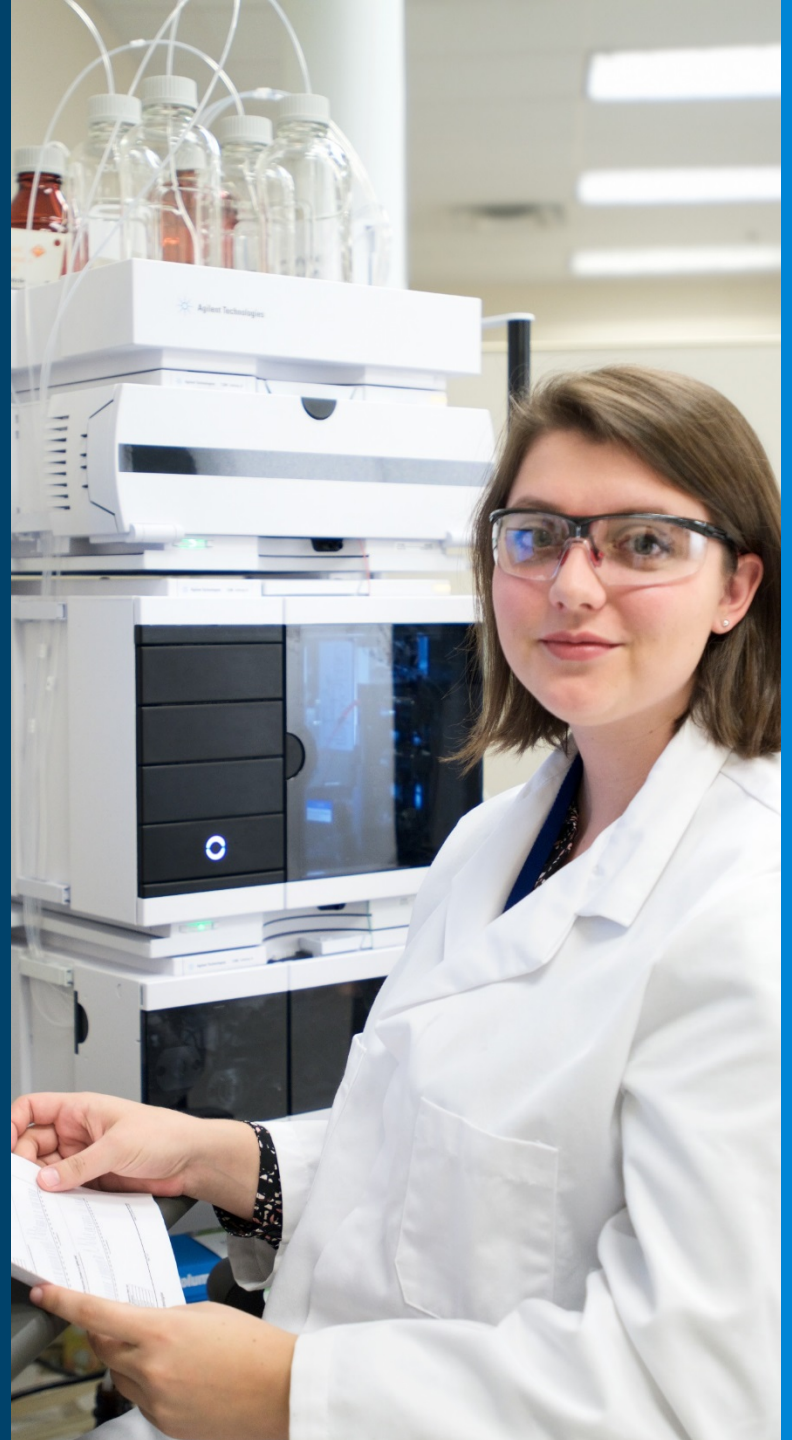

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Outline

Background and Analytical Challenges of Phosphorylation

Automated Phosphopeptide Sample Preparation

PME11 part II-Phosphoproteomics Study

Phosphorylation

Background

- Key intracellular signal transduction mechanism

Analytical approaches

- ATP-gamma P-32 labeling
- Fluorescent assays
- LC/MS of phosphopeptides

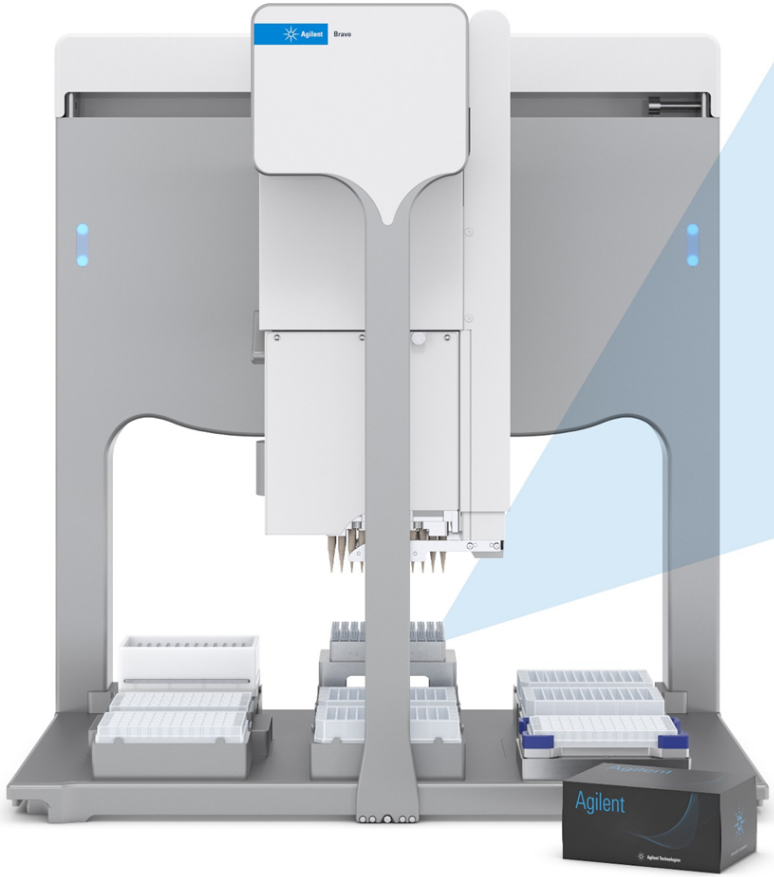
Analytical challenges

- Low abundance
- Labor intensive and variable sample preparation
- Complex bioinformatics

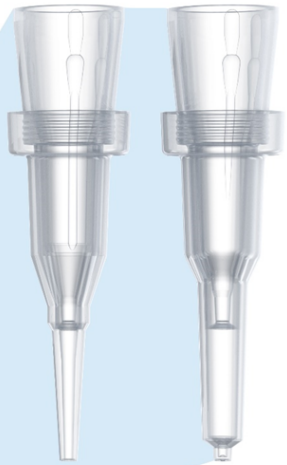
AssayMAP Platform

Simple User Interface

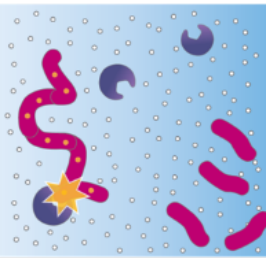
AssayMAP Bravo



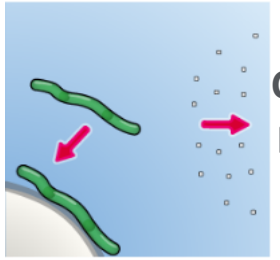
AssayMAP Cartridges



AssayMAP Application Portfolio



In-Solution Digestion

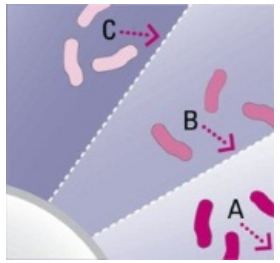


C18
RPS

Peptide Clean Up

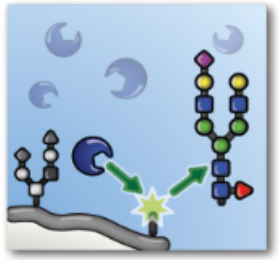
Fe(III)-NTA
TIO_2

Phosphopeptide Enrichment



SCX
RPS

Peptide Fractionation

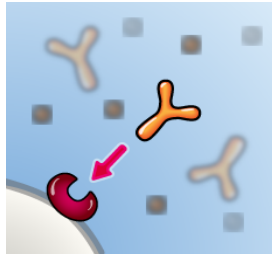


2-AB
InstantAB
APTS
InstantPC

N-Glycan Sample Prep

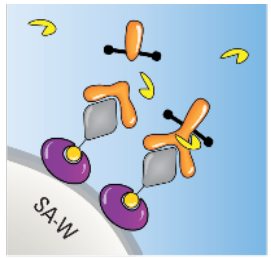
RPW

Protein Cleanup



PAW
PGW
SAW

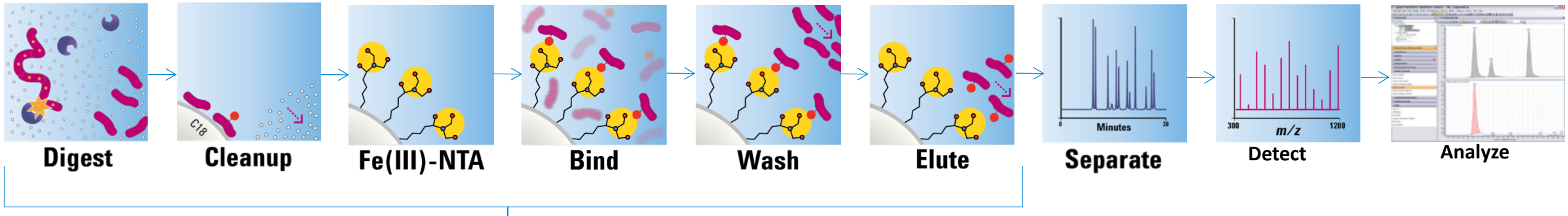
Affinity Purification



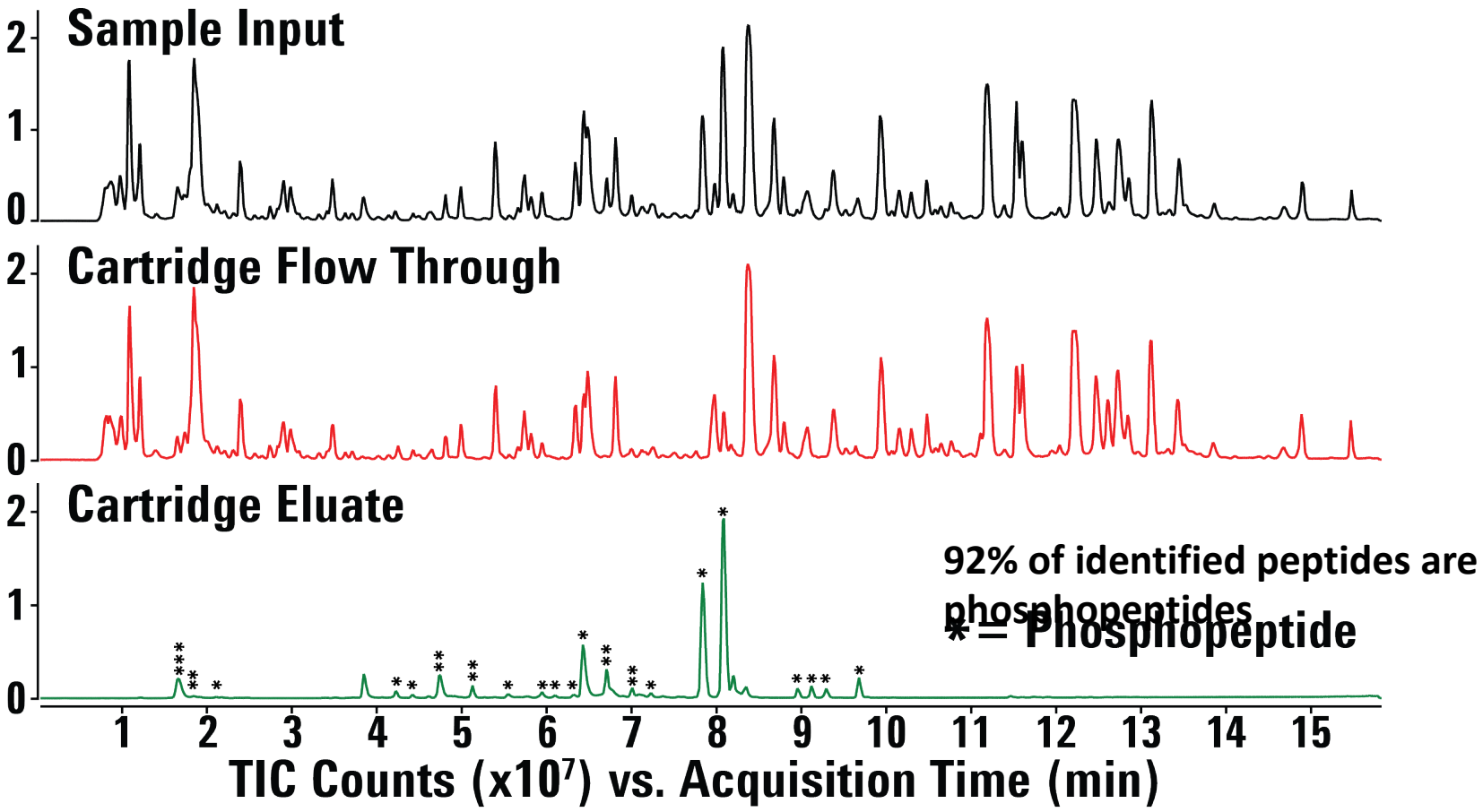
PAW
PGW
SAW

On-Cartridge Reaction

Phosphopeptide Enrichment Workflow



Fe(III)-NTA Enrichment



92% of identified peptides are phosphopeptides

* = Phosphopeptide

150 µg α-casein digest loaded

European Proteomics Association PME11 part II Phosphoproteomics

Overview

- Multiple sites were provided with phosphopeptide samples to compare phosphopeptide enrichment results

Materials provided

- Digested and cleaned up (C18) cell lysate spiked with 20 phosphopeptide standards
- 20 heavy phosphopeptide controls

Goals

- Compare phosphopeptide enrichment using different techniques at multiple sites
- Determine the affect of affinity medium to sample ratios on enrichment

Analytical endpoints

- Reproducibility and selectivity of the enrichment
- Number of phosphopeptides identified
- Yield of known phosphopeptides spiked into cell lysate
- Phosphorylation site identification

PME11 – Phosphoproteomics

Complex Mixture
+ Light Phosphomix

→

- Human MCF7 breast cancer cell line digest 125 µg /sample (C18 purified)
- Pre spiked, Phosphomix 1, 2 200 fmol each

↓

Phosphopeptide enrichment

→

- AssayMAP Bravo enrichment
- Fe(III)-NTA cartridges

↓

+ spike Heavy Phosphomix (200 fmol)

- Number of Phosphopetide IDs
- Phosphosite assignment
- Phosphomix recovery

→

- LC/MS Analysis
  - Discovery, 6550 iFunnel Q-TOF
  - Quantitation, 6495B TQ

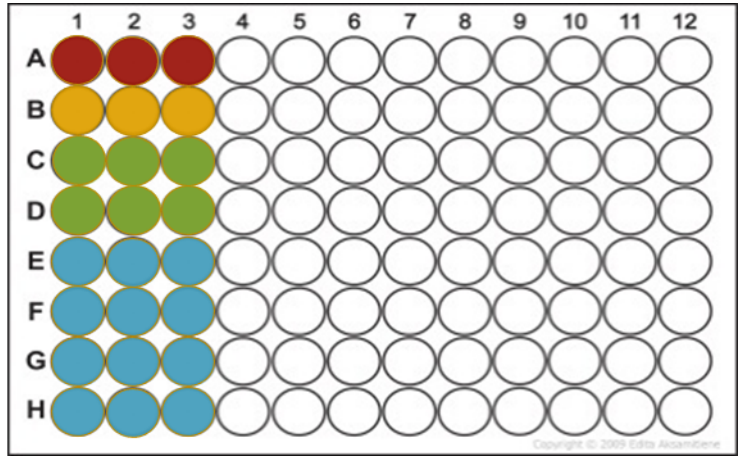
Phosphopeptide Enrichment

5 µL : 125 µg **Sample R1**

5 µL : 62.5 µg **Sample R2**

5 µL : 31.25 µg **Sample R3**

5 µL : 15.625 µg **Sample R4**



AssayMAP User Interface

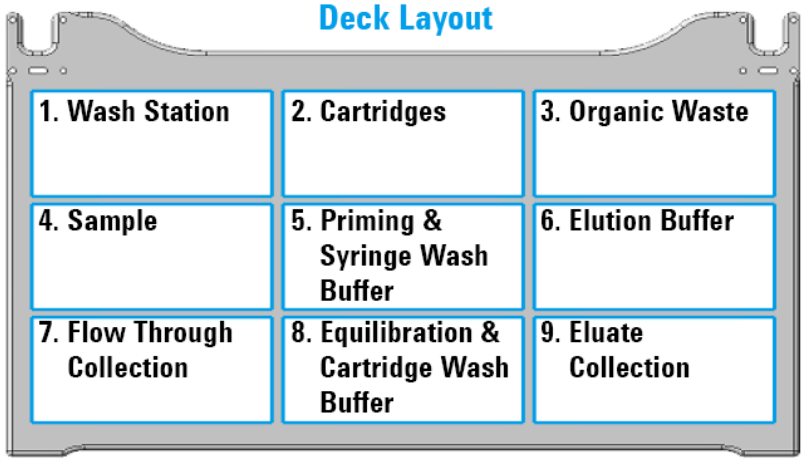
Phosphopeptide Enrichment: Using AssayMAP v2.0

Application Settings

Number of Full Columns of Cartridges	3

Step	Conduct Step?	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Initial Syringe Wash	☑			3
Prime	☑	100	300	1
Equilibrate	☑	50	10	1
Load Sample	☑	150	3.5	3
Collect Flow Through	☑			
Cup Wash	☑	25		1
Internal Cartridge Wash	☑	50	10	3
Collect Flow Through	☑			
Stringent Syringe Wash	☑	50		1
Elute	☑	20	5	1
Eluate Discard	☐	0		
Add to Flow Through	☐			
Existing Collection Volume		5		
Final Syringe Wash	☑			3

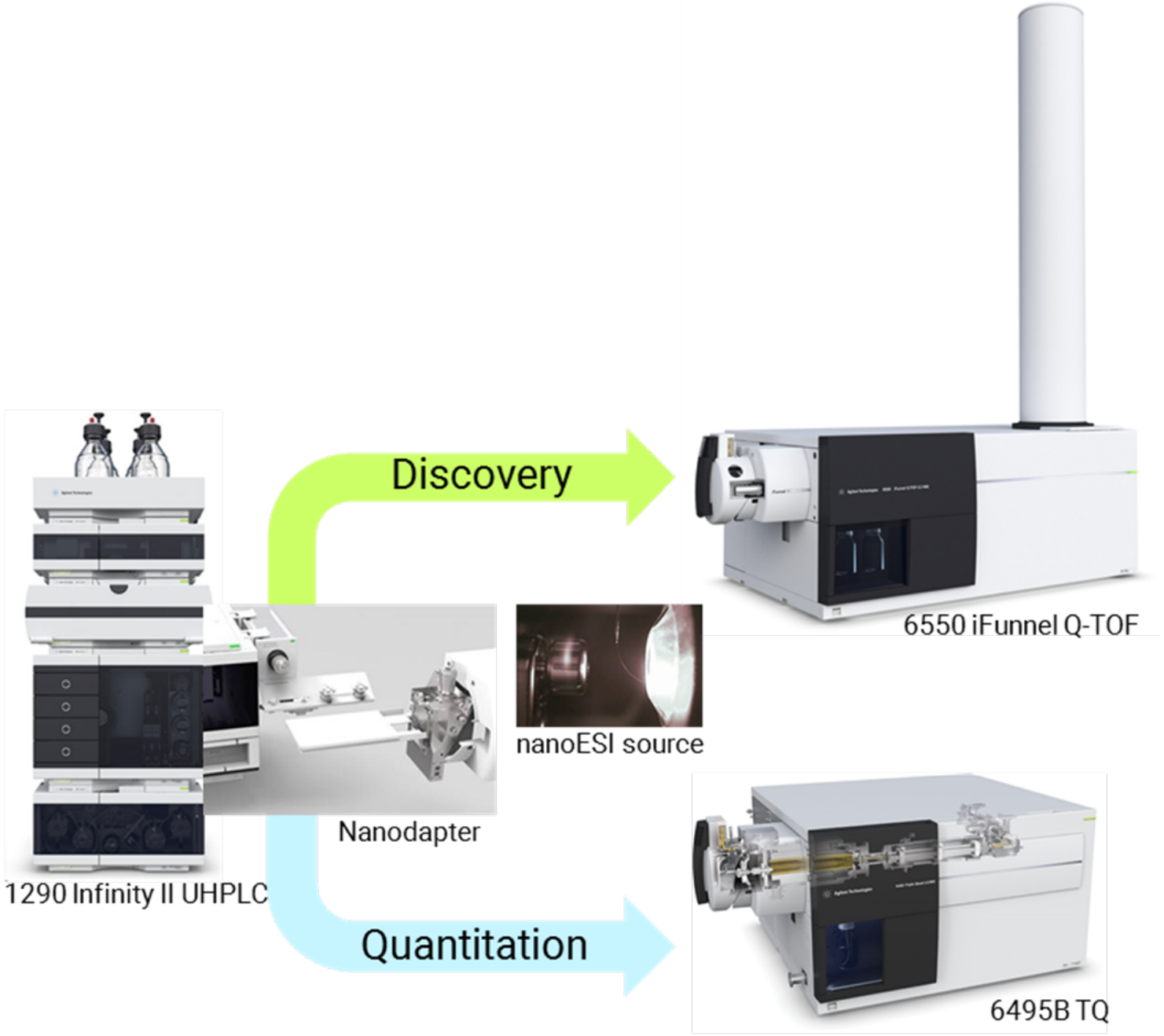
Deck Layout



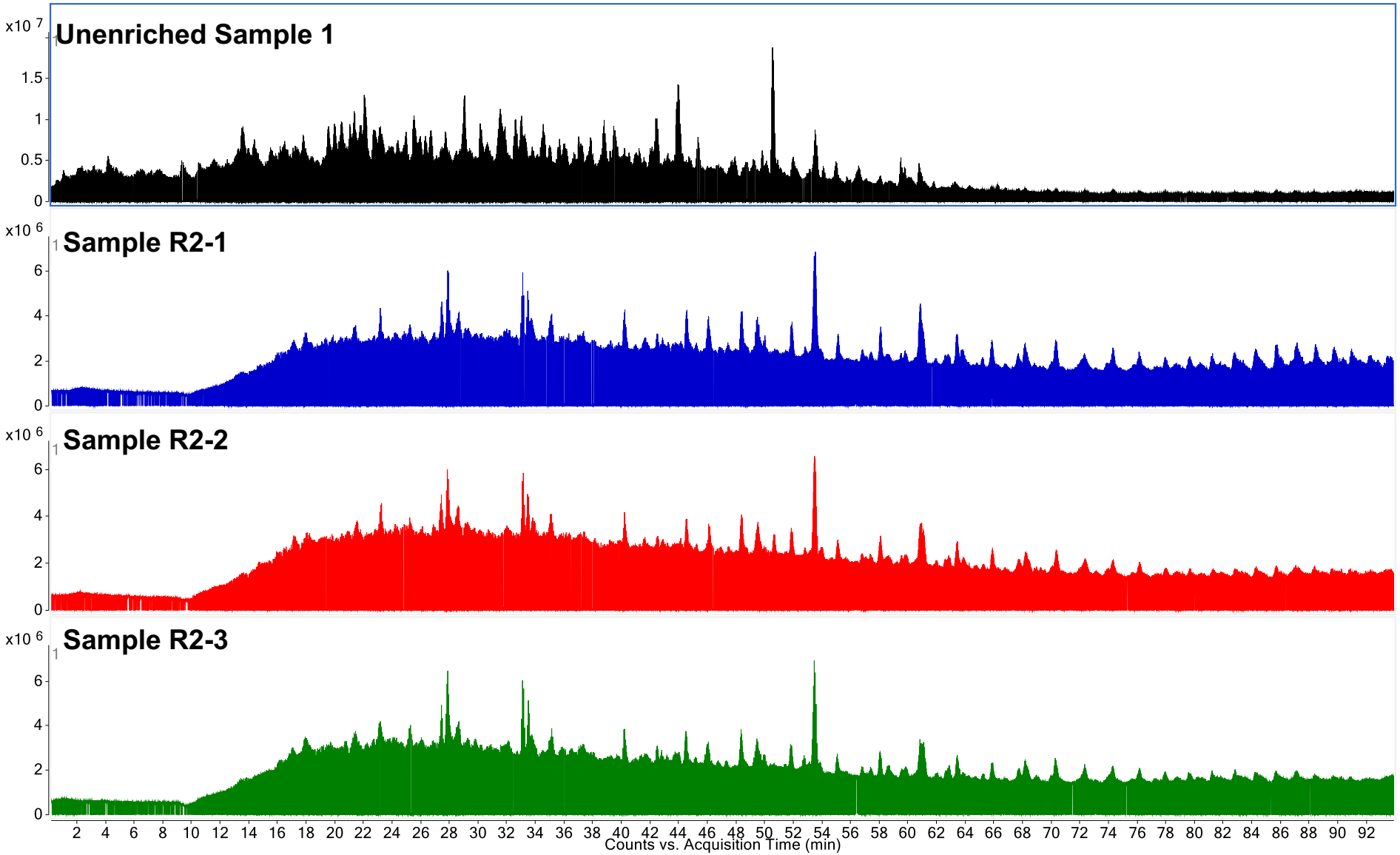
Labware Table

Deck Location	Labware Type
1	96AM Wash Station
2	96AM Cartridge & Tip Seating Station
3	12 Column, Low Profile Reservoir, Natural PP
4	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
5	12 Column, Low Profile Reservoir, Natural PP
6	12 Column, Low Profile Reservoir, Natural PP
7	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
8	12 Column, Low Profile Reservoir, Natural PP
9	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro

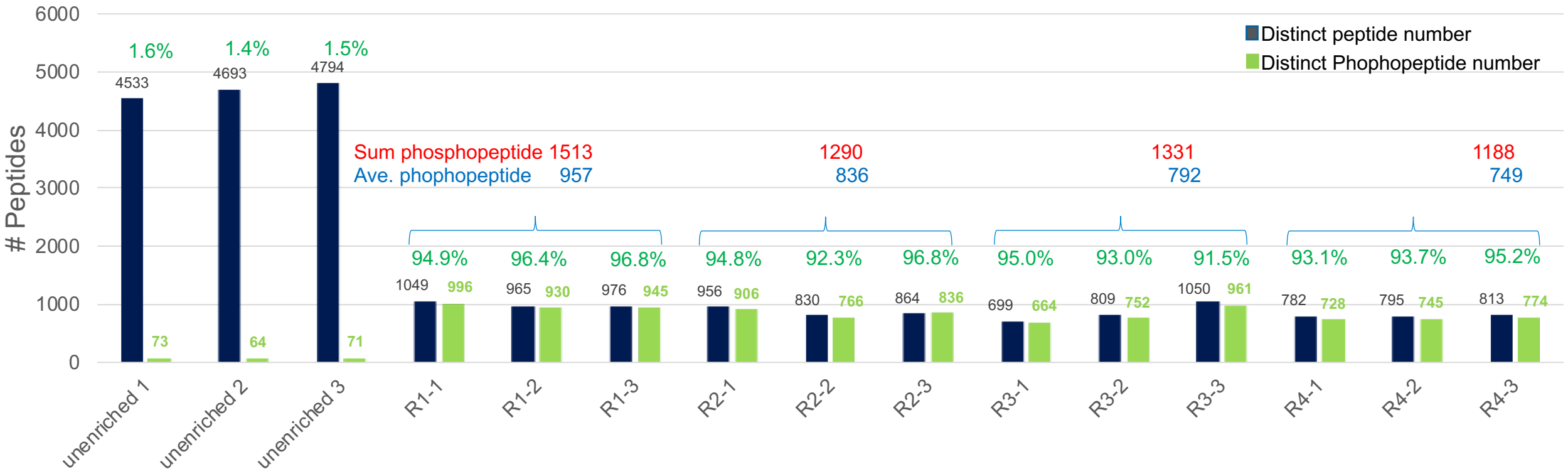
Nano-LC/MS



TIC Using a 90-min LC Gradient



Distinct Phosphopeptides Identified Before and After Enrichment



Enrichment for the Phosphopeptide Standards

Sequence	PhosphoMix	phospho site	Average Yield of Enrichment (%) Sample R1	Average Yield of Enrichment (%) Sample R2	Average Yield of Enrichment (%) Sample R3	Average Yield of Enrichment (%) Sample R4	RSD (%)
VLHSGsR	1_1	S6	not found	not found	not found	not found	0.0
RSysRSR	1_2	Y3, S4	not found	not found	not found	not found	0.0
RDSLGtYSSR	1_3	T6	54.3	51.9	58.0	58.7	3.2
tKLItQLRDAK	1_4	T1, T5	82.0	78.9	83.7	87.7	3.6
EVQAEQPSSsSPR	1_5	S10	94.7	95.7	94.0	96.0	0.9
ADEPsSEESDLEIDK	1_6	S5	73.6	67.1	73.7	76.7	4.0
ADEPSsEEsDLEIDK	1_7	S6, S9	95.7	92.3	99.0	101.3	3.9
FEDEGAGFEESsETGDYEEK	1_8	S12	79.7	77.3	81.0	92.0	6.5
ELSNsPLRENSFGsPLEFR	1_9	S5, S14	87.3	83.3	88.0	88.0	2.3
SPTEYHEPVyANPFYRPTtPQR	1_10	Y10, T19	84.7	79.5	84.7	74.0	5.1
LPQEtAR	2_1	T5	53.0	50.5	56.7	52.0	2.6
RYssRSR	2_2	S3, S4	not found	not found	not found	not found	0.0
EtQSPEQVK	2_3	T2	not found	not found	not found	not found	0.0
VIEDNEyTAR	2_4	Y7	48.3	46.5	51.3	50.7	2.2
sRSPsSPELNNK	2_5	S1, S5	65.0	71.3	58.3	65.0	5.3
ADEPSSEEsDLEIDK	2_6	S9	61.0	56.6	68.3	66.3	5.3
HQYSDYDyHSSsEK	2_7	Y8, S12	6.0	5.3	5.7	4.0	0.9
NTPsQHSHsIQHSPER	2_8	S4, S9	12.0	18.0	14.3	not found	3.0
ELsNsPLRENSFGSPLEFR	2_9	S3, S5	70.3	67.0	70.0	77.3	4.4
LGPGRPLPTFPtSECTSDVEPDTR	2_10	T12	35.7	33.3	36.3	35.0	1.3

Conclusions

Phoshopeptide enrichment using the AssayMAP Bravo for sample preparation

- Enrichment
  - is consistently greater than 90%
- Reproducibility
  - CV is generally 5% or less (average of 3.5% in this experiment)
- Yield
  - Ranged from approximately 5 to 100% with an average of approximately 60%
- Phospho-sites
  - Approximately 60% were identified
- The number of phosphopeptide identifications generally increased with sample mass but not proportionally.