Speeding up the cancer biomarker discovery: Advanced Clinical Proteomics workflow with High Resolution Accurate Mass (HRAM) MS

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Protein Biomarker Development

- **Precision medicine**
  - Validated biomarkers to better classify patients
    - Disease risk
    - Prognosis
    - Response to treatment
  - Critical need for better biomarkers, especially in oncology field

- **Biomarkers**
  - Correlational (*i.e.*, only associated with the disease)
  - Functional (*i.e.*, with identified mechanism of action)
  - Single biomarker *versus* biomarker panel
  - Systematic, accurate, and precise quantification in bodily fluids

Challenges of Biomarker Development

- **Candidate biomarkers by discovery LC-MS/MS (data dependent acquisition)**
  - ○ Numerous candidates, differential regulation in tissues / cell lines
  - ✓ Simple experimental design
  - ✓ Fast, generate large data sets
  - × Bias towards high-abundance components
  - × Under-sampling issue (missing values)

- **Validated biomarkers by immunoassays (ELISA)**
  - ○ Limited set of validated biomarkers, precise quantification in bodily fluids
  - ✓ Well established, robust technique
  - ✓ Very sensitive
  - × Costly and tedious to develop (for each target)
  - × Difficult to multiplex analytes

Biomarker Development Pipeline

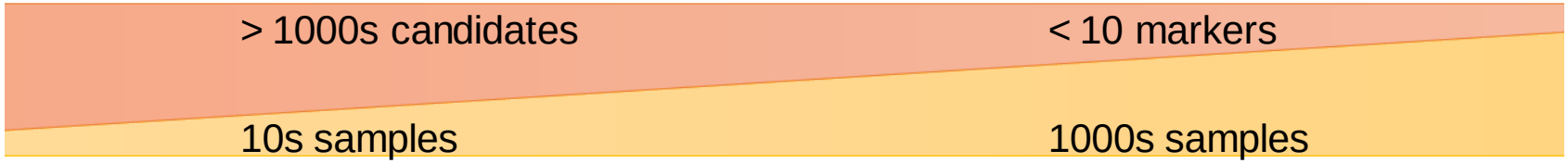
- **Challenge: reliable and high-throughput evaluation of the numerous candidates**
- **Evaluation stage to aid the transition between discovery and clinical stages**

(Prior knowledge:
genomics, literature)

Tissues/cell lines
Discovery LC-MS/MS (DDA)

Bodily fluids

Bodily fluids
Immunoassays (ELISA)



Biomarker Development Pipeline

- Challenge: reliable and high-throughput evaluation of the numerous candidates
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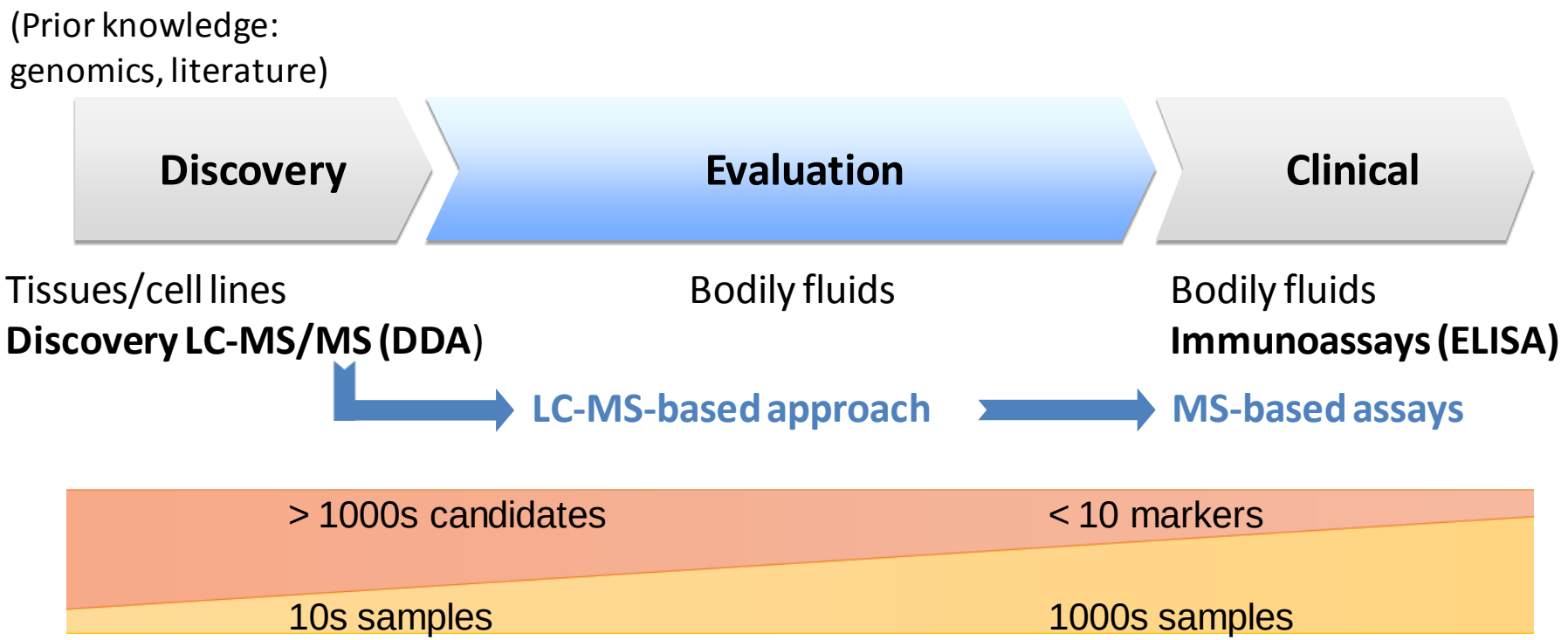


Biomarker Evaluation Stage

- **Systematic quant. of proteins in clinical samples (*e.g.*, plasma) is challenging**

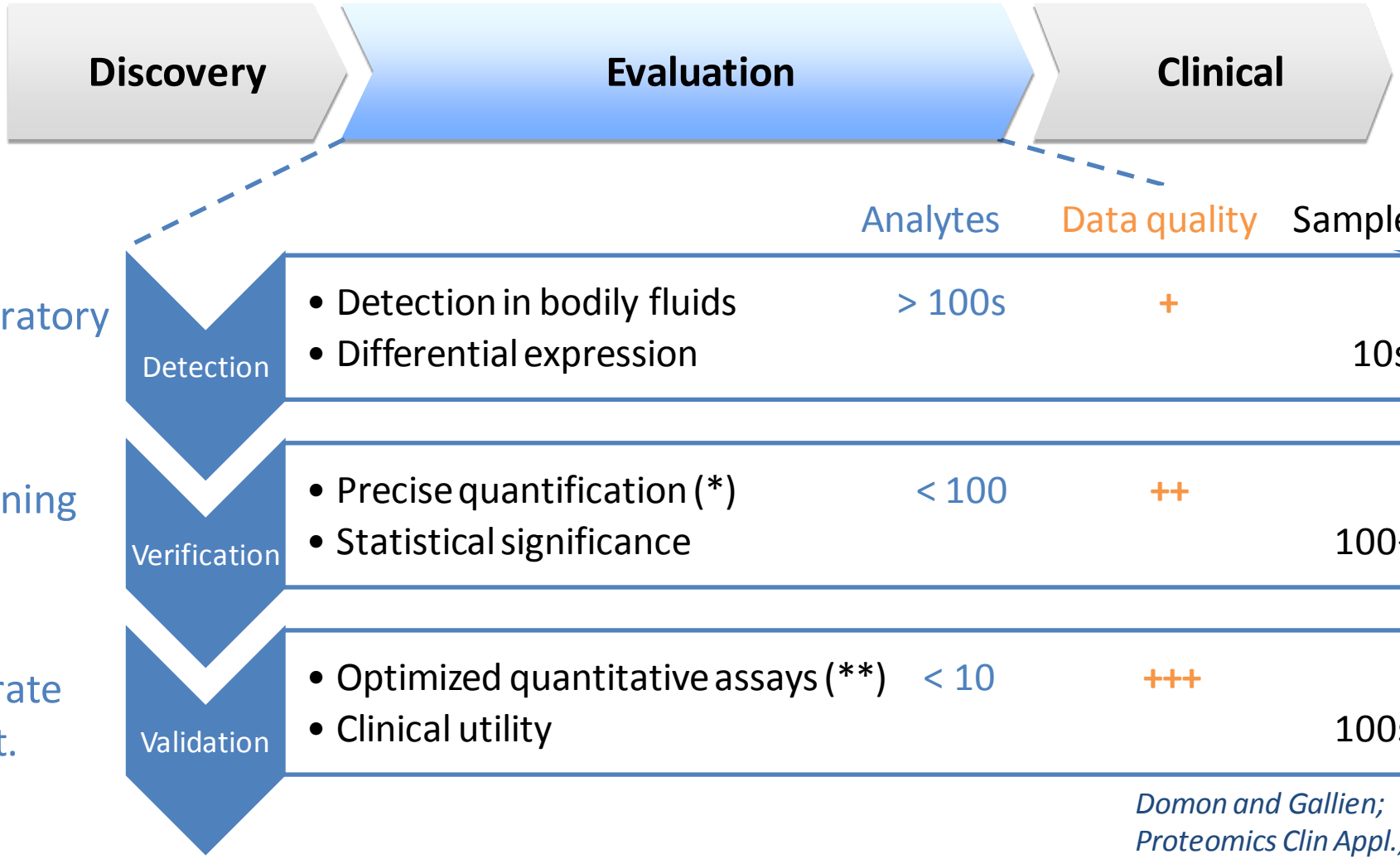
- **High performance LC-MS instruments**
  - Wide dynamic range (many orders of magnitude)
  - High sensitivity (low amol range)
  - Selectivity (to cope with the massive biochemical background)

- **Current practice**
  - Targeted LC-MS/MS analysis

    Predefined set of peptides surrogates for the proteins of interest
  - Analytical performance ∝ acquisition time

    Trade-off sensitivity, selectivity, and scale -> fit-for-purpose approaches

Fit-For-Purpose Targeted LC-MS approaches



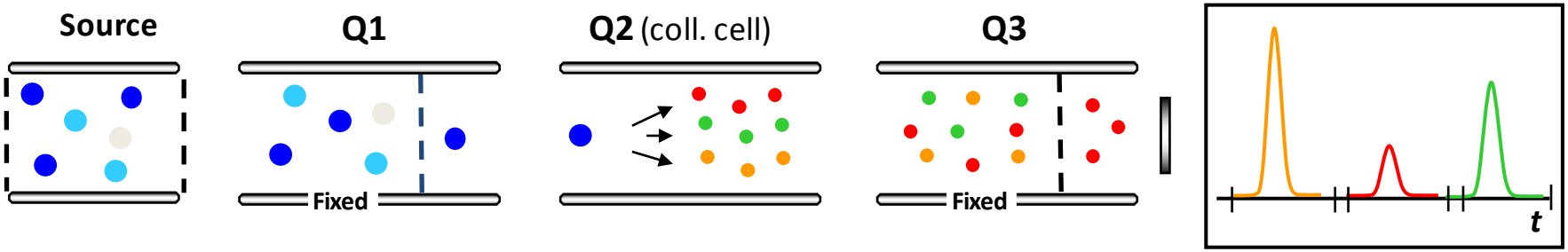
Domon and Gallien; Proteomics Clin Appl., 2015

(*) Use of internal standards, confirmation of identity

(**) Quantification using standard curves, calibrated amounts of internal standards

Targeted Proteomics

- **Selected reaction monitoring (SRM):** triple quadrupole instrument – ref method
  - Reproducible quantification of proteins
  - High sensitivity because of high duty cycle (Q1 and Q3 are static)
  - Wide dynamic range of measurements



Triple quadupole MS

Serial acquisition of transitions

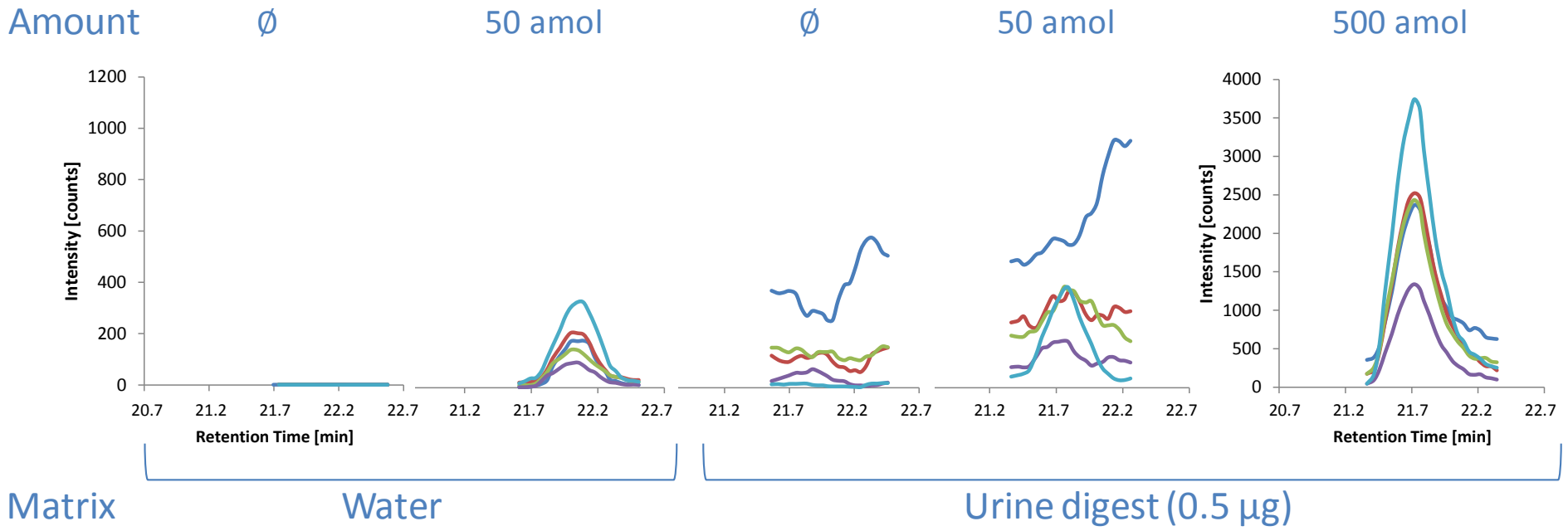
- **Limitations**
  - Complex and rigid workflow
  - Selectivity driven by the two levels of mass selection (at low resolution)

Gallien et al.; J. Mass Spectrom., 2015

Selectivity issue in SRM analysis

- Peptide SDLVNEEATGQF**R** spiked in different types of samples
- Transitions
  - 738.35 [(M+2H)2+] -> 618.32 (y5+)
  - 738.35 [(M+2H)2+] -> 689.36 (y6+)
  - 738.35 [(M+2H)2+] -> 818.40 (y7+)
  - 738.35 [(M+2H)2+] -> 947.45 (y8+)
  - 738.35 [(M+2H)2+] -> 1061.49 (y9+)

Thermo Scientific™
TSQ Vantage™



Domon et al.; Proteomics Clin Appl., 2012

Towards Advances in Biomarker Development

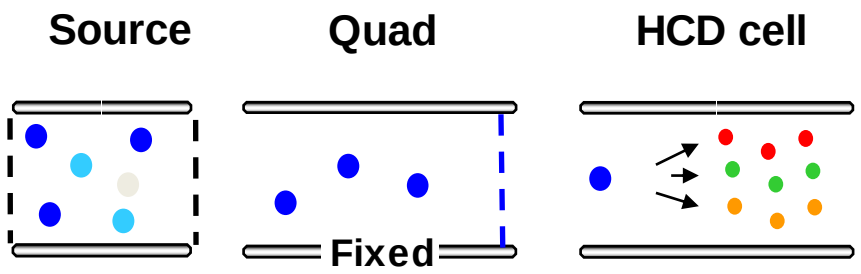
- **Alternative to SRM: Parallel reaction monitoring (PRM)**
  - Keep key advantages of targeted acquisition
  - Overcome limitations of SRM: low selectivity (unit mass resolution)

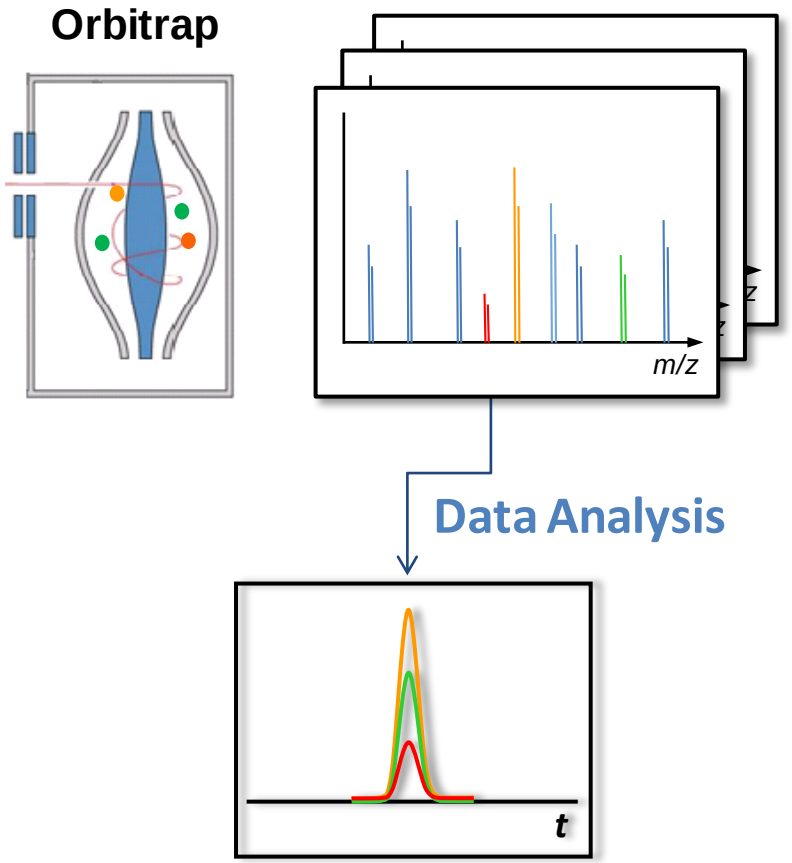
- **Benefits of high resolution accurate mass (HRAM) targeted methods on Quadrupole-Orbitrap**
  - High selectivity – high resolution: discriminate matrix / analytes
  - Trapping capabilities: exquisite sensitivity

- **Advanced PRM acquisition schemes**
  - Expedite the execution of biomarker evaluation stages
  - Improved acquisition efficiency
  - Progress towards the implementation of MS-based clinical assays

Parallel Reaction Monitoring (PRM)

HRAM -> Thermo Scientific™ Quadrupole – Orbitrap™ instrument



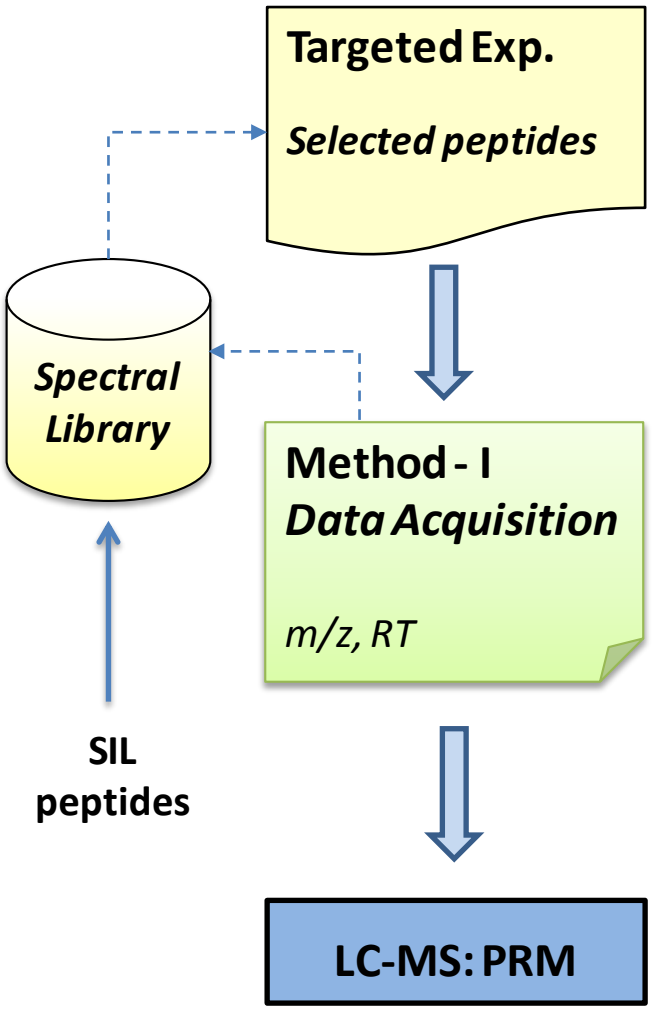


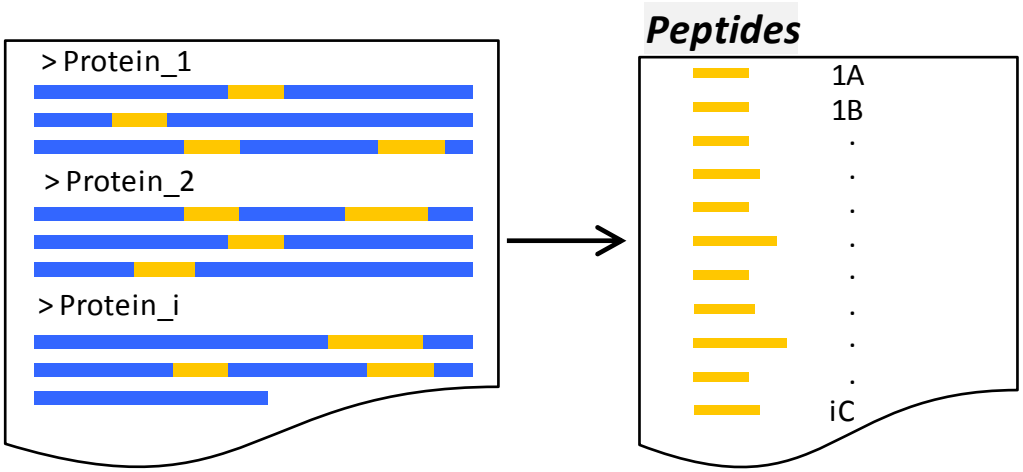
Immediate benefits of full MS/MS

- **Simplified experimental design**
  Few parameters required (m/z, RT)
- **Flexible processing method**
  Iterative analysis
  Fragment selected post-acquisition

Gallien et al.; Mol. Cell. Proteomics, 2012
Peterson et al.; Mol. Cell. Proteomics, 2012

PRM Analytical Workflow /1

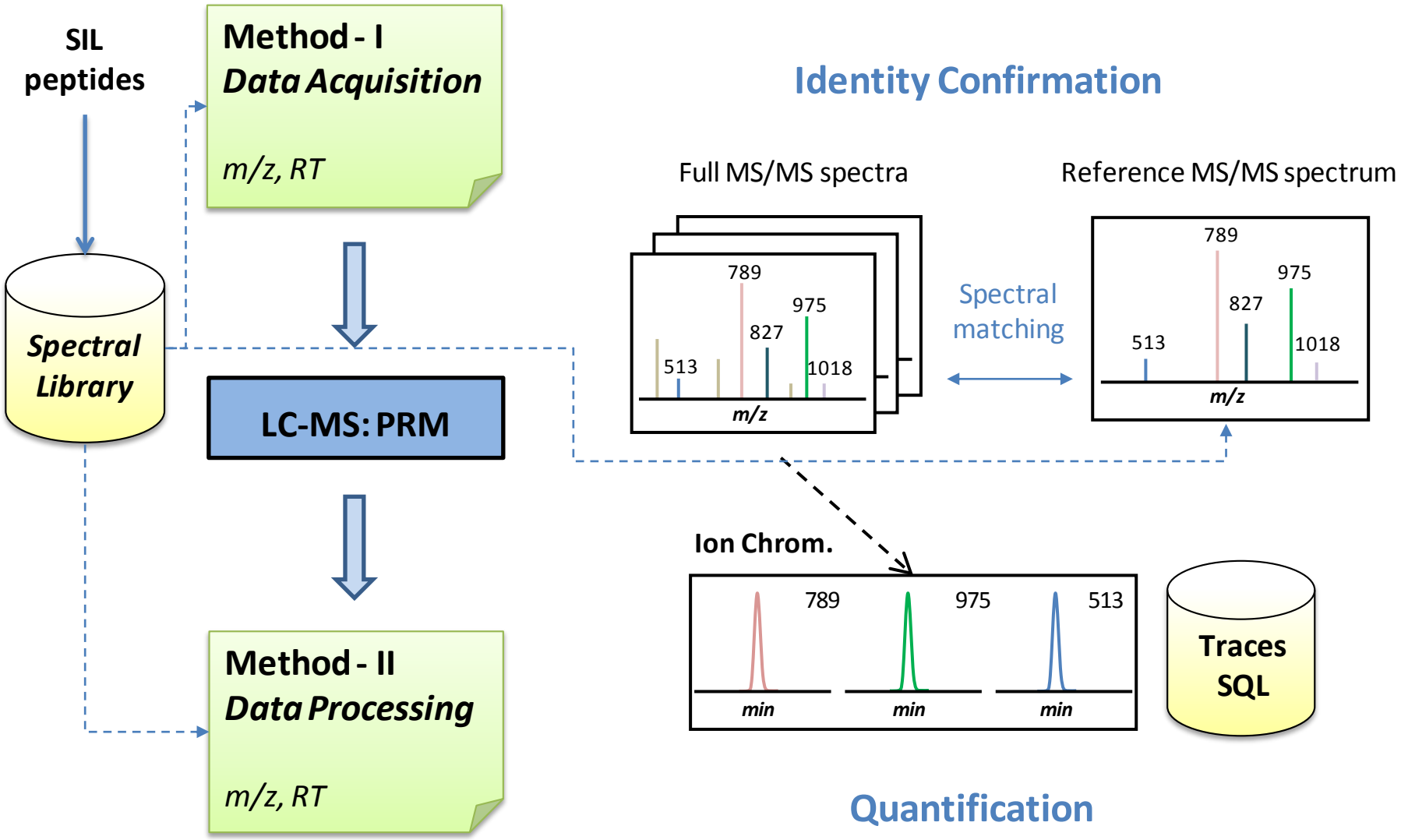




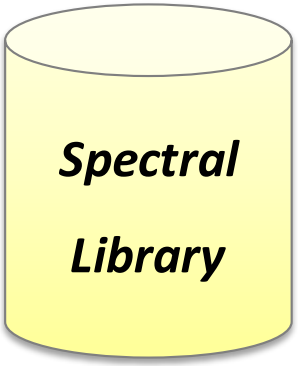
Peptide	Q1 m/z	RT [min]	Isol. win.	Orbitrap Res.	Fill Time [ms]	CE
Pep_1A	400	15	1	35 k	100	25
Pep_1B	500	20	1	35 k	100	25
Pep_1C	550	35	1	35 k	100	25
Pep_2A	700	17	1	35 k	100	25
.....	.....	.....	.....	.....	.....	.....
Pep_iC	900	25	1	35 k	100	25

Gallien et al.; Methods, 2015

PRM Analytical Workflow /2

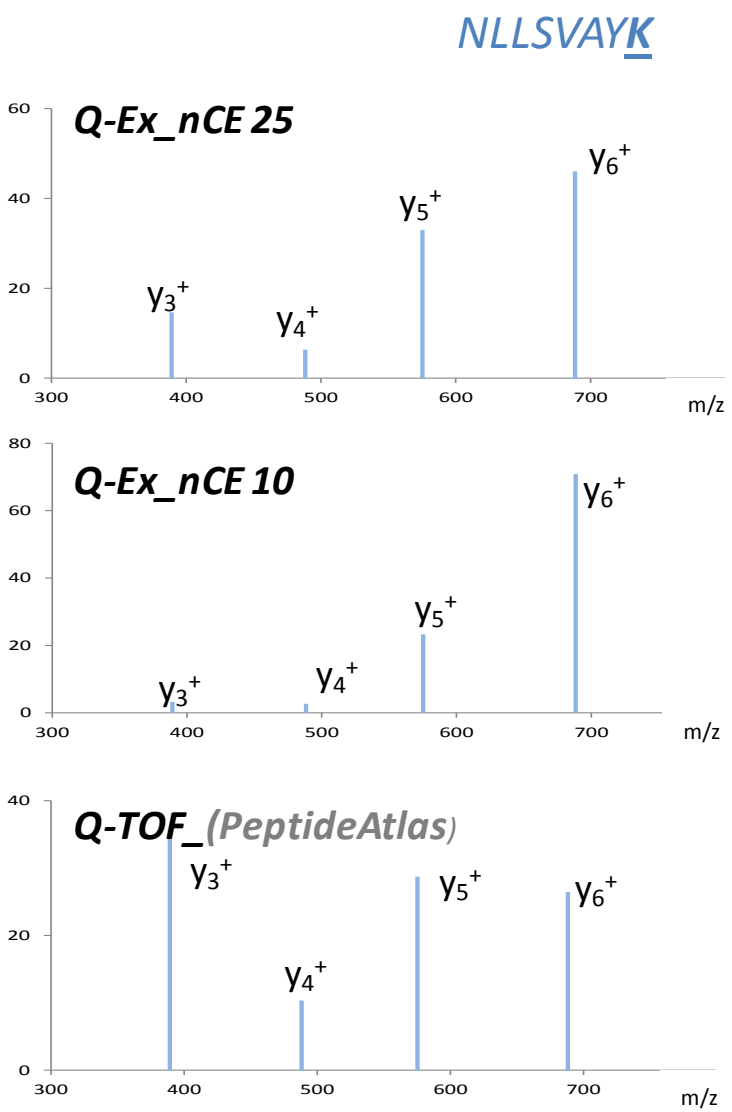


Reference Libraries



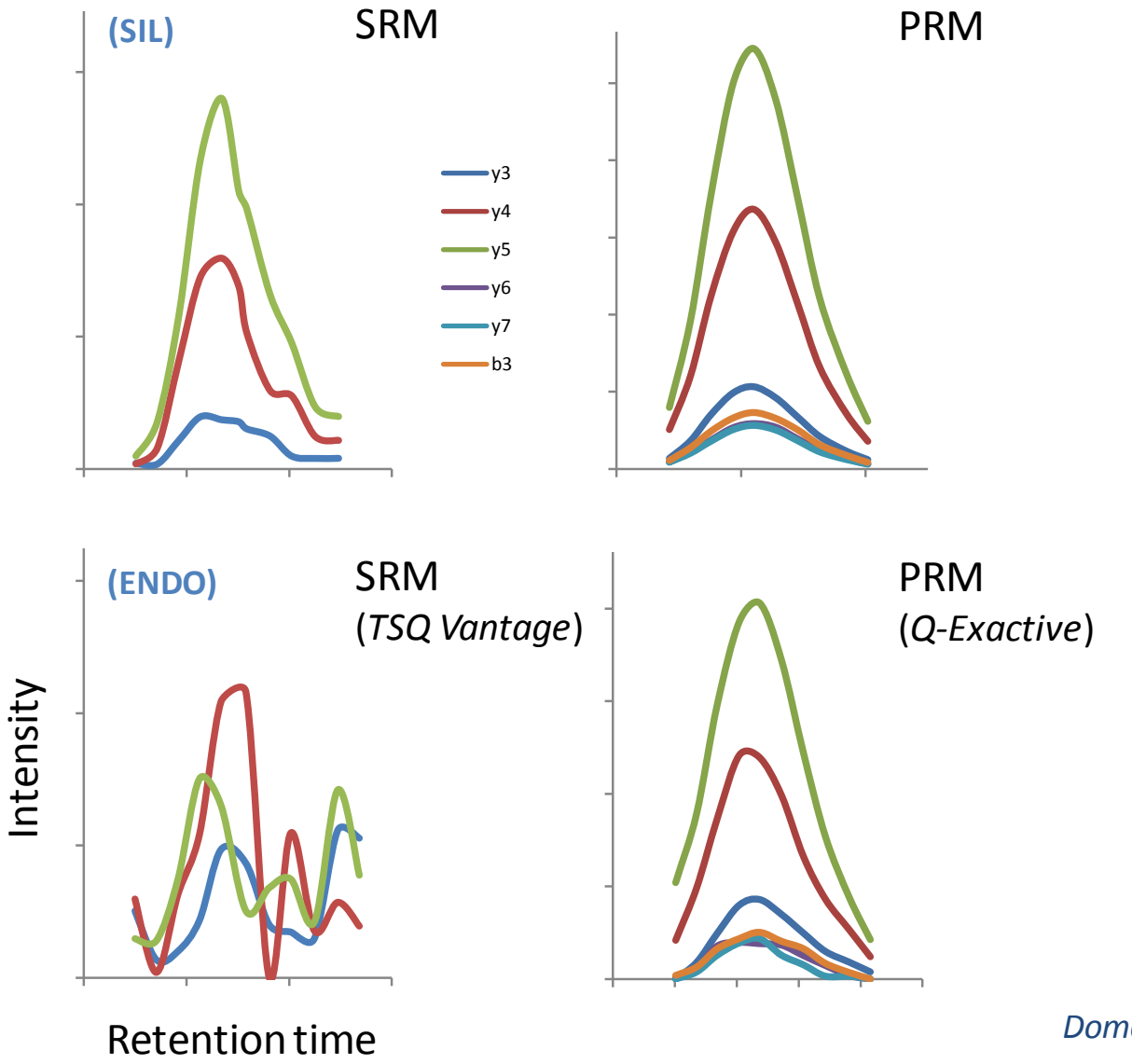
Reference Library

- Project specific *(SIL peptides)*
- MS/MS: Controlled fragmentation patterns (***m/z and rel. intensities***) *(gas pressure, collision energy)*
- LC: Defined elution times
- MS: Response factor (estimation)



Kim, Gallien, Van Oostrum, and Domon; Proteomics Clin Appl., 2013

PRM Measurements in Plasma Samples



GDSVVYGLR (*Osteopontin*) in plasma samples

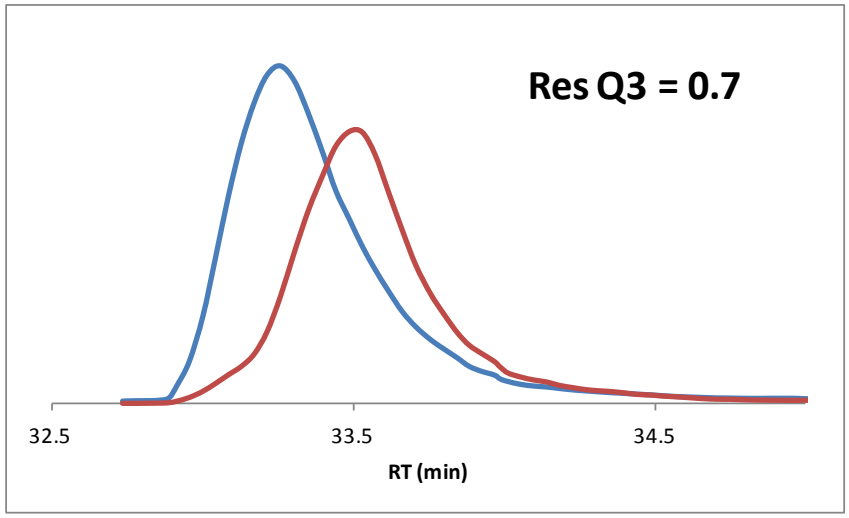
PRM

- Higher sensitivity
- Better confidence in measurements
- Improved selectivity

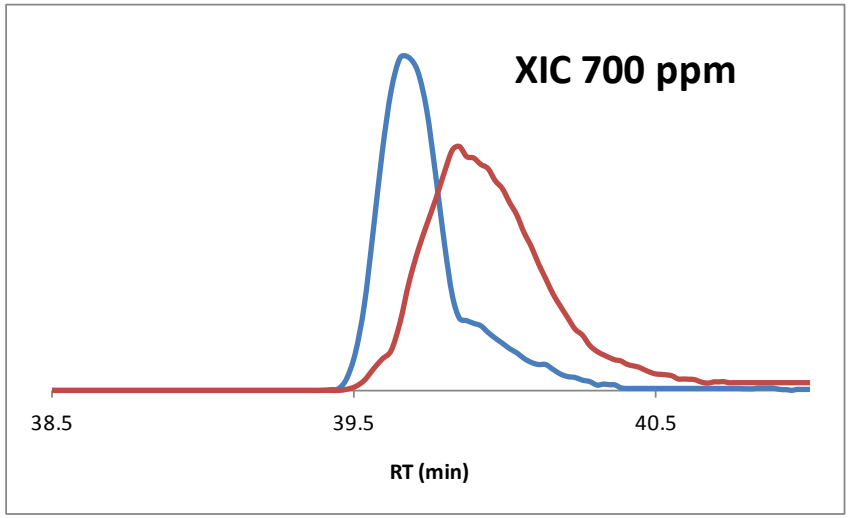
Domon and Gallien; Proteomics Clin Appl., 2015

Selectivity in Orbitrap HRAM PRM

SRM on *Thermo Scientific™ TSQ Vantage™*

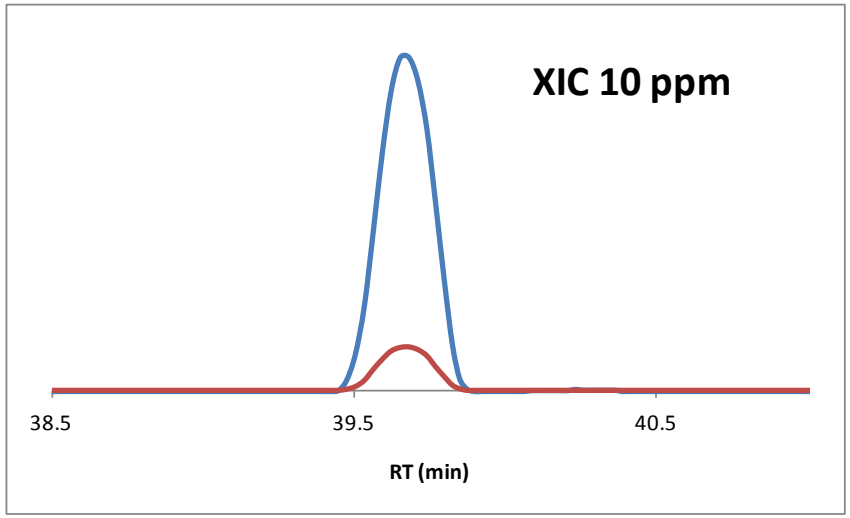


PRM on *Thermo Scientific™ Q Exactive™*



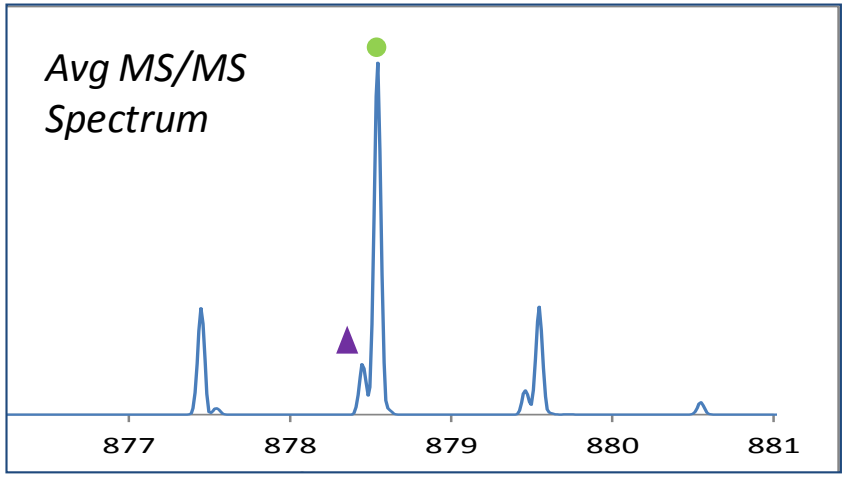
SDLAVPSELALLKY**K**
spiked in urine sample

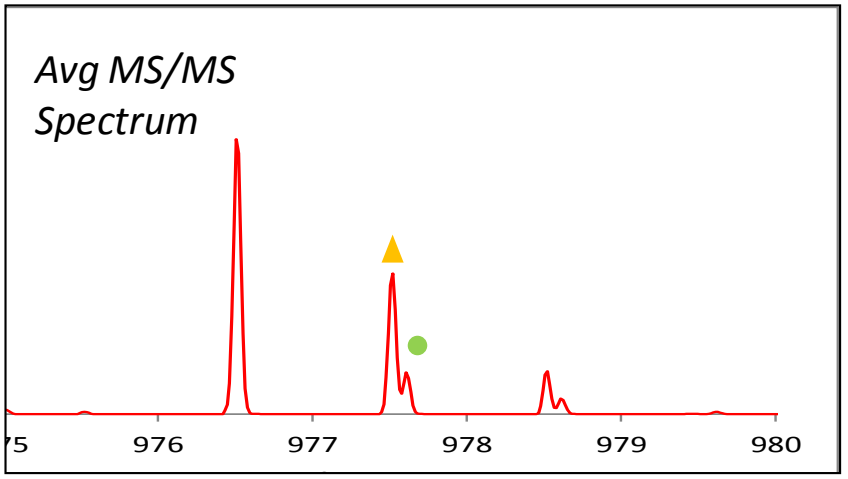
Transitions : 682.40->878.54
682.40->977.61



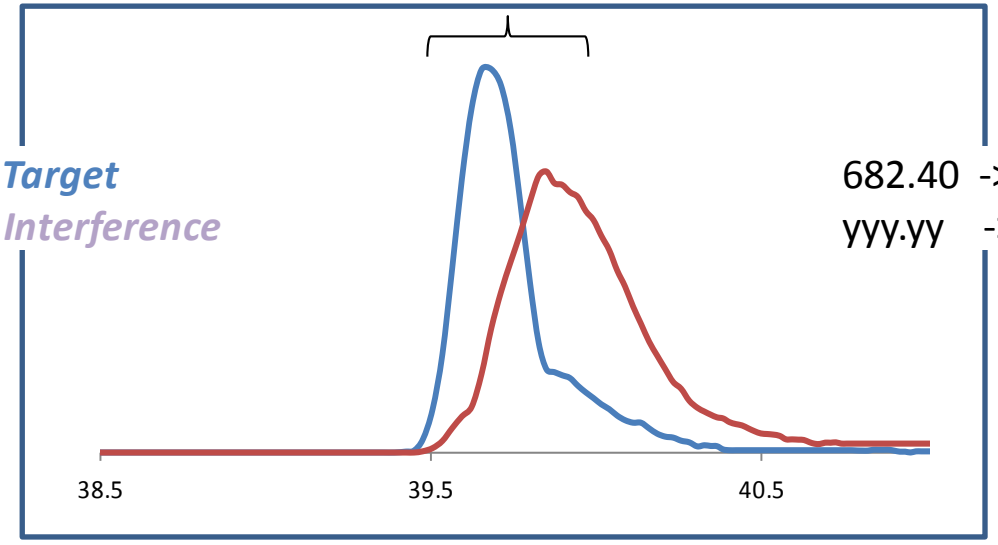
Gallien et al.; J. Proteomics , 2013

Interferences





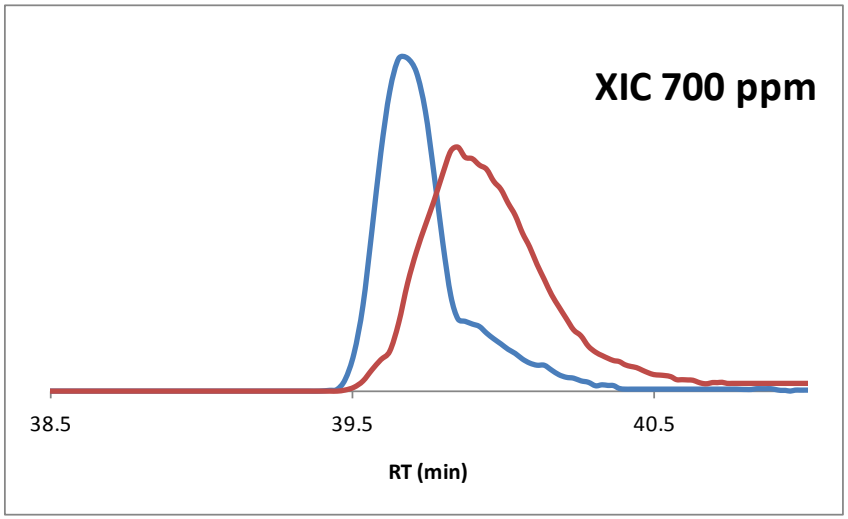
682.40 -> 878.54, ***Target***
xxx.xx -> 878.45, ***Interference***

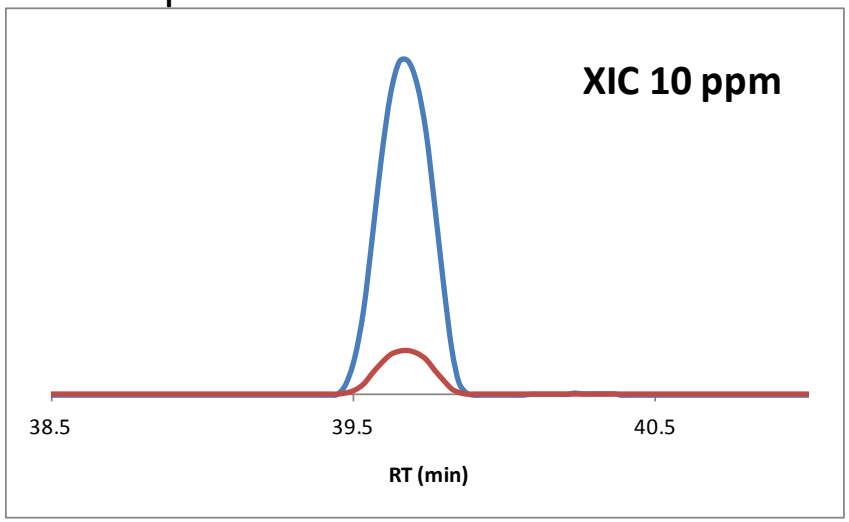


682.40 -> 977.61, ***Target***
yyy.yy -> 977.52, ***Interference***

Selectivity in Orbitrap HRAM PRM

PRM on Q Exactive Orbitrap MS

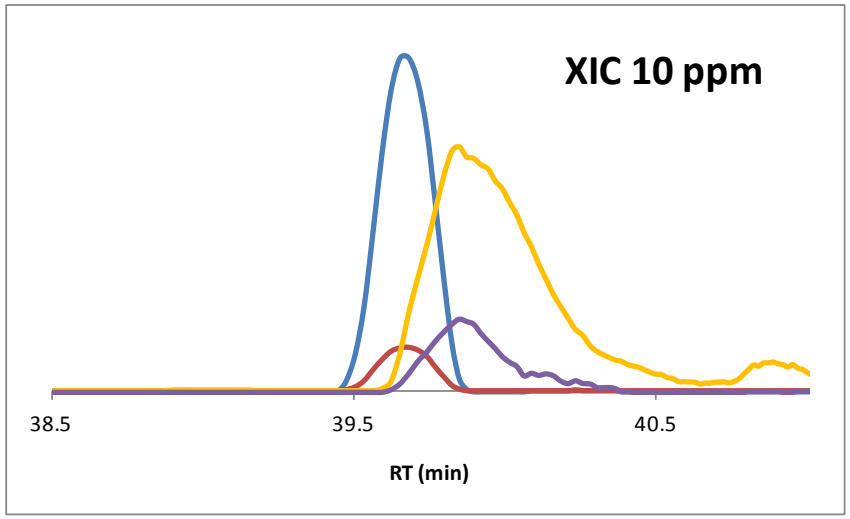




SDLAVPSELALLKY**K**
spiked in urine samples

Transitions :

- 682.40 -> 878.54
- xxx -> 878.45 , ***Interference***
- 682.40 -> 977.61
- yyy -> 977.52, ***Interference***



Quantification of SAA1/SAA2 in Lung Cancer Plasma

Analytical challenge for immunoassays and MS-based assays

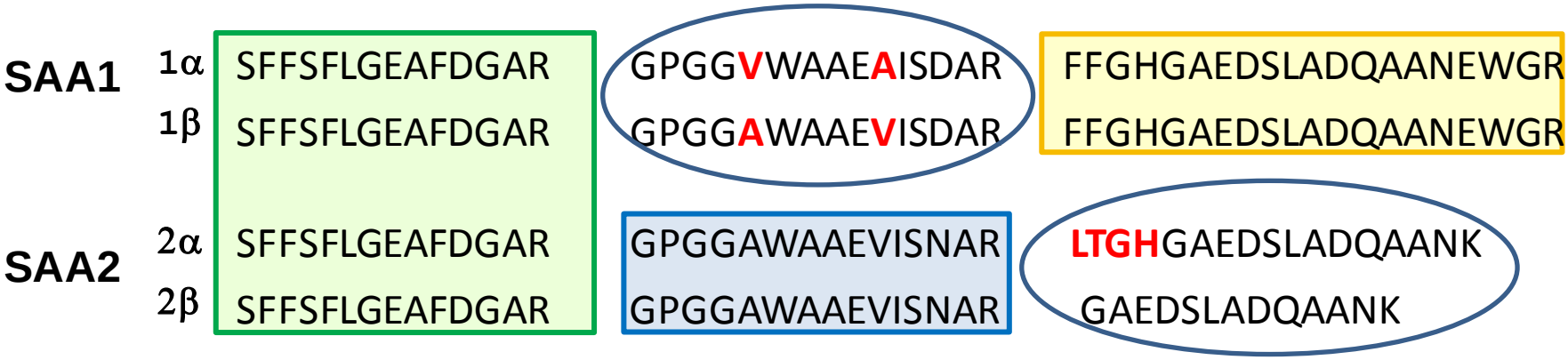
- Serum amyloid A proteins (SAA1 and SAA2)
- Elevated plasma levels in patients diagnosed with lung cancer
- Highly homologous proteins; several allelic variants

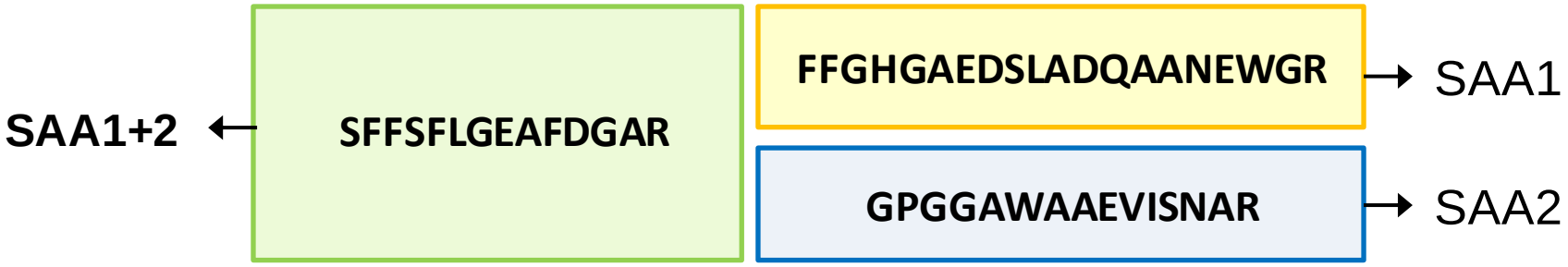
```
1α   MKLLTGLVFCSLVLGVSSRSFFSFLGEAFDGARDMWRAYSDMREANYIGSDKYFHARGNYD
1β   MKLLTGLVFCSLVLGVSSRSFFSFLGEAFDGARDMWRAYSDMREANYIGSDKYFHARGNYD
2α   MKLLTGLVFCSLVLGVSSRSFFSFLGEAFDGARDMWRAYSDMREANYIGSDKYFHARGNYD
2β   MKLLTGLVFCSLVLGVSSRSFFSFLGEAFDGARDMWRAYSDMREANYIGSDKYFHARGNYD

1α   AAKRGPGGVWAAEAISDARENIQRFFGHGAEDSLADQAANEWGRSGKDPNHFRPAGLPEKY
1β   AAKRGPGGAWAAEVISDARENIQRFFGHGAEDSLADQAANEWGRSGKDPNHFRPAGLPEKY
2α   AAKRGPGGAWAAEVISNARENIQRLTGHGAEDSLADQAANKWGRSGKDPNHFRPAGLPEKY
2β   AAKRGPGGAWAAEVISNARENIQRLTGRGAEDSLADQAANKWGRSGKDPNHFRPAGLPEKY
```

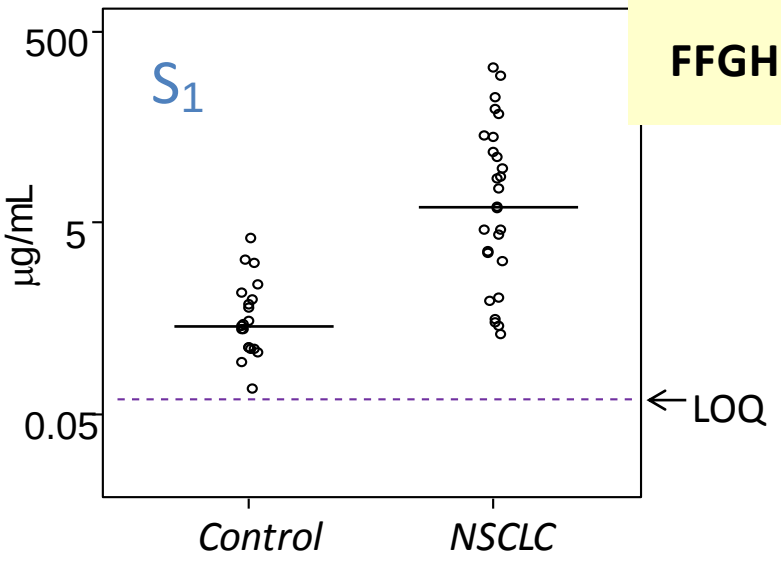
Kim et al.; Proteomics , 2015

Development of PRM Assays



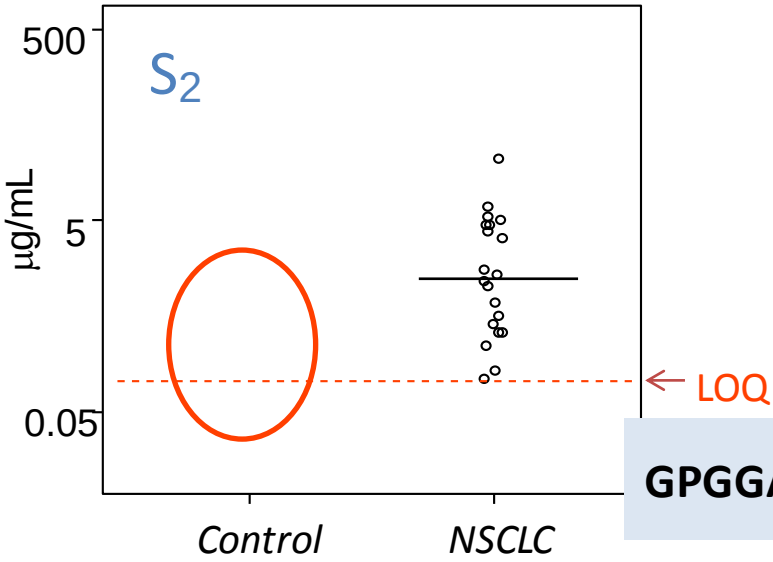


PRM Assays: SAA1 and SAA2



FFGHGAEDSLADQAANEWGR

	Control samples	*NSCLC samples*
Samples	20	27
S1 > LOQ	20	27
S2 > LOQ	0	20



Missing values: below quantification limit

GPGGAWAAEVISNAR

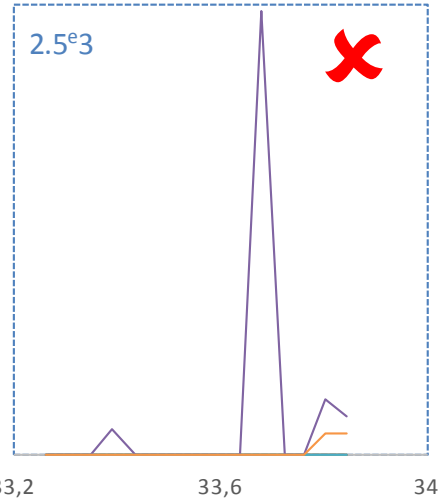
PRM Assays: SAA2 Isotypes (LOQ)

S_2: GPGGAWAAEVISNAR

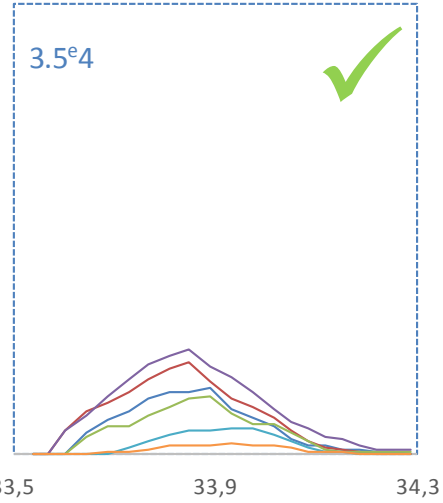
$S_{2\alpha}$ LTGHGAEDSLADQAANK

$S_{2\beta}$ GAEDSLADQAANK

0.01 µg/mL



0.1 µg/mL

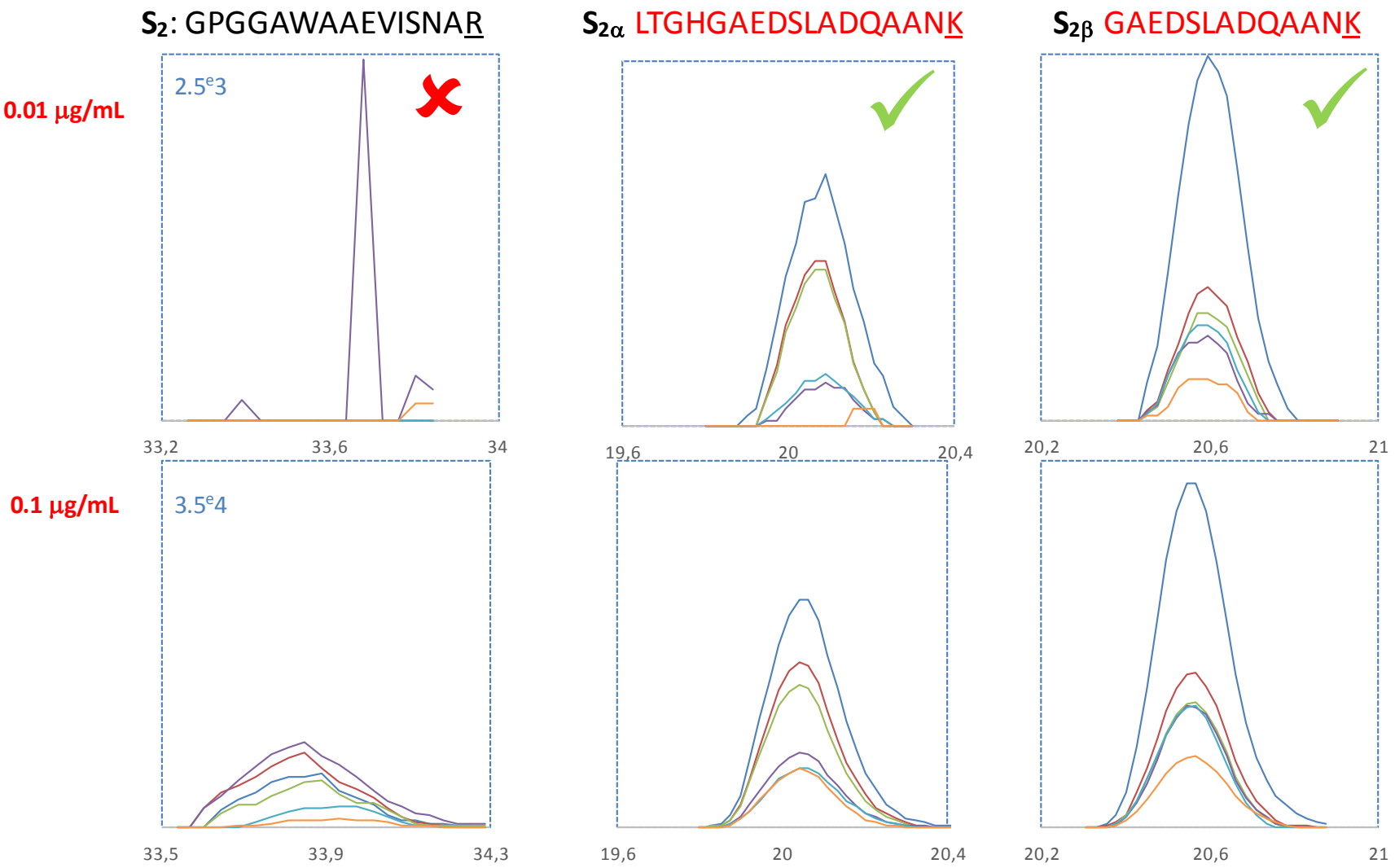


GPGGAWAAEVISNAR
GPGGAWAAEVISNAR

LTGHGAEDSLADQAANK
GAEDSLADQAANK

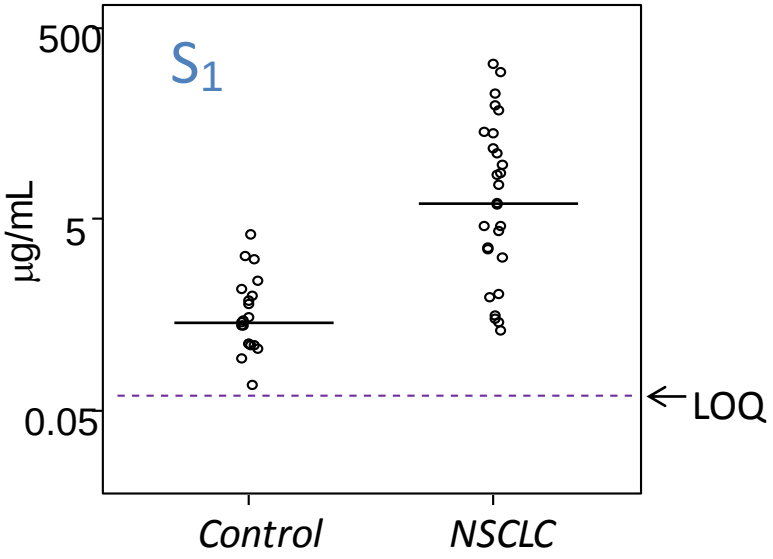
(*Q-Exactive MS*)

PRM Assays: SAA2 Isotypes (LOQ)

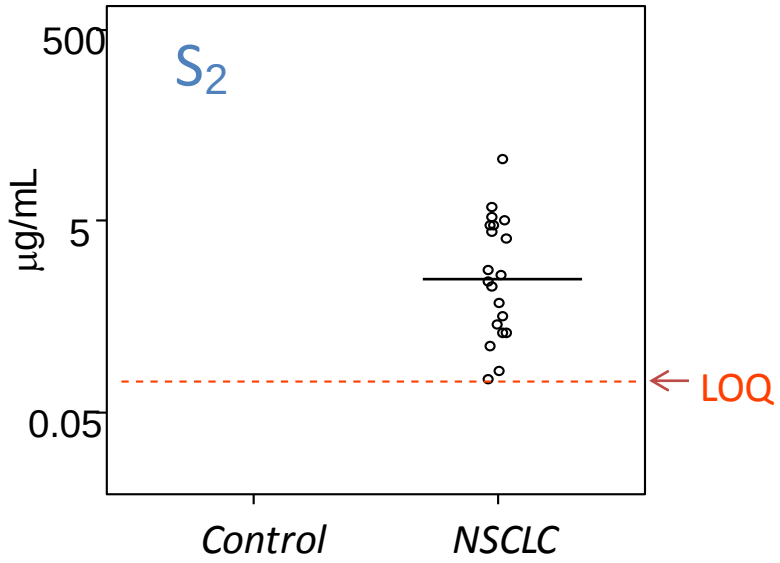


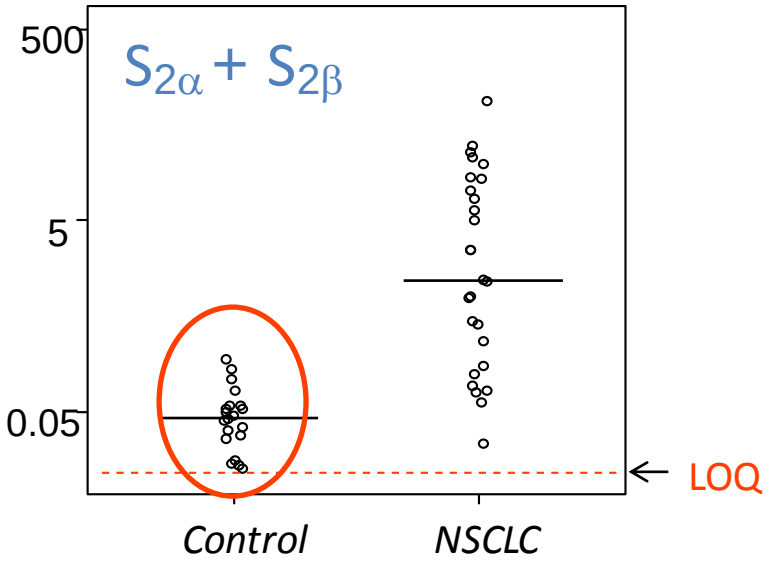
(*Q-Exactive MS*)

Quantification of SAA1/SAA2 in Lung Cancer Plasma

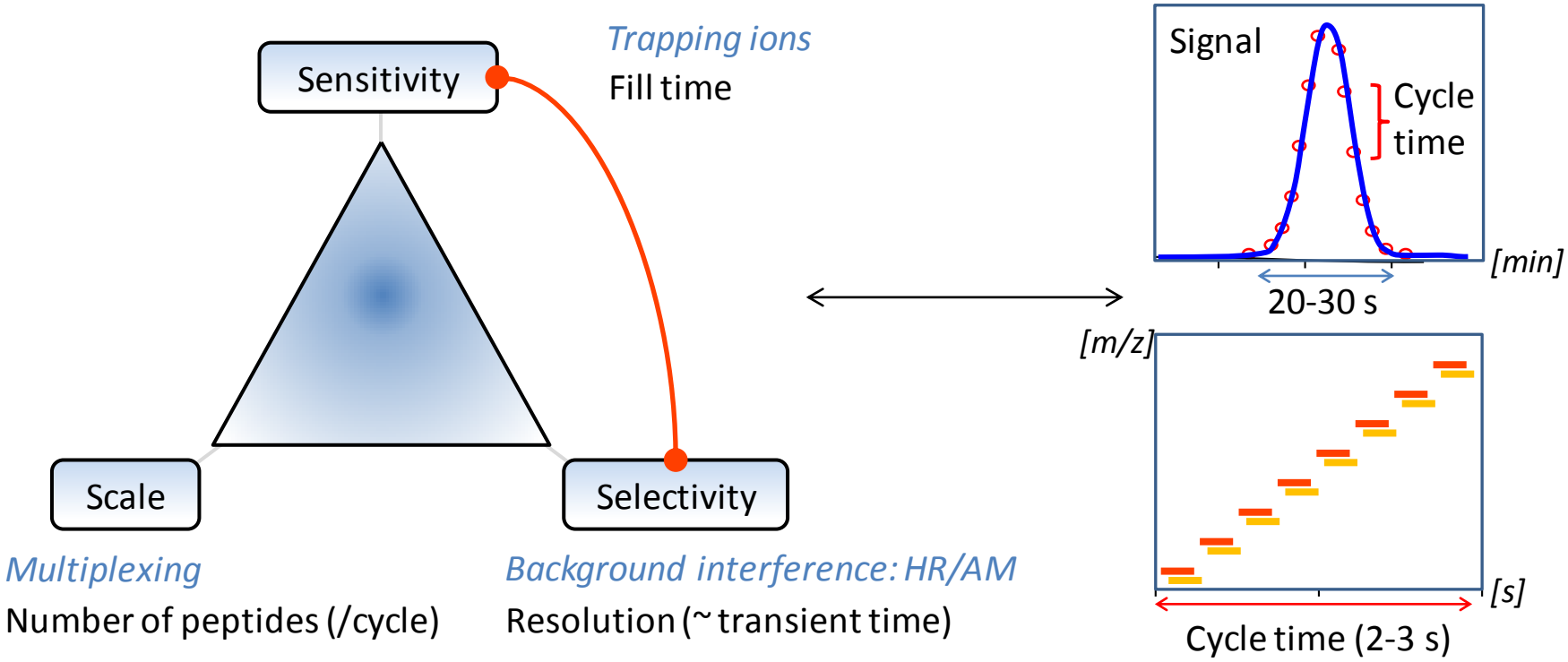


	Control samples	*NSCLC samples*
Samples	20	27
S1 > LOQ	20	27 (↑)
S2α+S2β > LOQ	20	27 (↑)





PRM Parameter Settings and LC Constraints



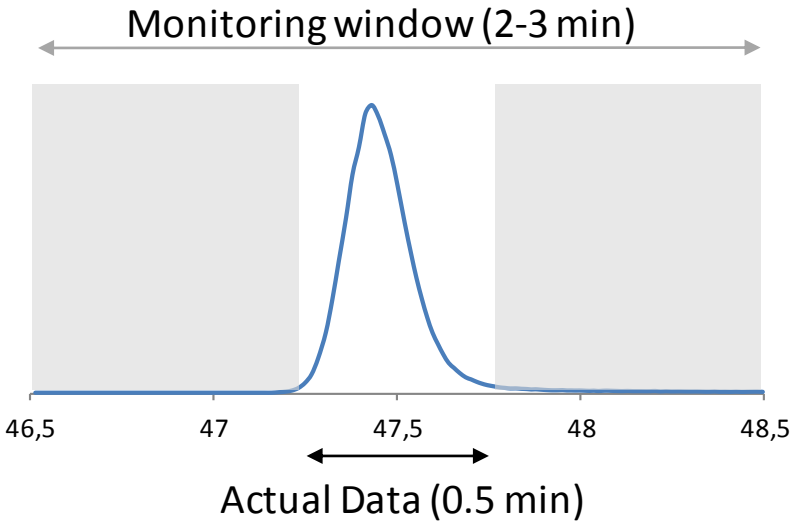
	Scale	Quality
Resolution	17 500	**70 000**
Fill time [ms]	60	**250**
Number of pep. / cycle	**40**	10

[Thermo Scientific™ Q Exactive™ Plus MS]

Acquisition Efficiency in Targeted Exp.

- Time-scheduled acquisition
- Long monitoring windows
- Elution time drift
- Conventional PRM

  Efficiency: 15-25 %



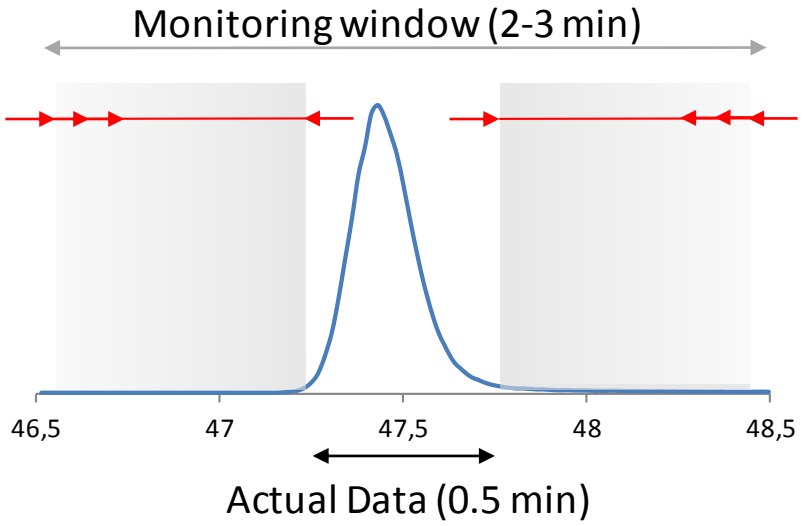
Acquisition Efficiency in Targeted Exp.

- Time-scheduled acquisition
- Long monitoring windows
- Elution time drift
- **Aim:**

  ↘ Wasted resources

  (↘ LC windows and acquisition time)

  ↗ Acquisition efficiency



IS Triggered-PRM: a Two-Step Dynamic Process

- Rational
  - Use internal standards to drive acquisition
  - Use two distinct modes of acquisition: WATCH and QUANT modes

- Control the monitoring windows *(Gallien et al.; J Proteomics, 2014)*
  - Use a few reference peptides (landmarks)
  - On-the-fly correction for drifts in targeted peptide elution times
  - Narrow monitoring windows (60 - 90 s)

- Internal Standards (IS) used to trigger the acquisition of ENDO peptides
  - WATCH mode: monitor IS (stable isotopically labeled peptide, SIL)

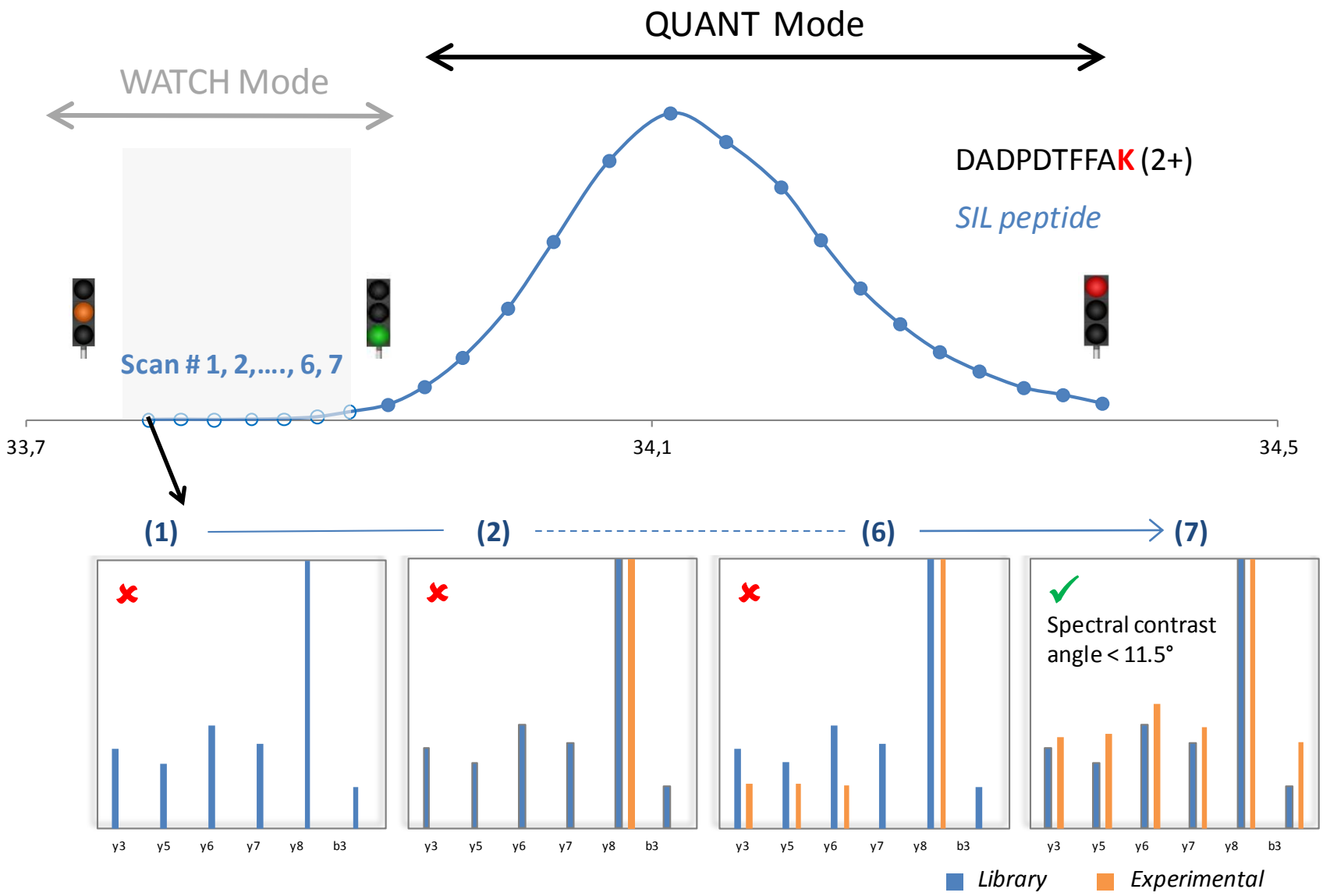
LoRes.; short fill time; real time data analysis

  - Switch to QUANT mode : measure ENDO and SIL peptides

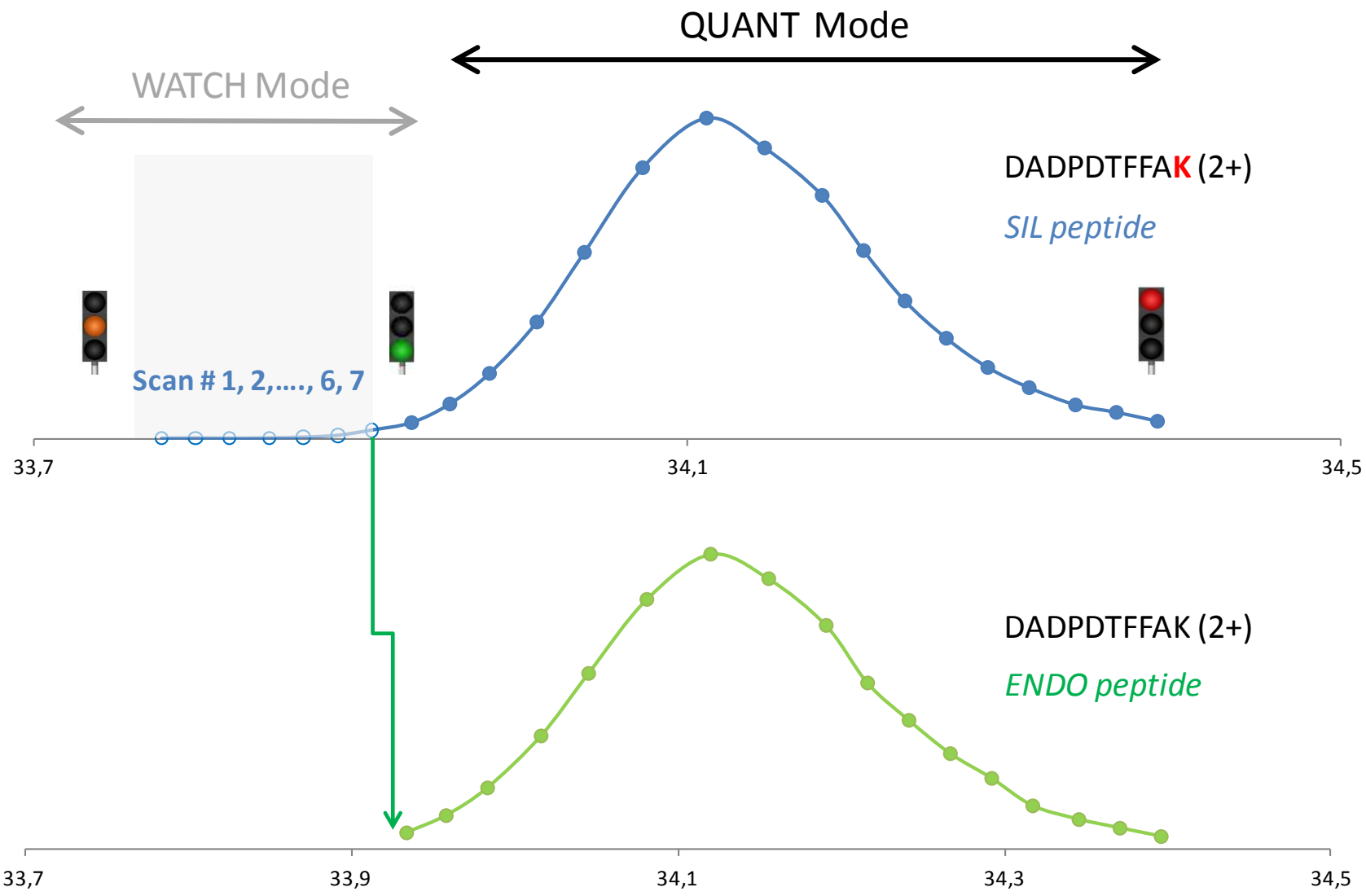
HiRes; long fill time; actual elution

Gallien , Kim, and Domon; Mol. Cell. Proteomics, 2015

IS Triggered-PRM: *WATCH* Mode (*IS-PRM)*

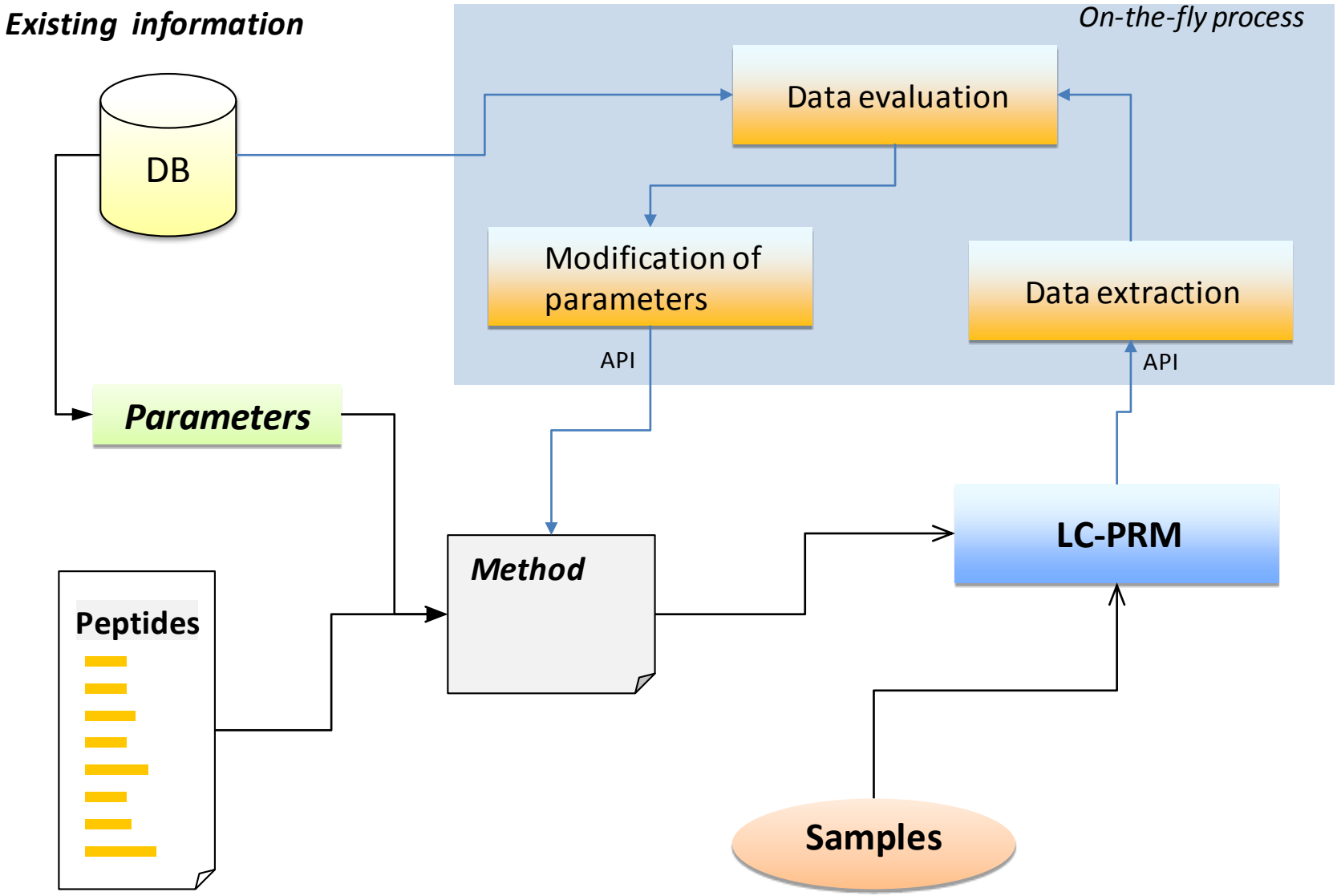


IS Triggered-PRM: *QUANT* Mode (*IS-PRM)*



Acquisition efficiency: 90 %

IS-PRM: Dynamic Parameter Setting



Application: Screening of Biomarkers in Plasma

Experiment

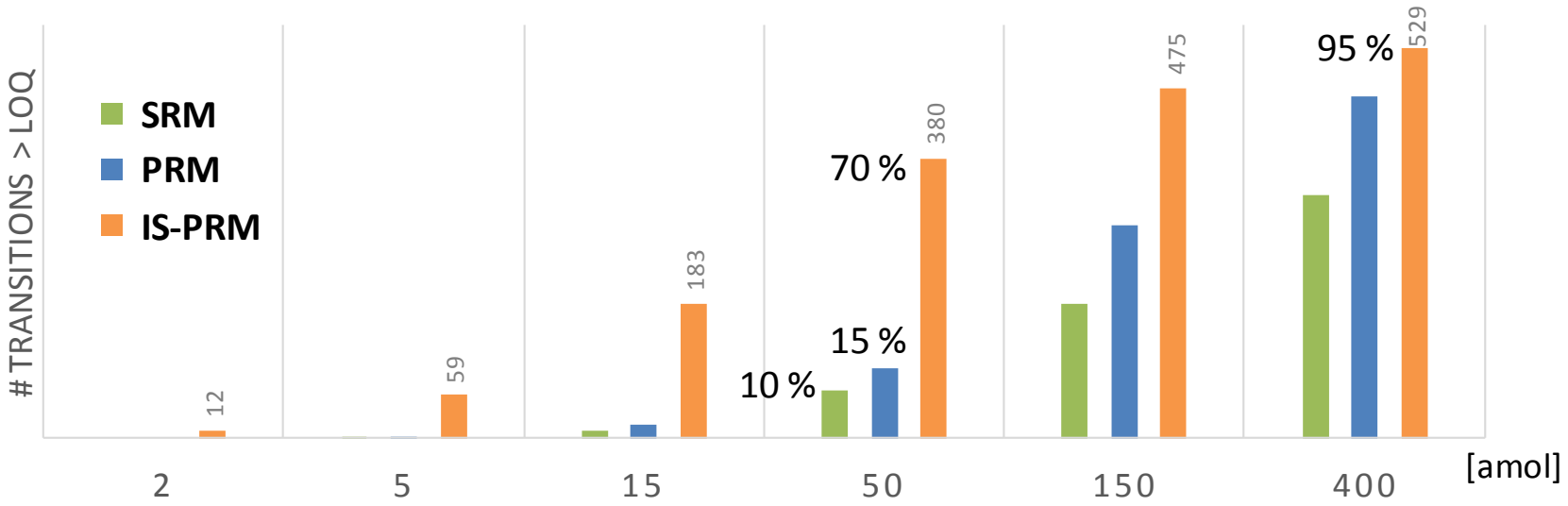
- 93 pairs (ENDO + SIL) of peptides (55 **lung cancer candidate biomarker proteins**)
- IS-PRM, PRM, and SRM analyses
- Plasma samples (digest: 0.5 µg/µL)
- 10 patients (lung cancer) + 10 controls
- Acquisition parameters adjusted for cycle time **<** 3 s

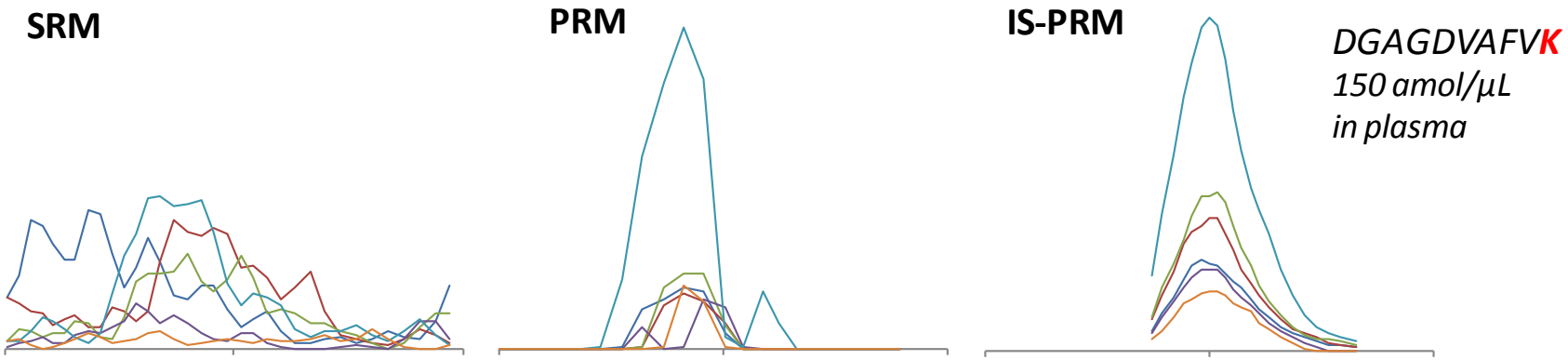
Parameters*	PRM	IS-PRM	
		QUANT	WATCH
Orbitrap resolution	17500	70000	17500
Max. fill time [ms]	60	360	60

(*Q-Exactive Plus MS)

IS-PRM – Performance

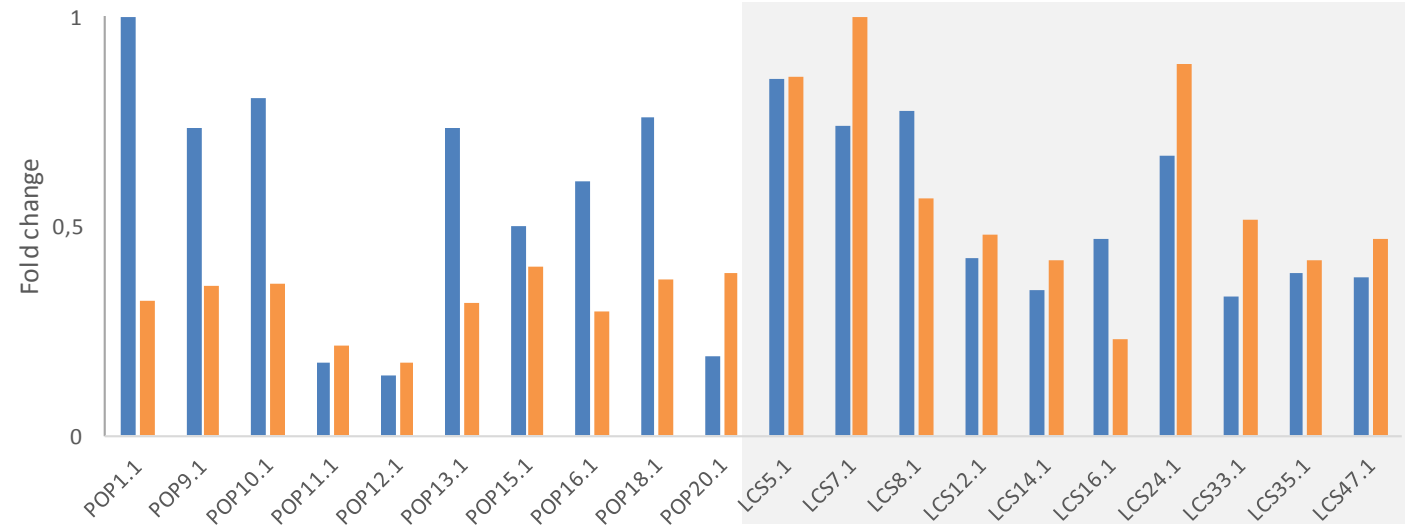
LOQ determined on 558 fragments (6 per peptide)





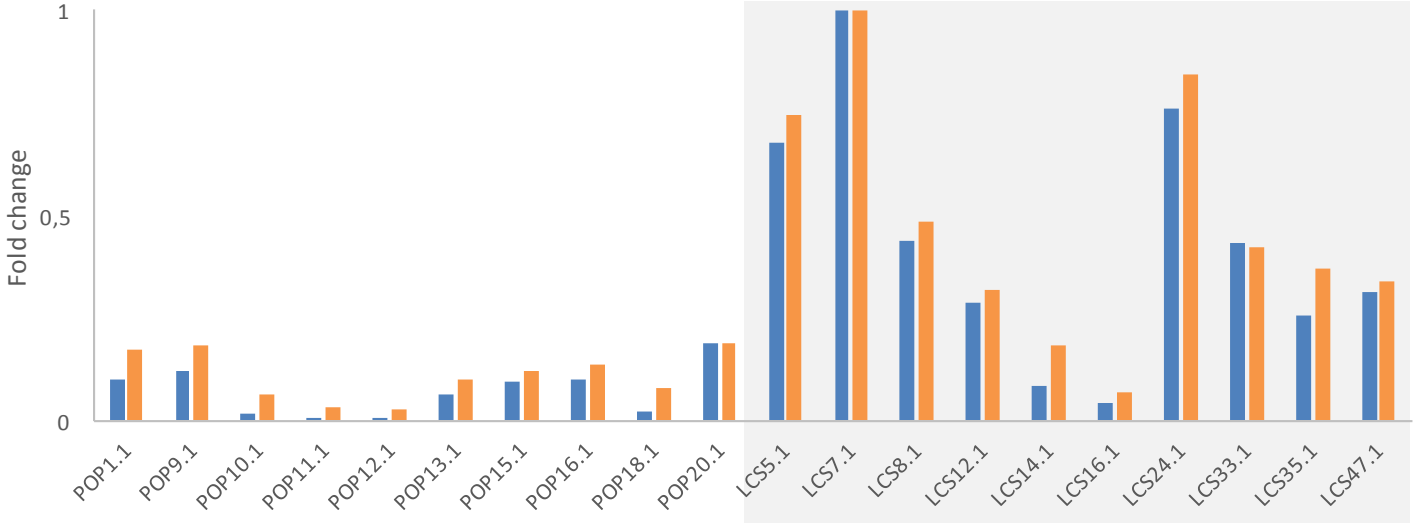
Screening of Biomarkers

Ex: *PGK1 (Phosphoglycerate kinase 1)*



10 controls

10 patients (lung cancer)



SRM ✘

Low consistency

YAEVTR

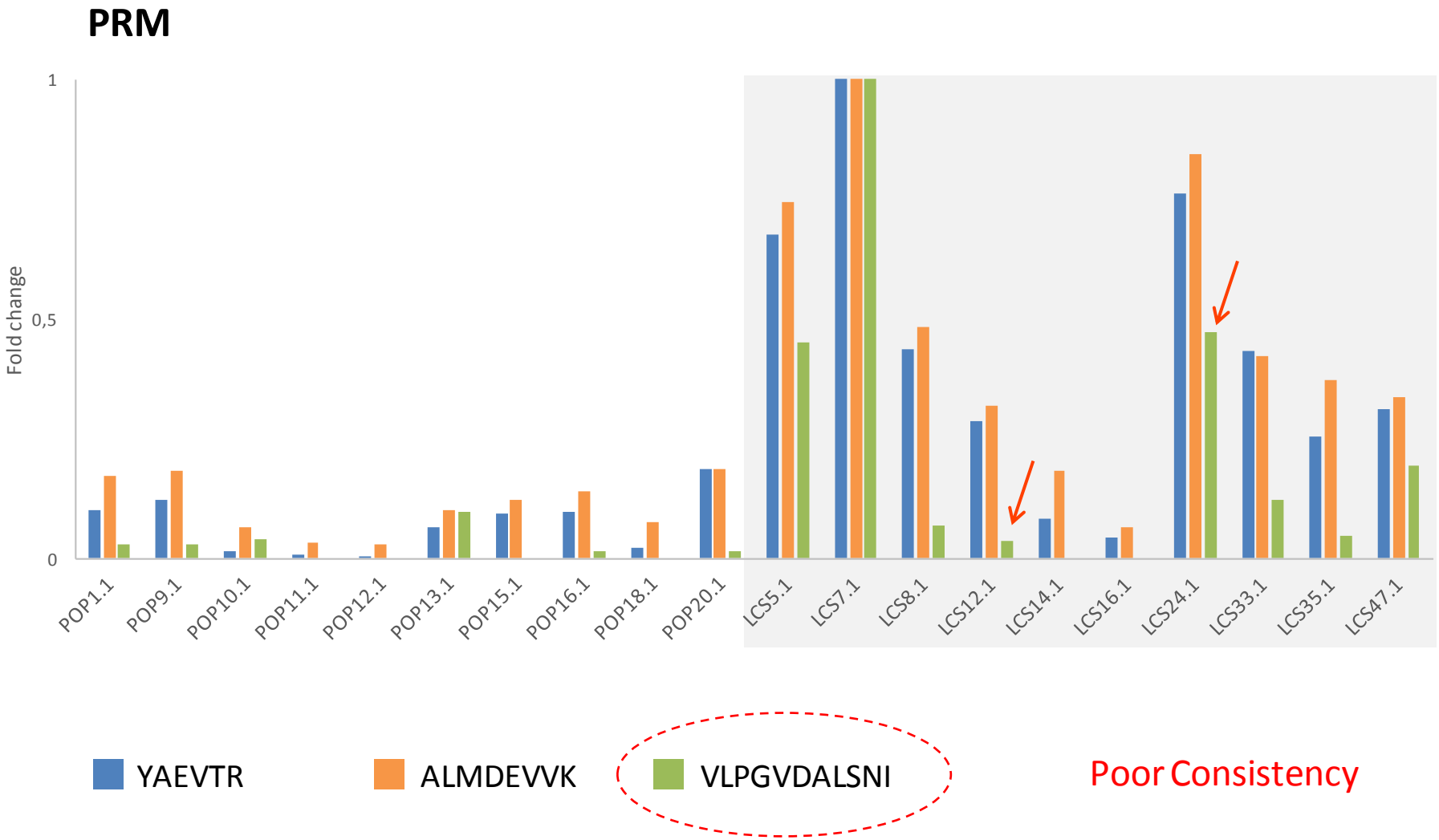
ALMDEVVK

PRM ✓

High consistency

Screening of Biomarkers

Ex: *PGK1 (Phosphoglycerate kinase 1)*



Screening of Biomarkers

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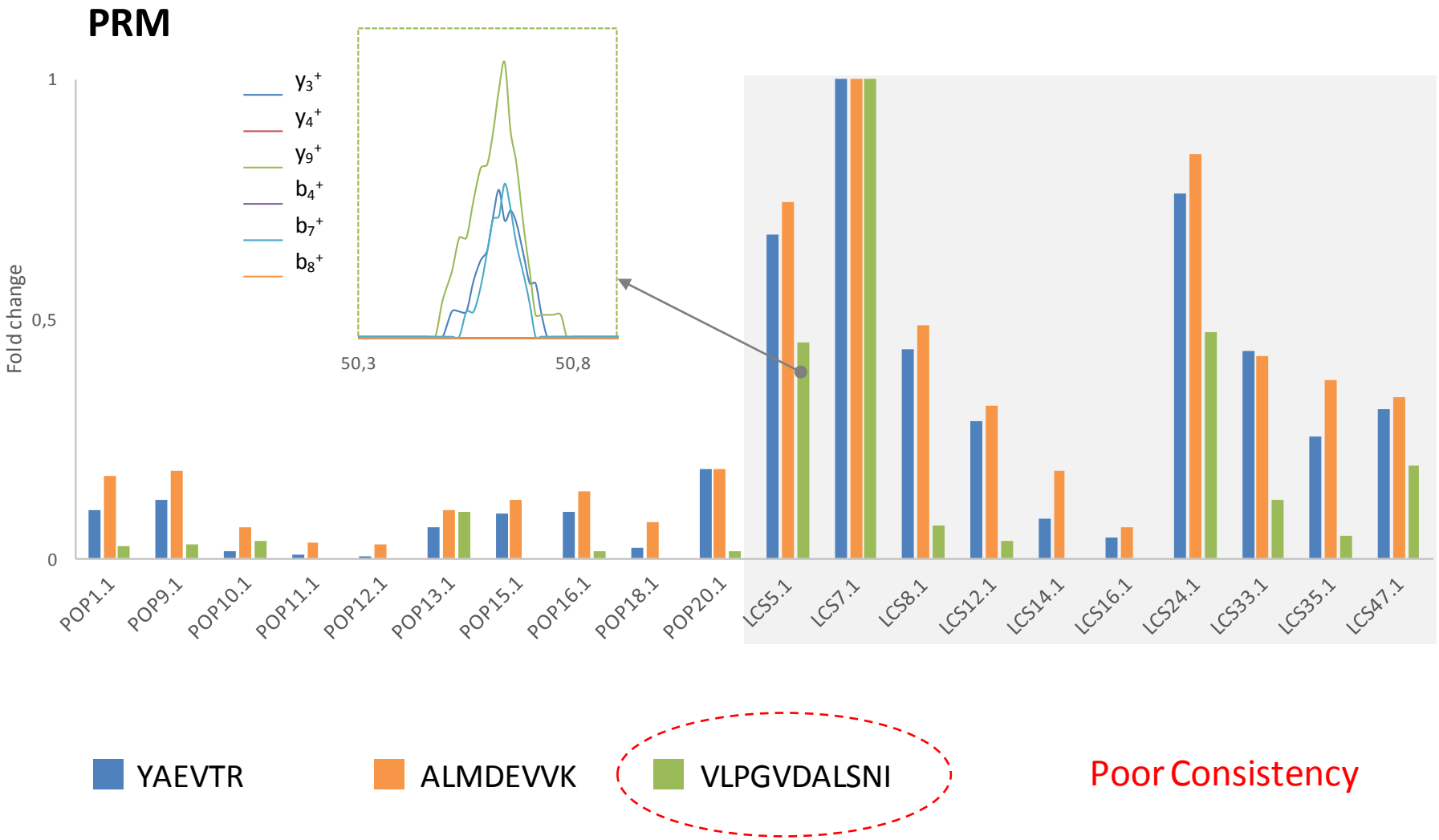
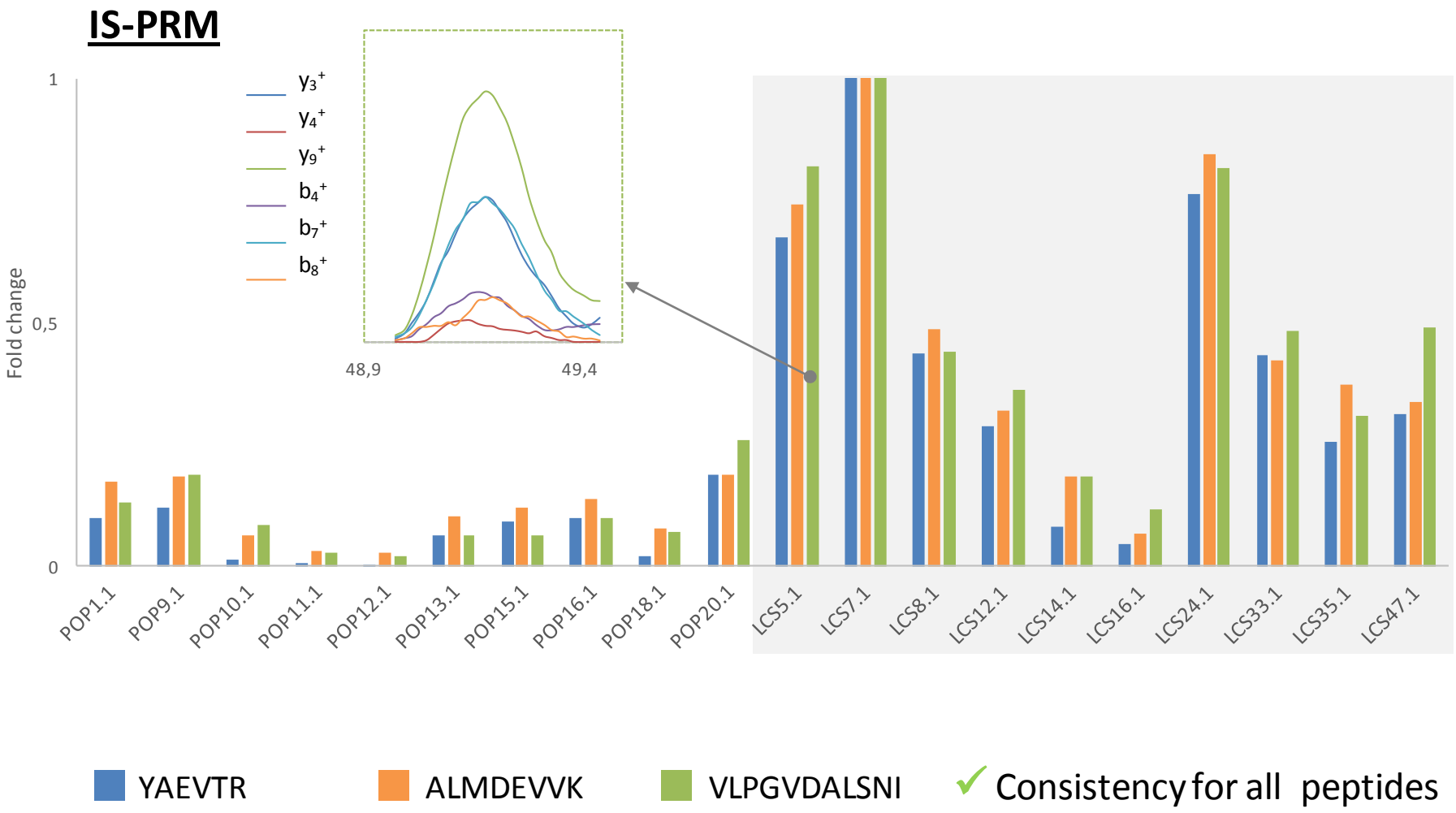
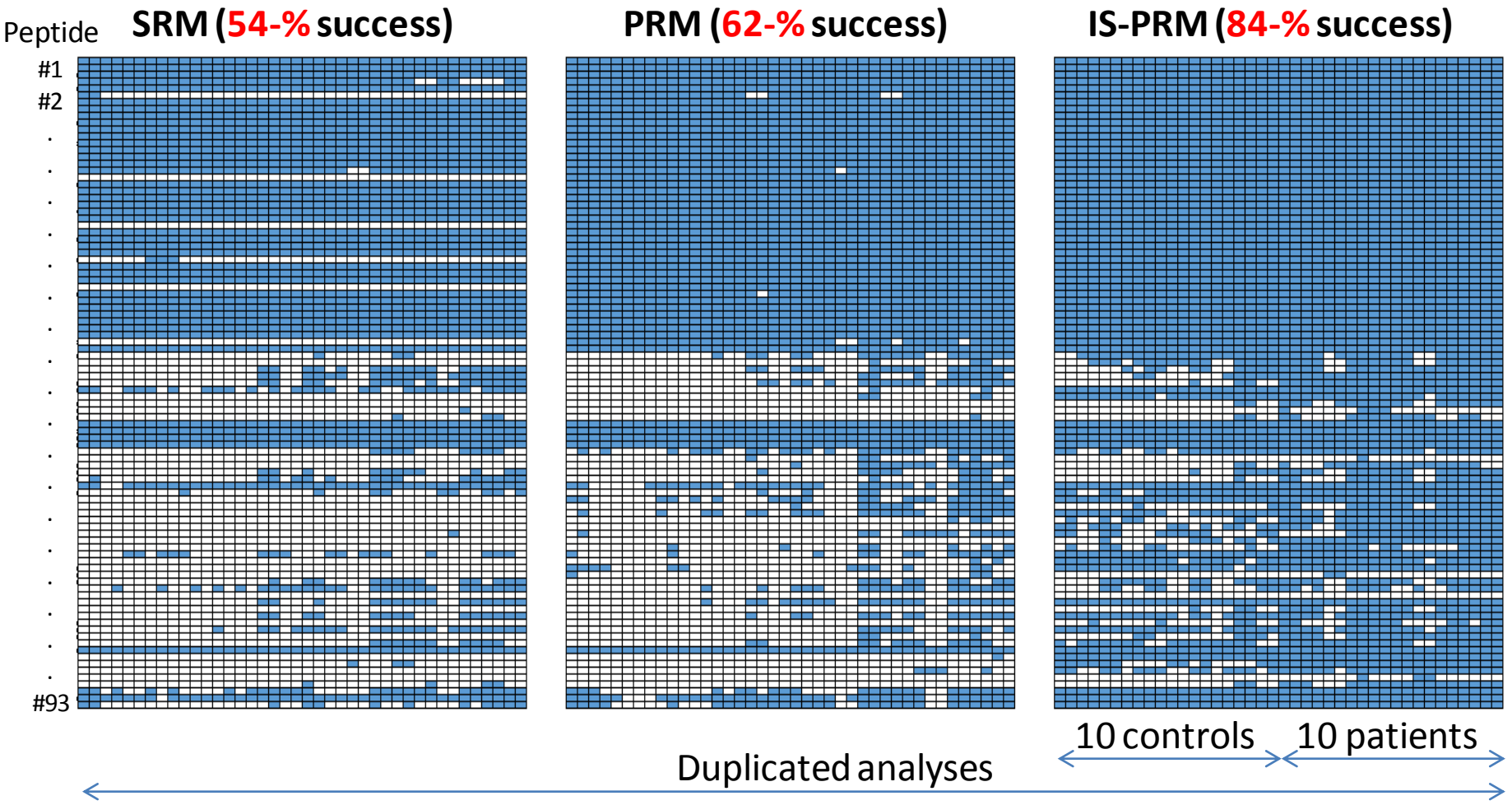


Screening of Biomarkers

Ex: *PGK1 (Phosphoglycerate kinase 1)*

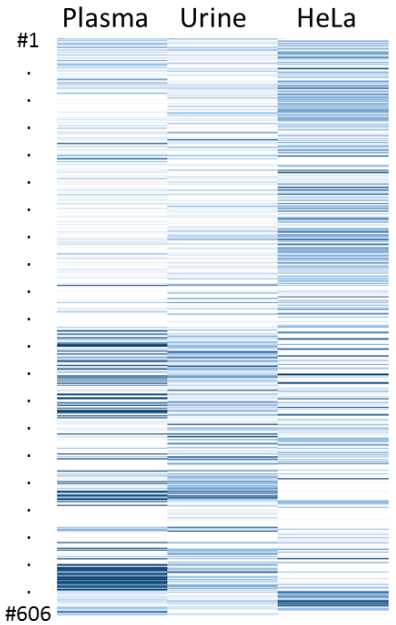


Screening of Biomarkers: Results Overview



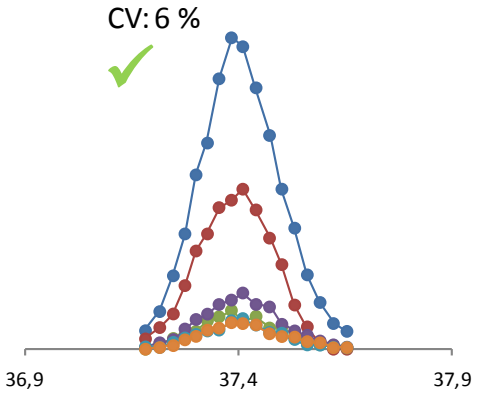
Large Scale Screening

- **Proof-of-principle: 600 pairs of SIL + ENDO peptides**
  - Plasma, urine, and HeLa cells digest samples
  - IS-PRM analyses on Thermo Scientific™ Q Exactive HF (WATCH: 15k / 20 ms ; QUANT : 60k / 110 ms)
  - 1-h LC gradient
  - **Detection of a total of 525 peptides (300 proteins)**

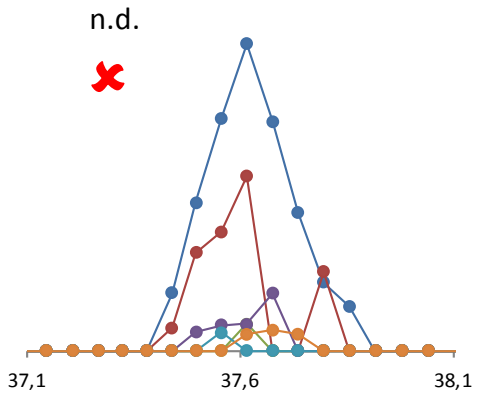


WPEPVFGR (m/z 494.256) in 0.5 µg/µL plasma

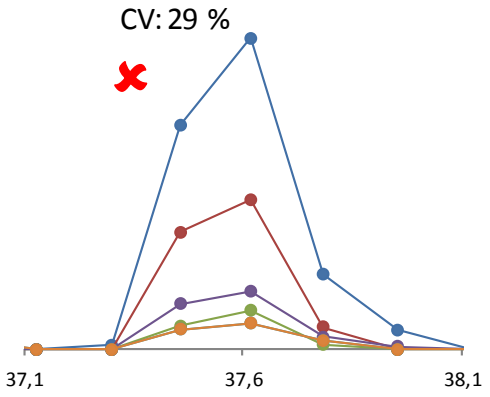
IS-PRM



PRM (15k / 20 ms)



PRM (60k / 110 ms)



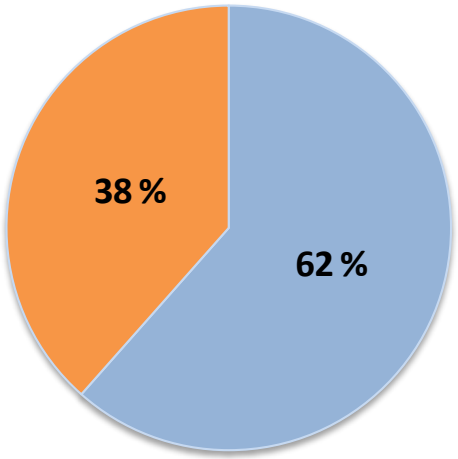
Signaling Pathway Monitoring

- **Proof-of-principle: 405 pairs of SIL + ENDO peptides (91 proteins)**
  - Samples: NSCLC cell lines (digest: 0.3 µg/µL); w/ or w/o resistance to *Erlotinib*
  - IS-PRM and DIA analyses on Q Exactive HF MS
    ***IS-PRM**: WATCH 15k / 20 ms ; QUANT 60k / 110 ms*
    ***DIA**: 32 segments @ 25 Th; m/z 400-1200 (30k / 60 ms)*
  - 1-h LC gradient

  - **Quantification of 295 peptides (70 proteins) in IS-PRM mode**
  - **Several peptides showed interferences in DIA mode**

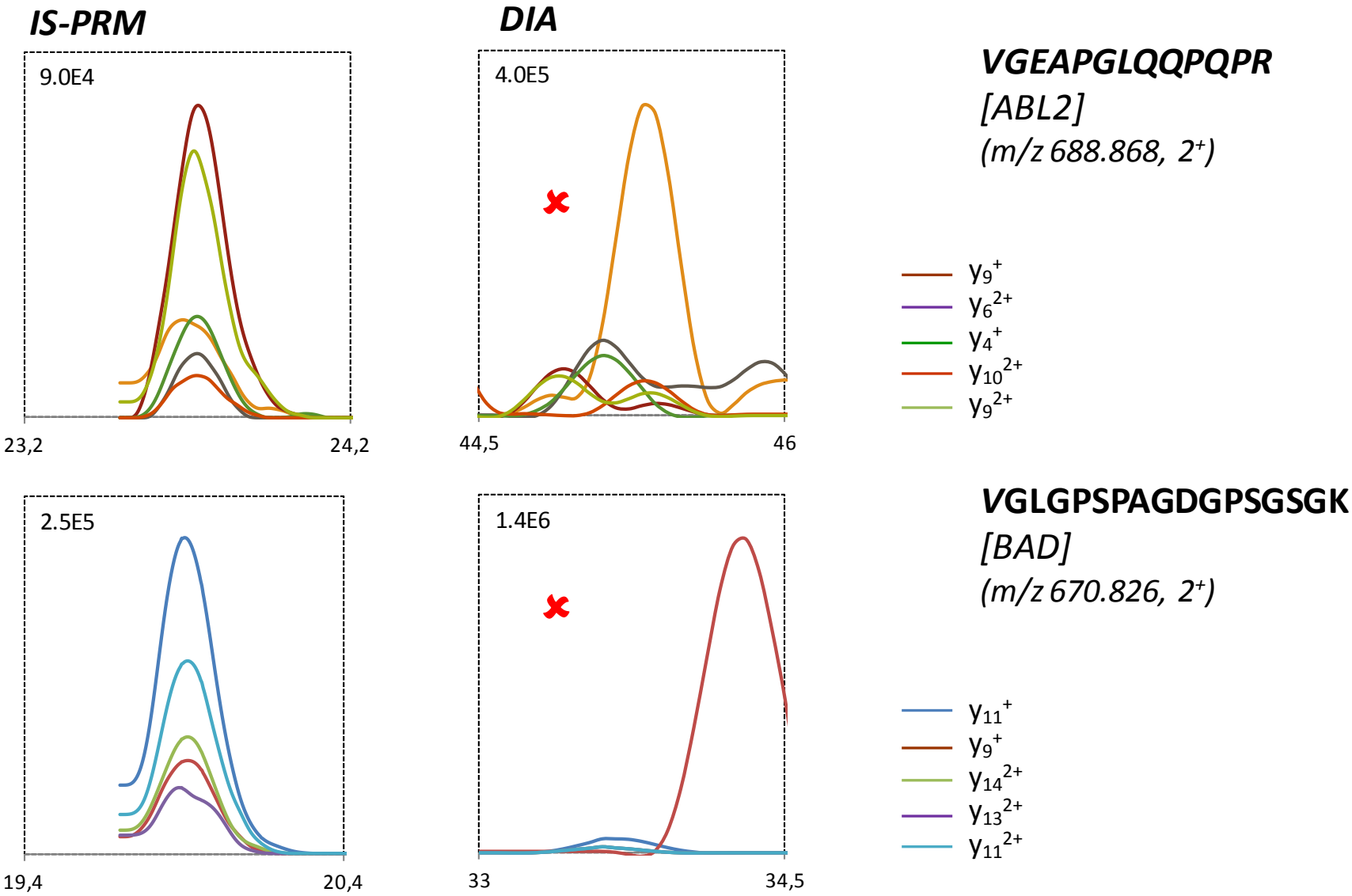
Proteins quantified

- IS-PRM only
- DIA and IS-PRM

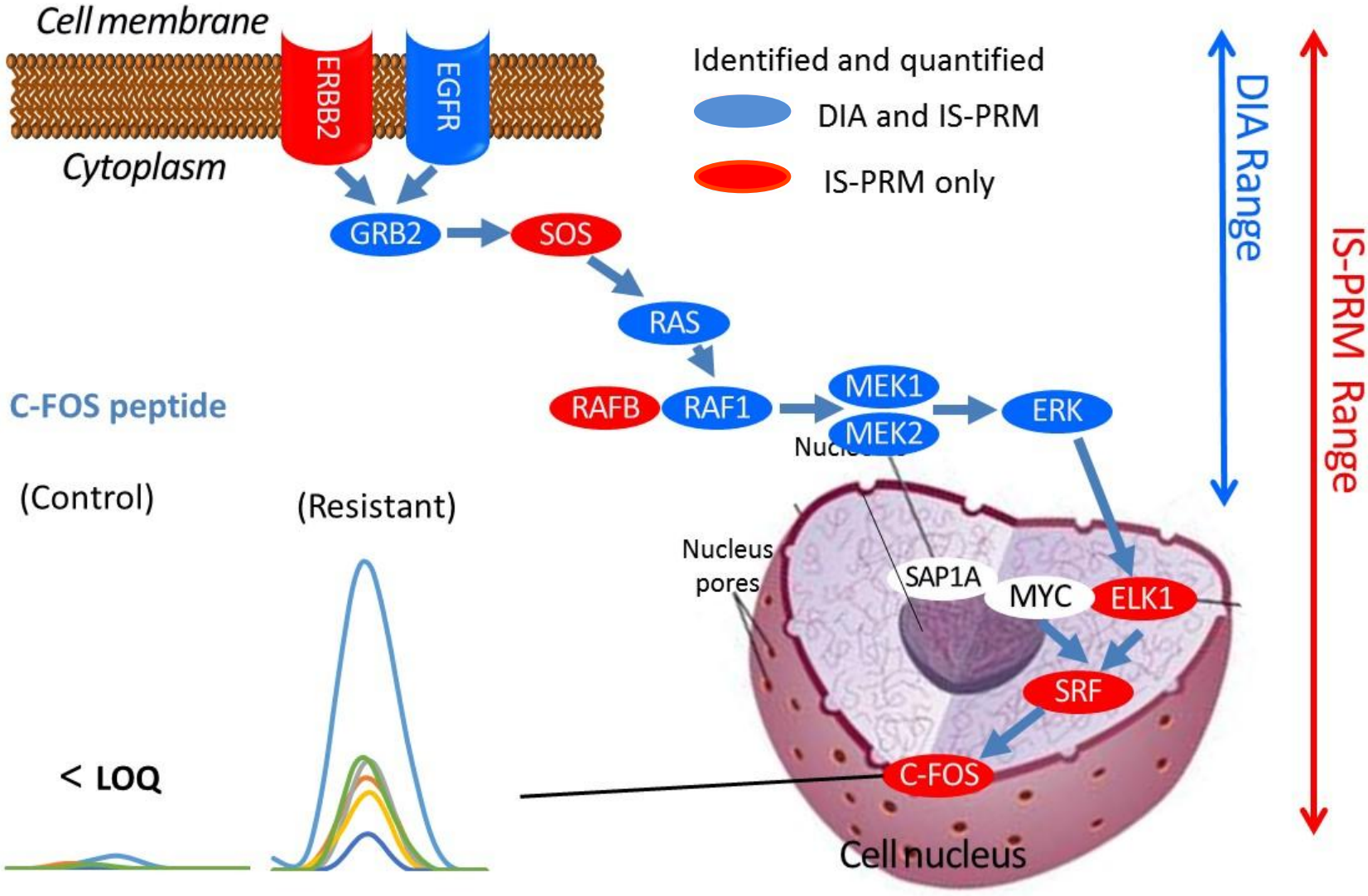


Blank et al.; Proteomics, submitted

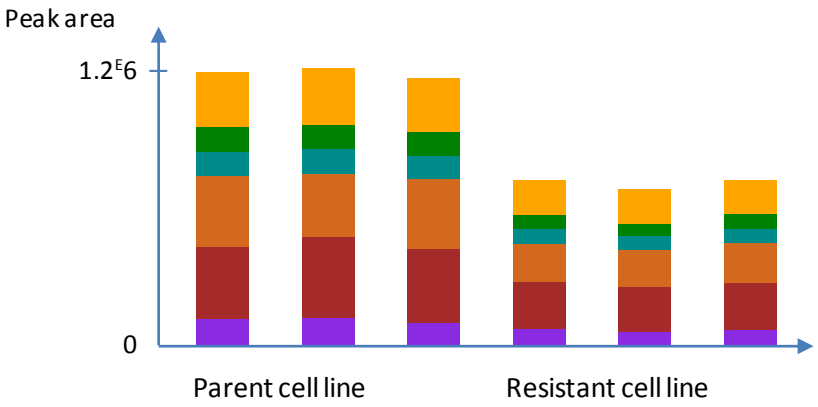
Signaling Pathway Monitoring

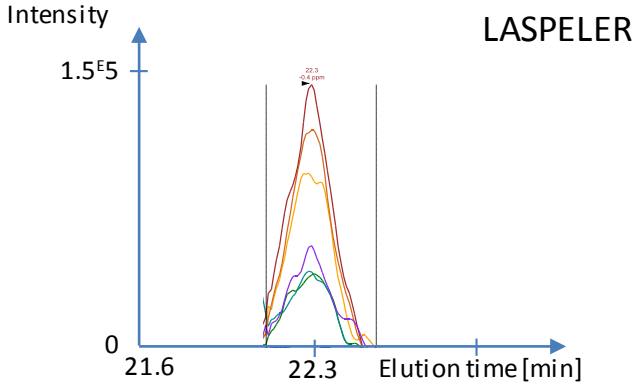


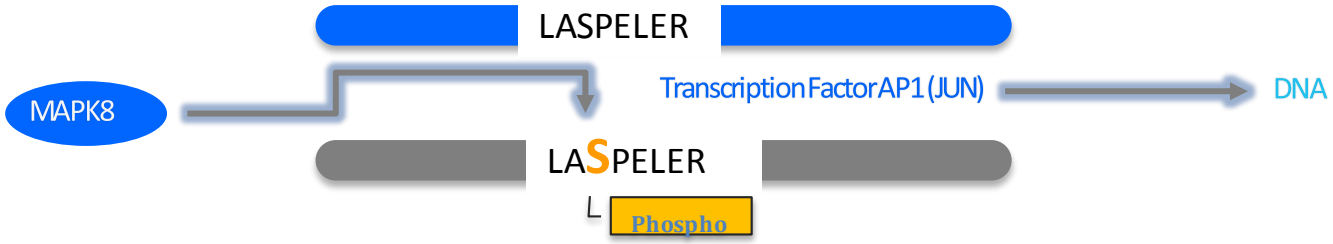
Analysis of MAPK Signaling Pathway

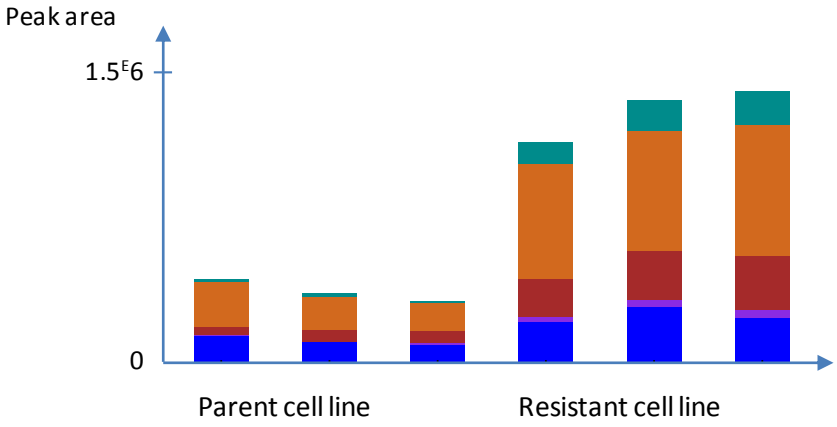


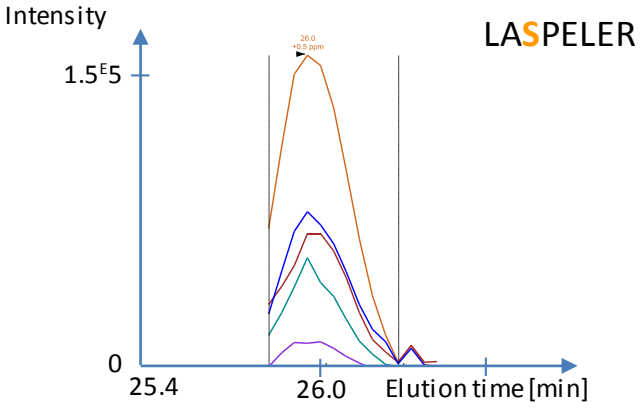
Differential Phosphosite Occupancy









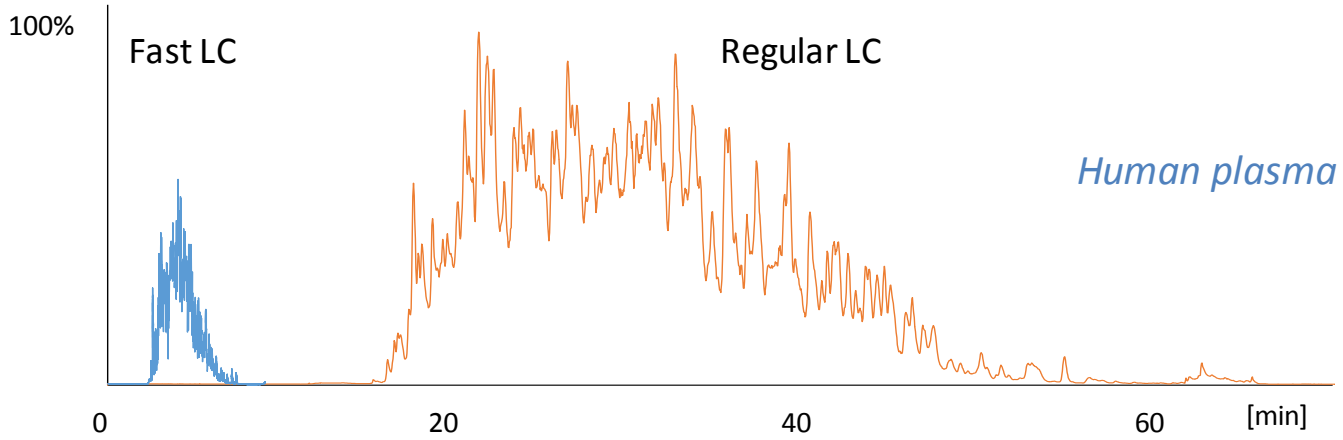


Fast Liquid Chromatography

Configurations

	Fast LC	Regular LC
Stationary phase	Syncronis C18	Pepmap C18
Particles	1.7 µm 100Å	2 µm 100Å
Column dimensions	150µm i.d. × 45 mm	75µm i.d. × 150 mm
Gradient	2-40% B* in 6 min	2-35% B* in 48 min
Flow rate	1500 nL/min	300nL/min

Analysis time



Human plasma tryptic digest

Fast Liquid Chromatography

Chromatographic features

- Peak capacity only moderately affected
- Analysis time significantly reduced
- Decrease in peak width (≈ 6 s)

	Fast LC	Regular LC
Stationary phase	Syncronis C18	Pepmap C18
Particles	1.7 µm 100Å	2 µm 100Å
Column dimensions	150µm i.d. × 45 mm	75µm i.d. × 150 mm
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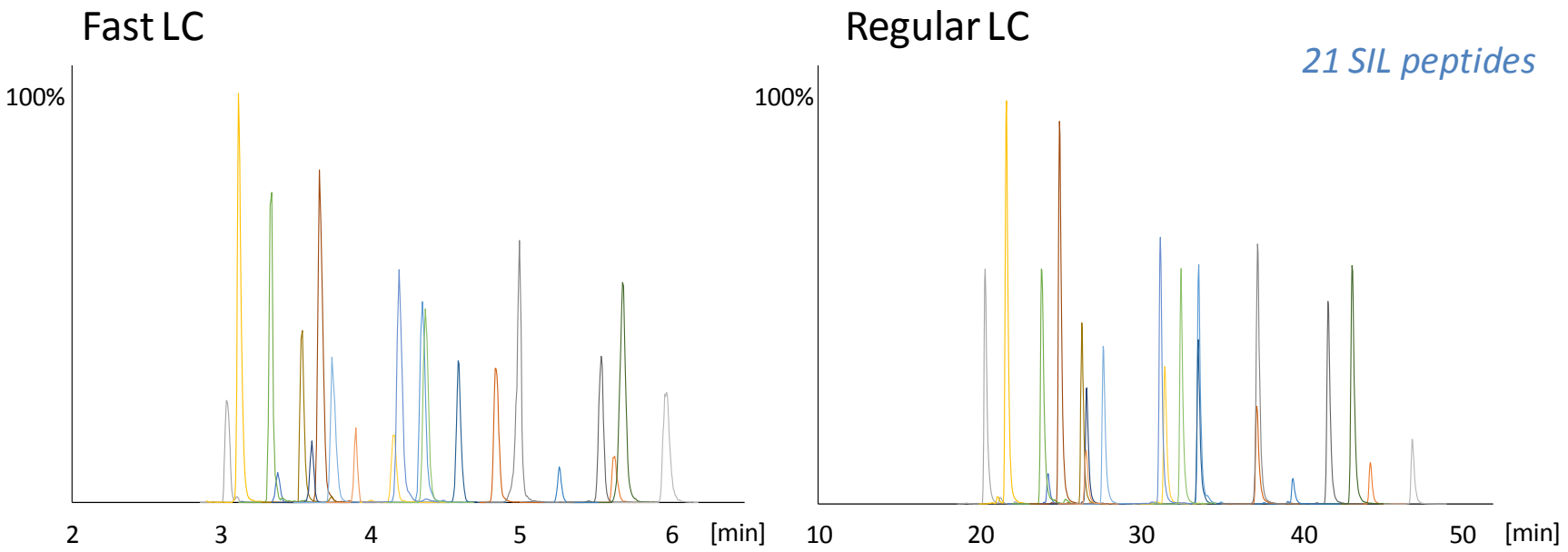
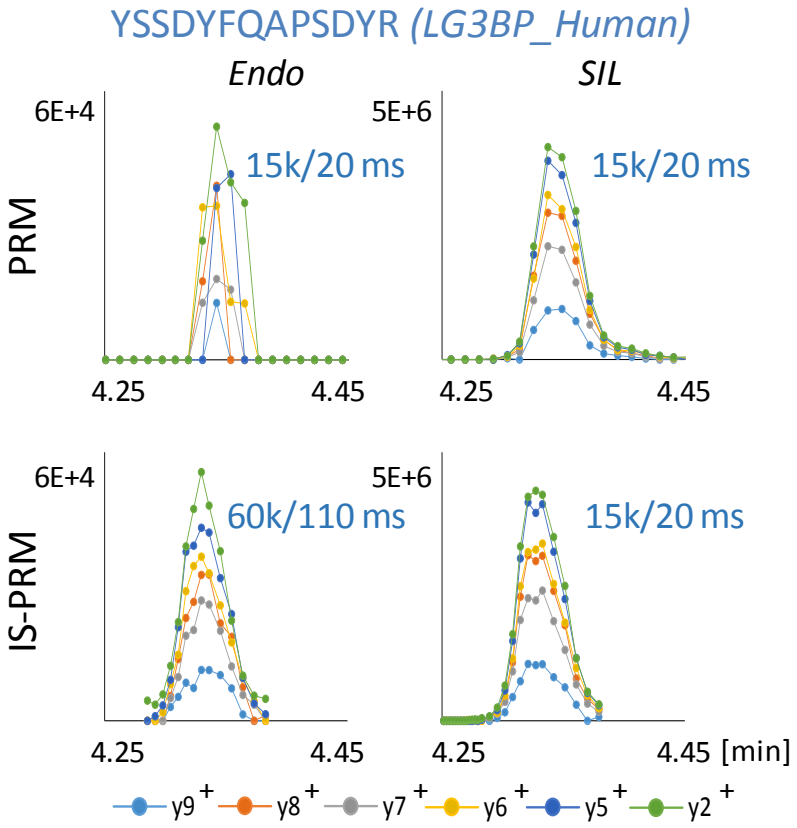


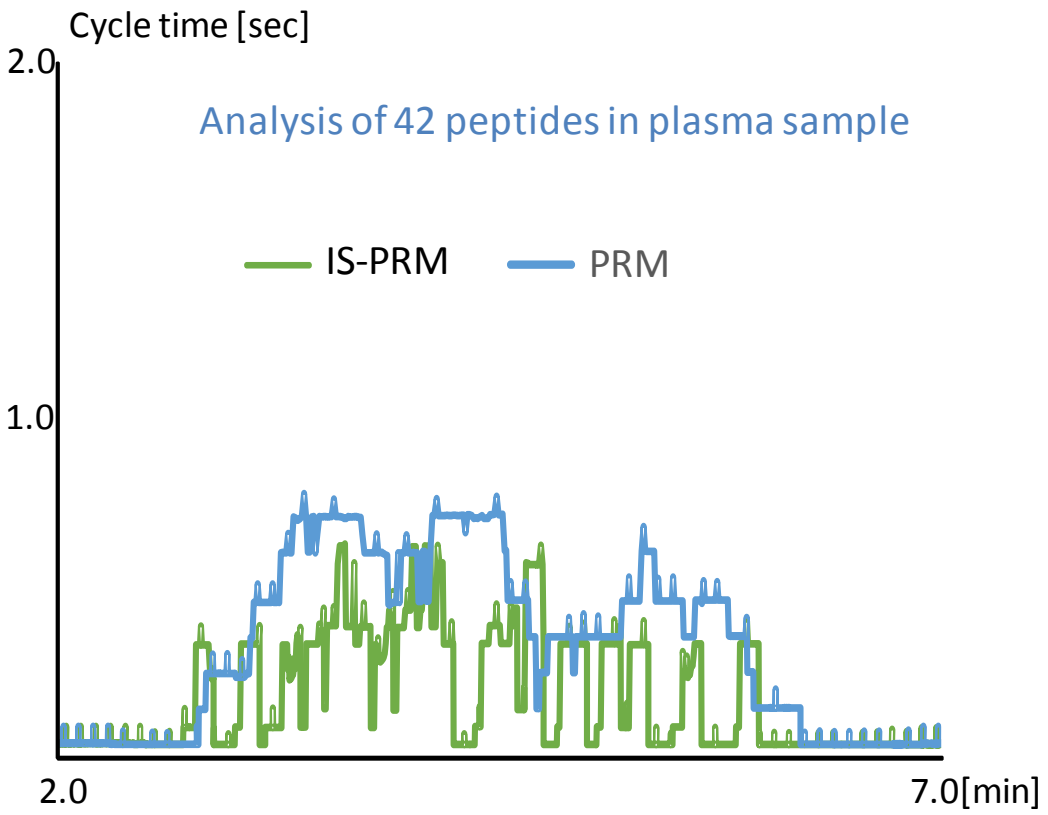
Lesur, Gallien, and Domon; TrAC, 2016

Fast LC / IS-PRM Analyses

Analytical throughput

- Short cycle time induced by fast LC separation (≈ 0.8 s)
- High acquisition efficiency of IS-PRM maintaining high selectivity/sensitivity
- Throughput capability : 50 peptides in 100 samples within one day



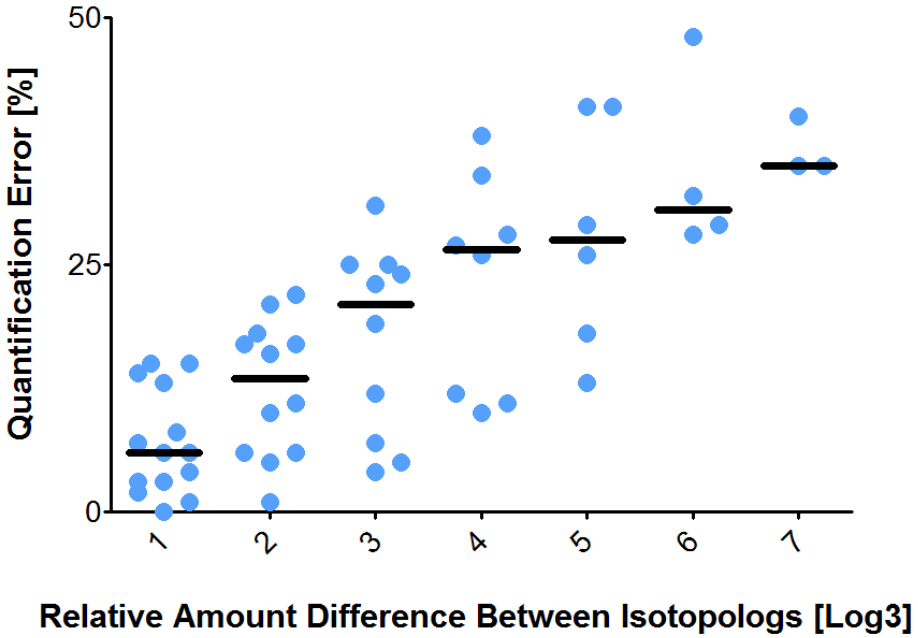


IS-PRM for Accurate Quantification Experiments

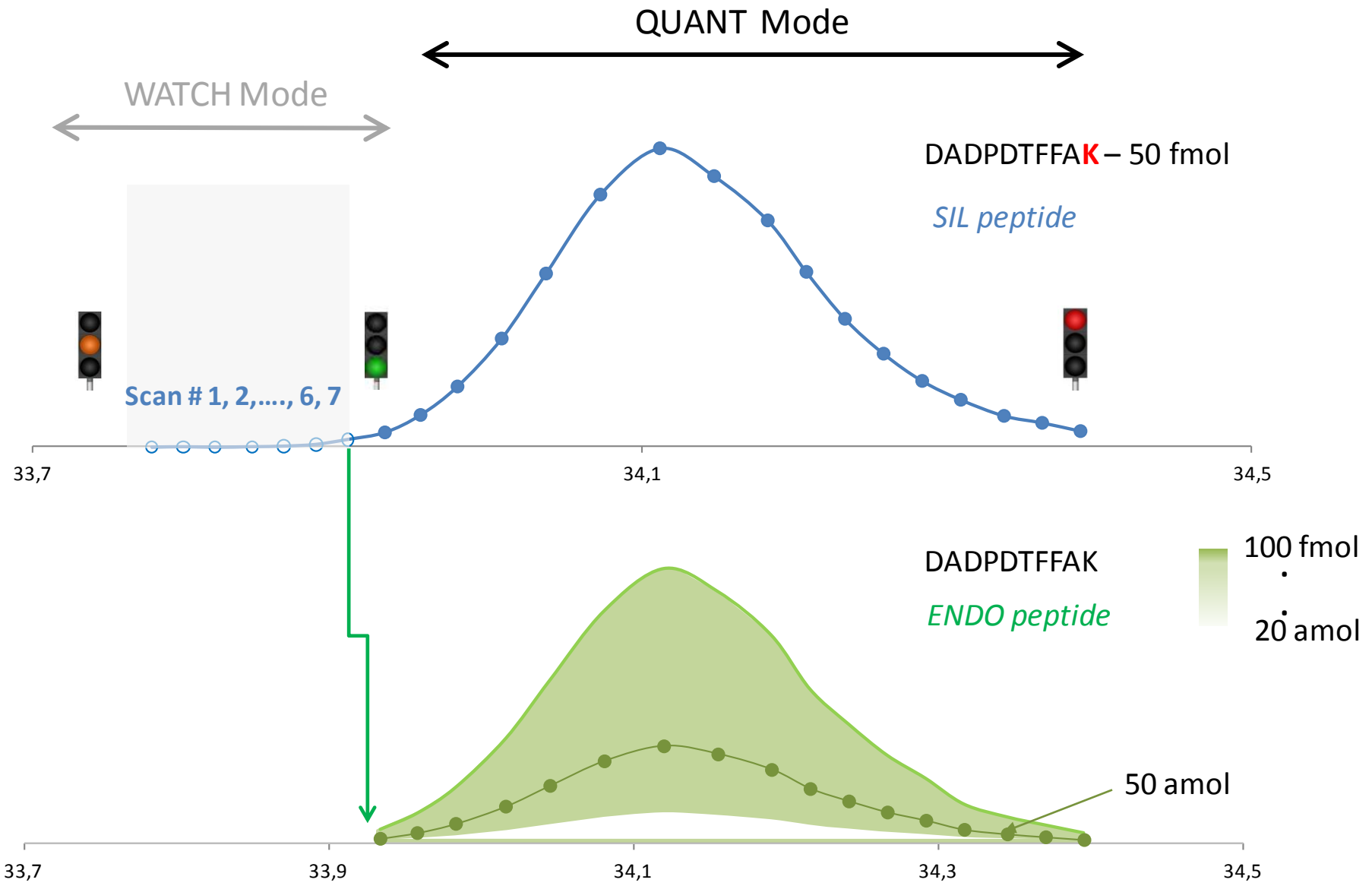
- **Additional constraints for "Quantification experiment"**
  - Accurate quantification of limited sets of peptides
  - Calibrated amount of IS peptides (SIL)
  - Balanced concentration of IS and ENDO peptides

- **Amount of IS affects quantification accuracy (single-point quantification)**

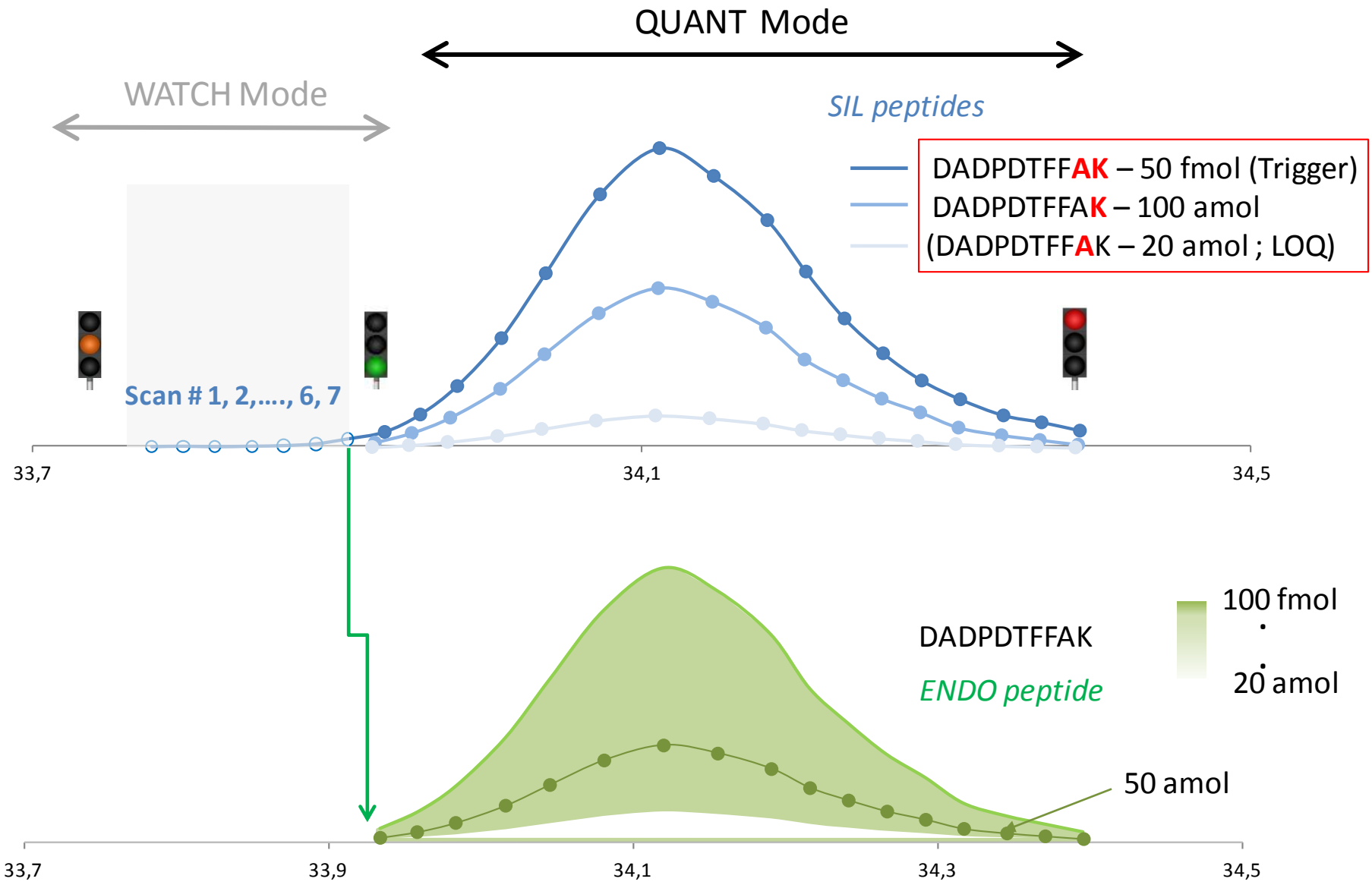
m/z [M+2H]$^{2+}$	*Peptide*	*Amount (fmol)*
418.287	AALPAAFK	0.004
415.28	AALPAAFK	0.01
412.775	AALPAAFK	0.03
410.273	AALPAAFK	0.08
407.765	AALPAAFK	0.3
405.263	AALPAAFK	0.82
402.751	AALPAAFK	2.4
394.737	AALPAAFK	7.9



IS Triggered-PRM



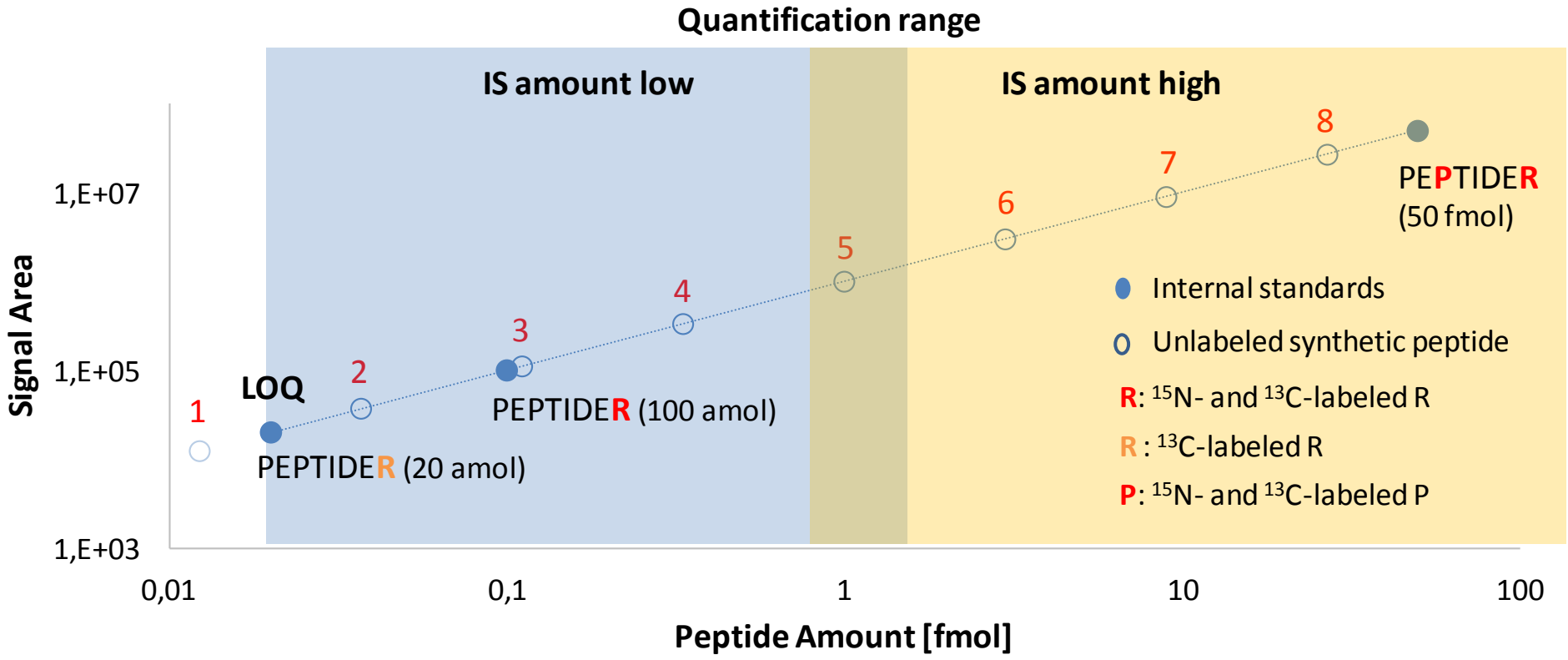
IS Triggered-PRM *V2.0* – Principle



IS Triggered-PRM *V2.0* – Proof of principle

Experiment

- 11 SIL peptides in 3 forms and amounts corresponding to five proteins, including **lung cancer biomarker candidates** (*e.g.,* ZYX, THBPS1, GSN)
- Different amounts of unlabeled synthetic peptides (from 0.01 to 27 fmol)
- Q Exactive HF MS



IS Triggered-PRM *V2.0* – Proof of principle

Experiment

- 11 SIL peptides in 3 forms and amounts corresponding to five proteins, including **lung cancer biomarker candidates** (*e.g.,* ZYX, THBPS1, GSN)
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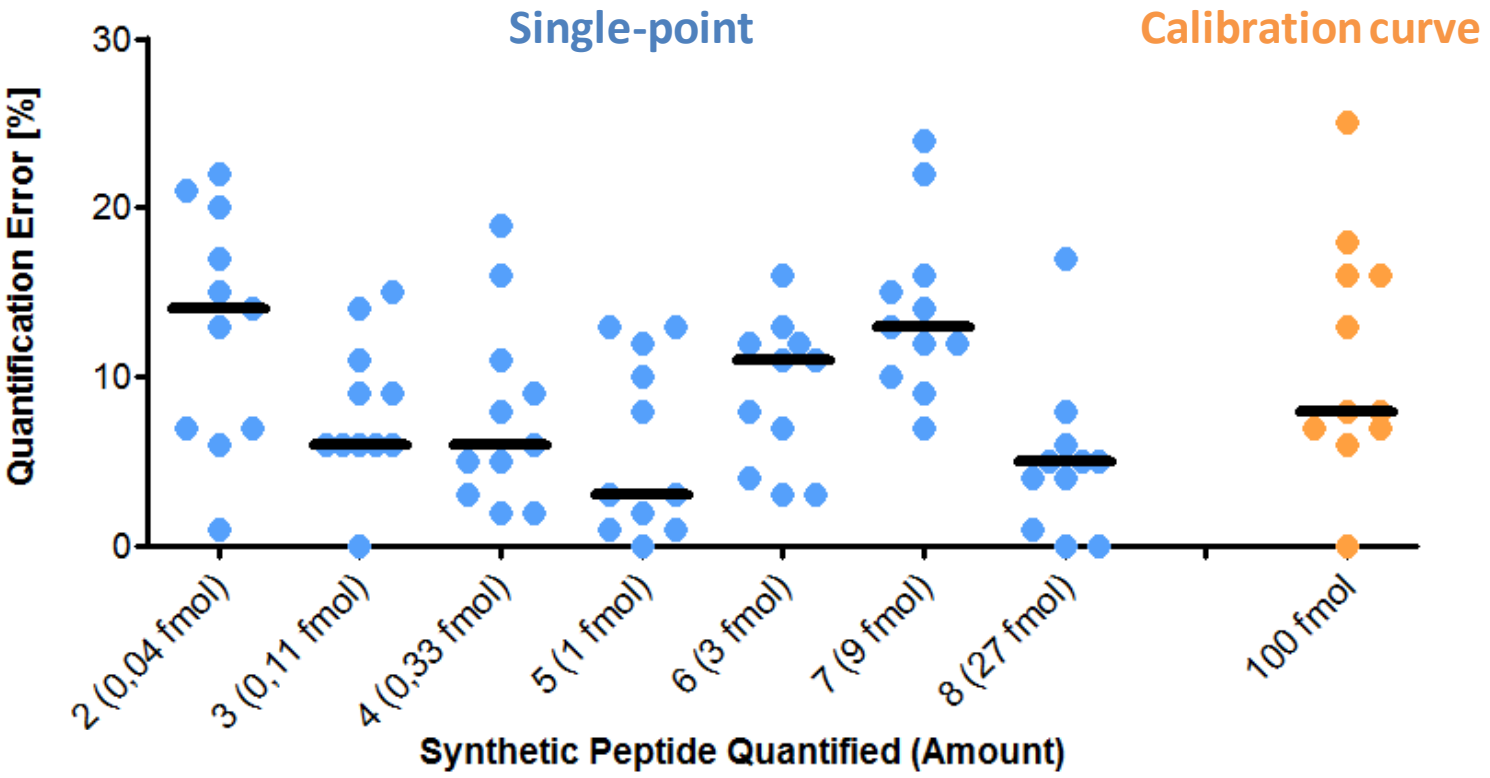
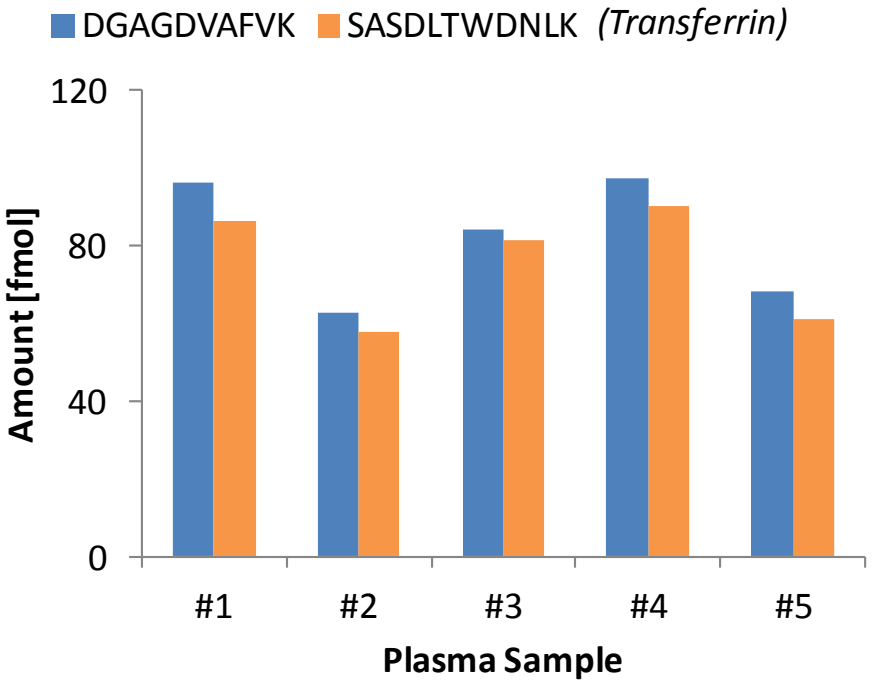
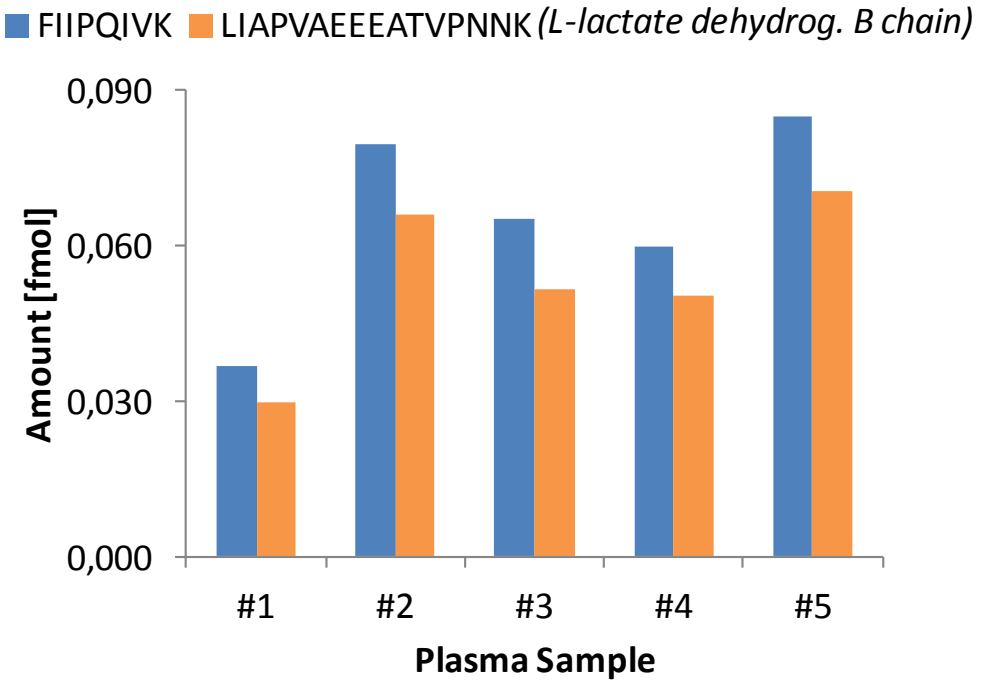


IS-PRM *V2.0* – Application to plasma samples

Experiment

- 11 SIL peptides in 3 forms and amounts corresponding to five proteins, including **lung cancer biomarker candidates** (*e.g.,* ZYX, THBPS1, GSN)
- Proteins consistently quantify over a wide range of abundance
- Broad applicability of the method





Conclusion

- **Internal standard triggered - parallel reaction monitoring (IS-PRM)**
  - Use internal standards to drive in real-time the acquisition
  - Two modes of operation: WATCH mode and QUANT mode
  - More effective use of instrument time
  - Implementation in progress in standard method editor

- **Figures of merit**
  - Expand the scale of PRM experiment (up to 600 pairs of peptides)
  - Retain high analytical performance of conventional small-scale studies
  - Portability across multiple instruments and applicability to a variety of samples

- **Applications**
  - Moderate to large-scale screening (*e.g.*, biomarkers, signaling pathways)
  - Accurate quantification of more limited peptide sets (IS-PRM V2.0, multi IS)
  - Large sample set studies (combined with fast LC separation)

Acknowledgements

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- Andreas Huhmer