- Tutorial Webinar #17

PRM Method Development and Data Analysis with Skyline

With

Brendan MacLean (Principal Developer, Skyline)
Eduard Sabidó, Ph.D. (Head of the UPF/CRG Proteomics Unit)
Cristina Chiva, Ph.D. (PRM researcher, CRG Proteomics Unit)

Agenda

- Welcome from the Skyline team!
- PRM Method Development and Data Analysis with Skyline
    - Introduction with Brendan MacLean
    - Theoretical concepts and benefits of PRM with Eduard Sabidó
    - Tutorial with Cristina Chiva
- Audience Q&A – submit questions to Google Form:

https://skyline.ms/QA4Skyline.url

It Began as Targeted MS/MS (pseudo-SRM)

- ASMS 2011 – Poster presentation (Birgit Schilling)
  - *Skyline: Targeted Proteomics with Extracted Ion Chromatograms from Full-Scan Mass Spectra*
- JPR May, 2012 – Sherrod, et. al (started by Amy Ham, 2009)
  - *Label-Free Quantitation of Protein Modifications by Pseudo-Selected Reaction Monitoring with Internal Reference Peptides*
- MCP Nov, 2012 – Peterson, et. al (Coon lab) – named **PRM**
  - *Parallel reaction monitoring for high resolution and high mass accuracy quantitative, targeted proteomics*
- Anal. Chem. 2015 – Schilling, et. al – latest on Skyline PRM for HRMS
  - *Multiplexed, Scheduled, High-Resolution Parallel Reaction Monitoring on a Full Scan QqTOF…*

Recorded Webinars

- 16 other webinars + this one *Coming Soon!*
- **Webinar #3 (2013)**
  PRM Targeted Proteomics Using Full-Scan MS and Skyline
  Bruno Domon
- **Webinar #9 (2015)**
  PRM for PTM studies with Skyline
  Research-grade targeted proteomics assay development
  Jacob D. Jaffe

Interest Remains High

- Modern instruments support PRM well
  - High resolution, faster cycle times and full scheduling
- Viable alternative to SRM with triple quadrupole instrument
- Appealing not to need an extra instrument for targeted

- Registration for first PRM webinar (431) v 2014 DIA webinar (342)
- Registration for this PRM webinar (434) v 2017 DIA webinar (409)

Eduard Sabidó

Webinar

PRM theoretical concepts, benefits and instrument acquisition settings

Eduard Sabidó
Cristina Chiva

CRG/UPF Proteomics Unit
Barcelona, Spain

Universitat Pompeu Fabra Barcelona

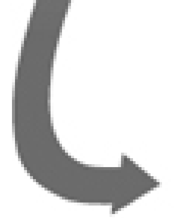
EXCELENCIA SEVERO OCHOA

Barcelona
Biomedical
Research
Park

Eduard Sabidó

Cristina Chiva

CRG Centre de Regulació Genòmica



PRM is a targeted proteomics workflow

"Targeted proteomics detects proteins of interest with high ***sensitivity****,* ***quantitative accuracy*** *and* ***reproducibility****"*

"By delivering ***precise****,* ***reproducible quantification*** *of proteins of interest in biological samples, targeted proteomics approaches are allowing researchers to apply the scientific method using mass spectrometry"*

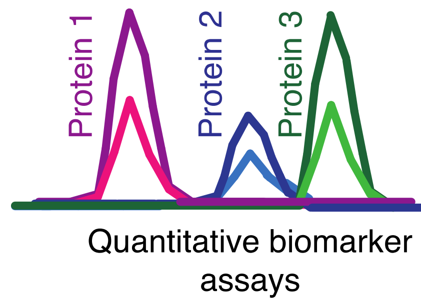
METHOD OF THE YEAR NEWS FEATURE | SPECIAL FEATURE

24 | VOL.10 NO.1 | JANUARY 2013 | NATURE METHODS

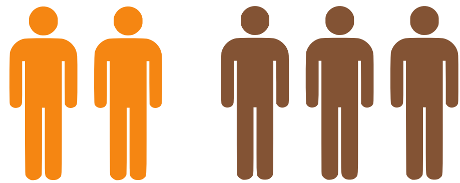
PRM is a targeted proteomics workflow

Types of projects suited for targeted proteomics

A **biomarkers**



Quantitative biomarker assays



Treatment A Treatment B

B **pathways**

C **interactions**

CRG
Centre de Regulació Genòmica

PRM is a targeted proteomics workflow

MS1 Targeted Methods
They rely on the mass of the entire molecule

MS2 Targeted Methods
They rely on the fragments of the molecule

Targeted Acquisition
They only acquire the molecules of interest

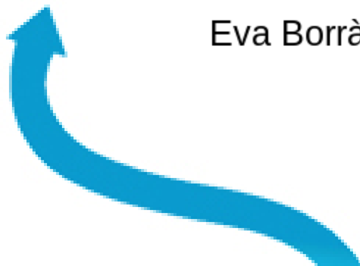
Targeted Data Analysis
They acquire everything and later specific information is extracted

Viewpoint

What is targeted proteomics? A concise revision of targeted acquisition and targeted data analysis in mass spectrometry

Eva Borràs[1,2] and Eduard Sabidó[1,2,*]

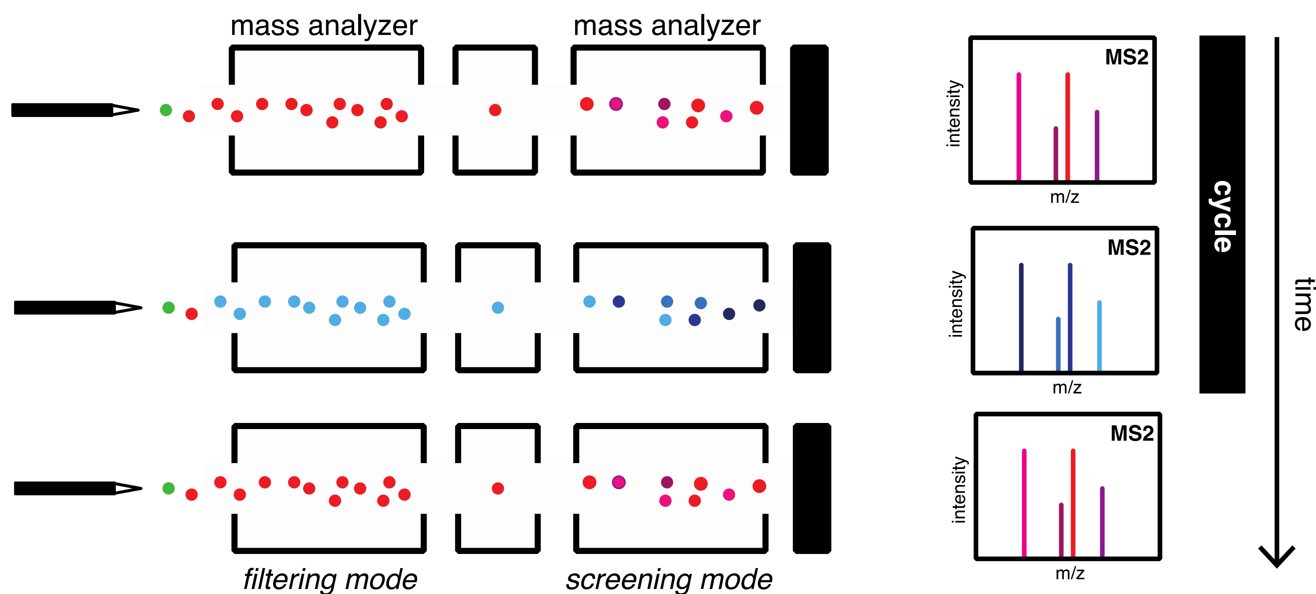
Issue — *Proteomics 17, 17–18*, 2017, 1700180



CRG
Centre de Regulació Genòmica

PRM is a targeted proteomics workflow

Parallel Reaction Monitoring (PRM)

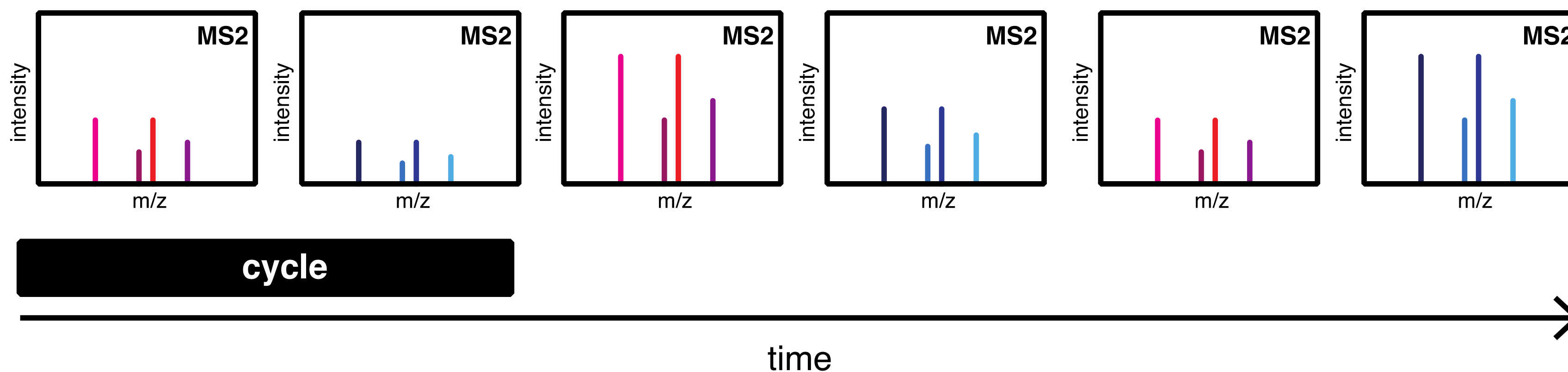


Peterson, ..., Coon J, Mol Cell Proteomics. 2012 Nov; 11(11): 1475-1488
Gallien S, ..., Domon B, Mol Cell Proteomics. 2012 Dec; 11(12): 1709-1723

CRG Centre de Regulació Genòmica

PRM is a targeted proteomics workflow

Parallel Reaction Monitoring (PRM)



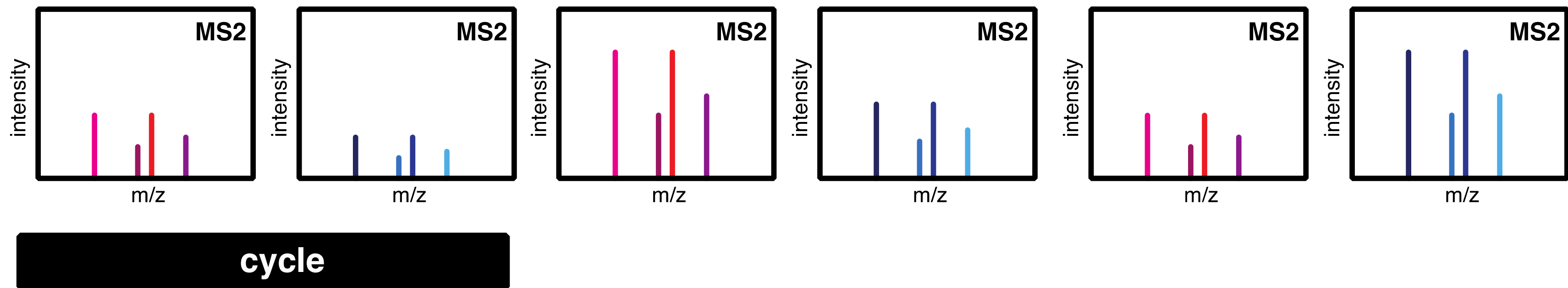
Peterson, ..., Coon J, Mol Cell Proteomics. 2012 Nov; 11(11): 1475-1488

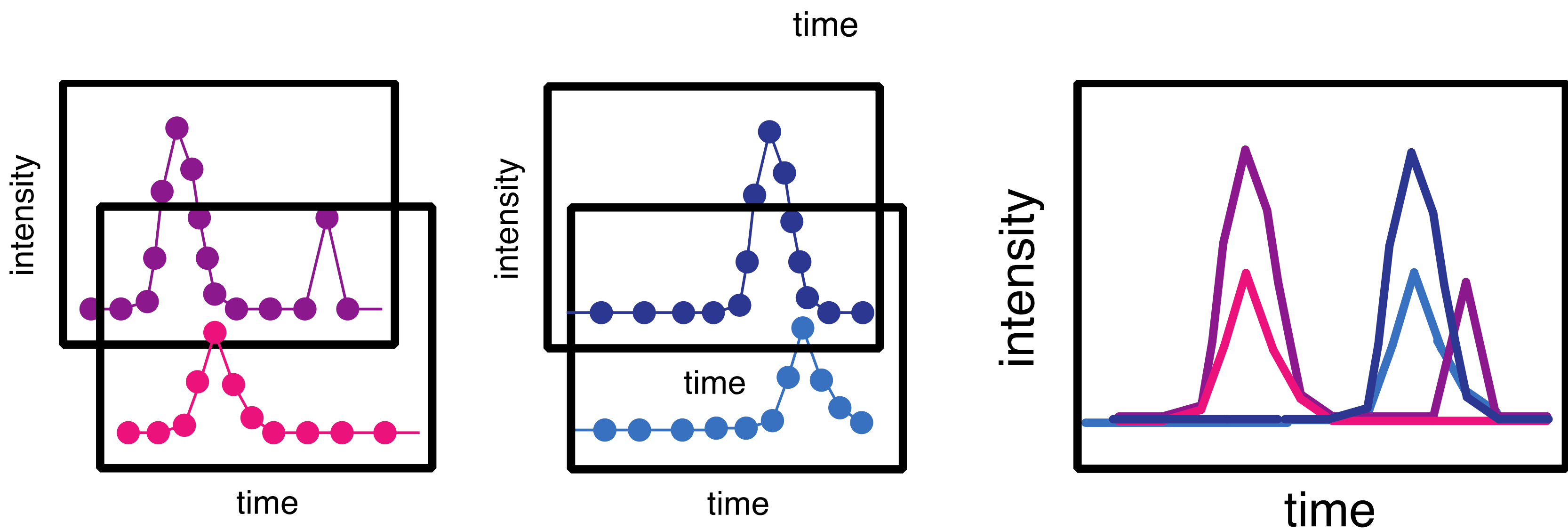
Gallien S, ..., Domon B, Mol Cell Proteomics. 2012 Dec; 11(12): 1709-1723

CRG Centre de Regulació Genòmica

PRM is a targeted proteomics workflow

Parallel Reaction Monitoring (PRM)



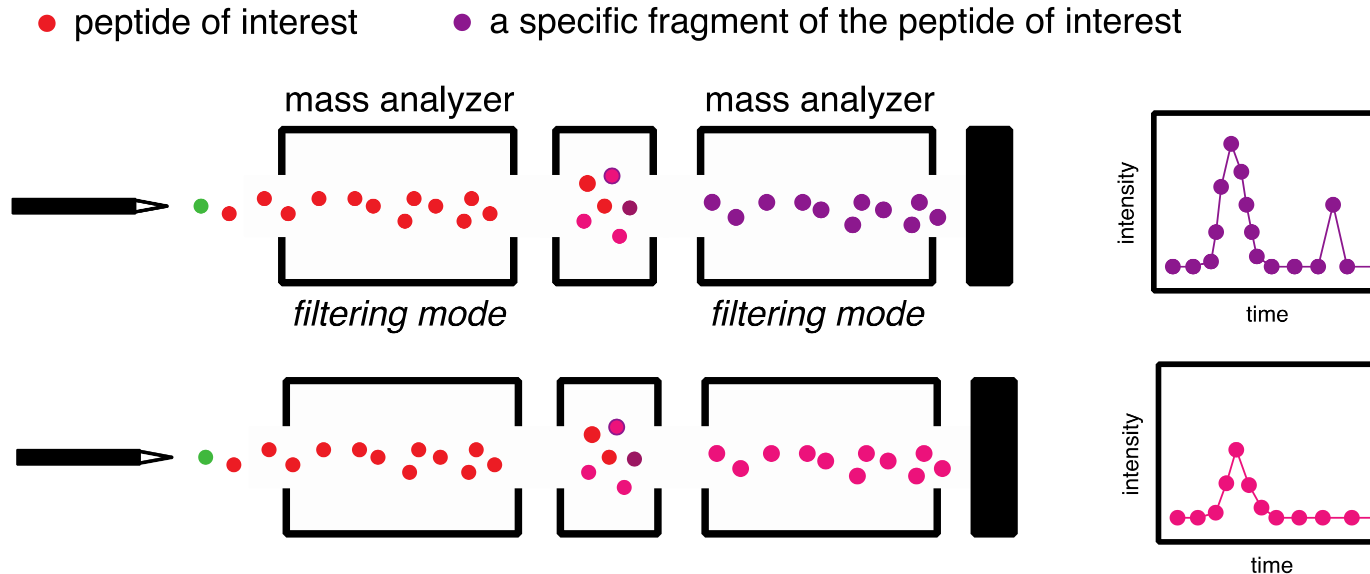


co-elution identification

CRG Centre de Regulació Genòmica

PRM is a targeted proteomics workflow

Selected Reaction Monitoring (SRM) is sequential (not parallel)



Centre de Regulació Genòmica

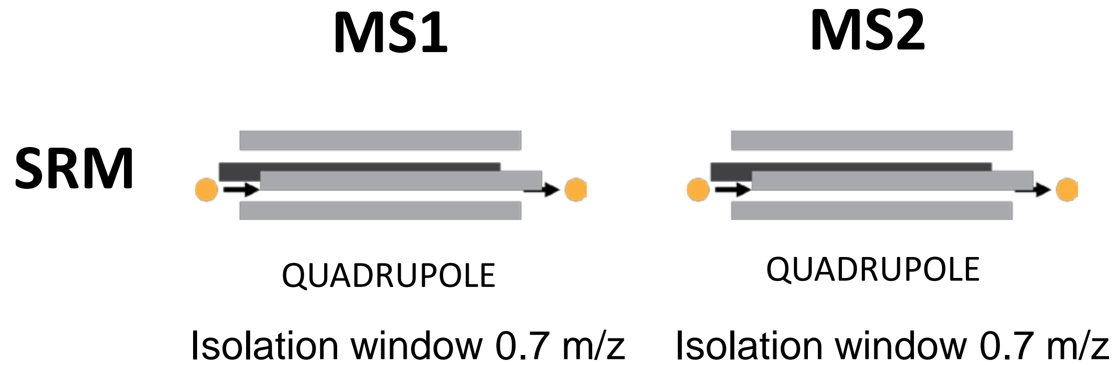
PRM is a targeted proteomics workflow

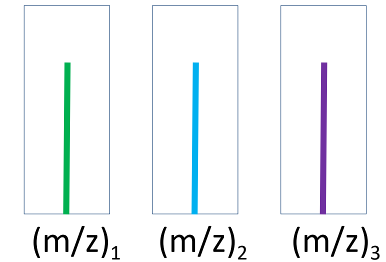
Mass spectrometry instruments

MS1 **MS2**

Low-resolution MS2 signal
One narrow scan per fragment
Hardware selected MS2 fragments

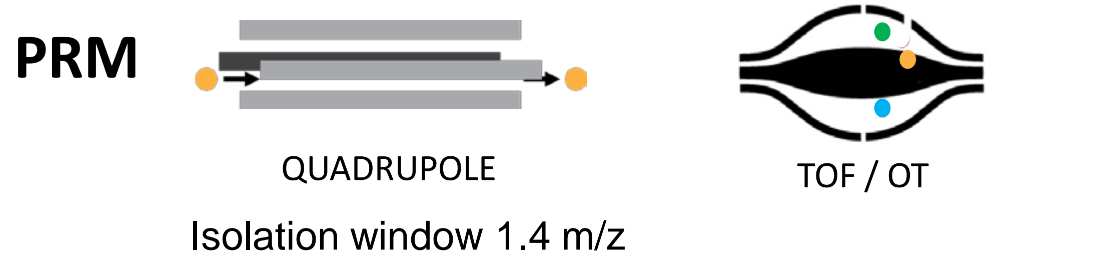
SRM

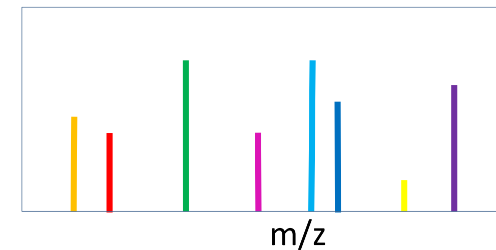




High-resolution MS2 signal
Full scan MS2
Software selected MS2 fragments

PRM

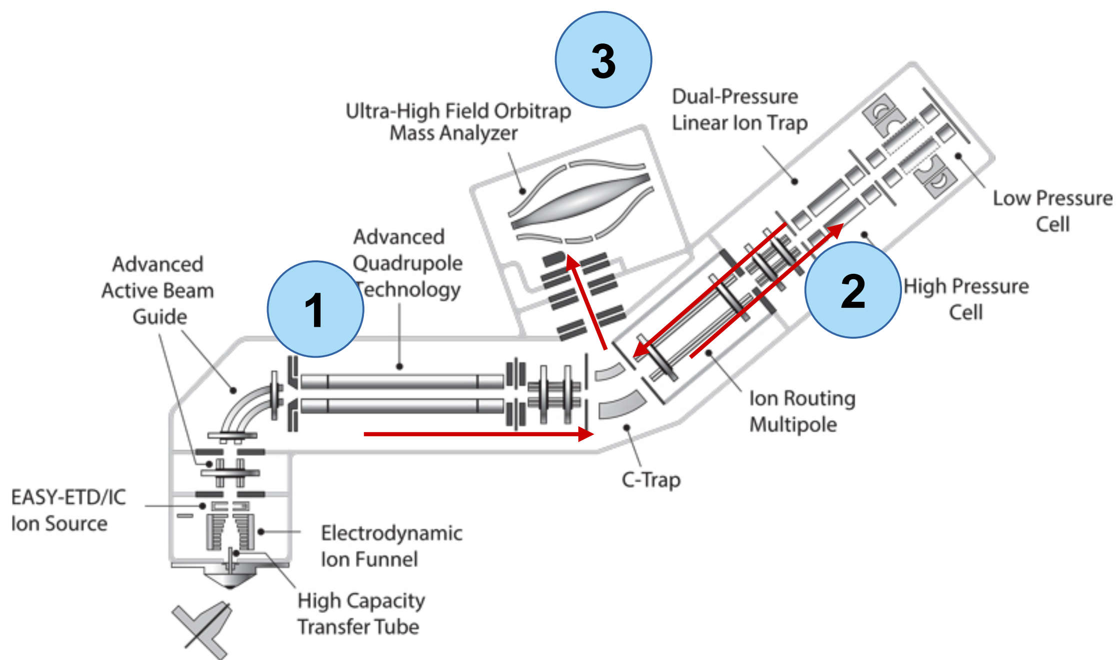




CRG Centre de Regulació Genòmica

PRM is a targeted proteomics workflow

PRM in the Orbitrap Fusion Lumos



1. Precursor selection in quadrupole
   - Isolation window
2. Precursor fragmentation in collision cell
   - Fill time
   - Collision energy
3. Fragment ion detection in the Orbitrap
   - Resolving power

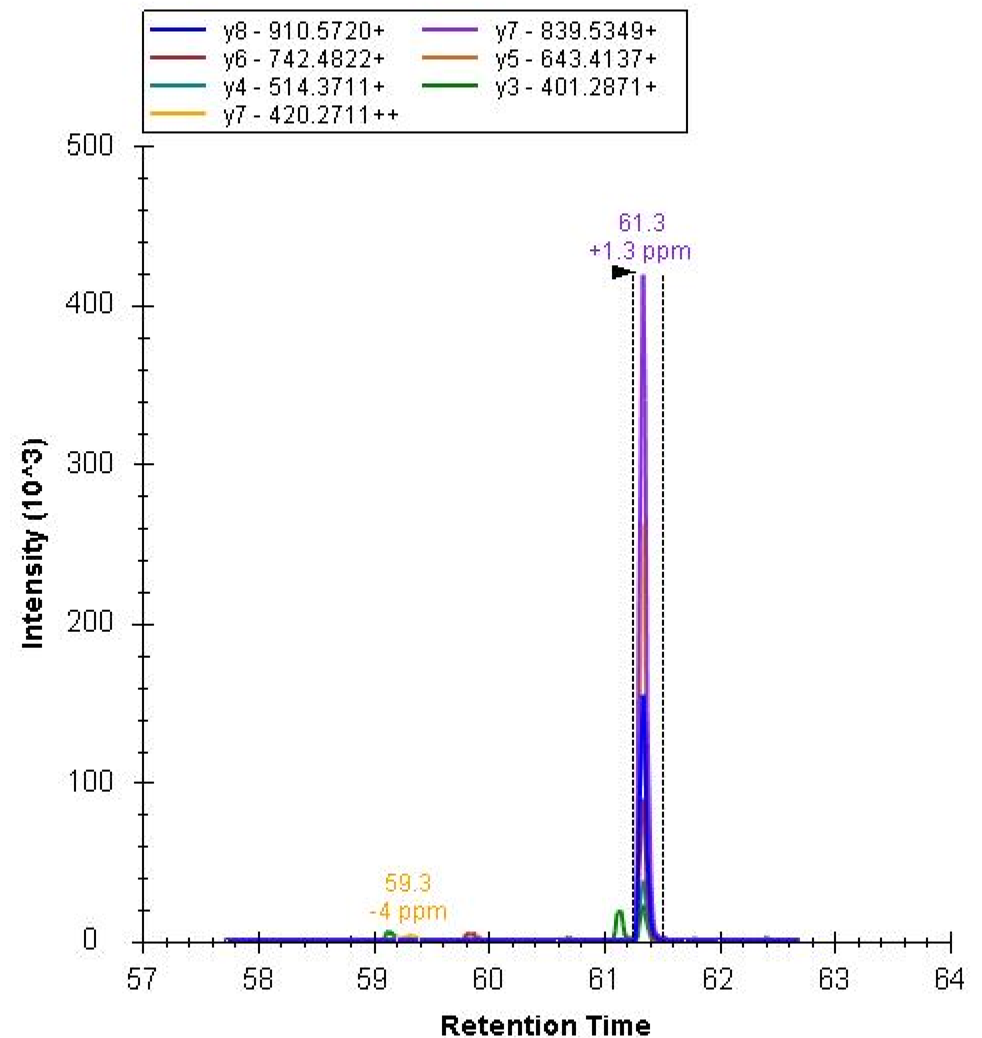
Peptide verification

Making sure that the integrated signal corresponds to the targeted peptide

Peptide verification

Making sure that the integrated signal corresponds to the targeted peptide

1. Co-elution of concurrent transitions

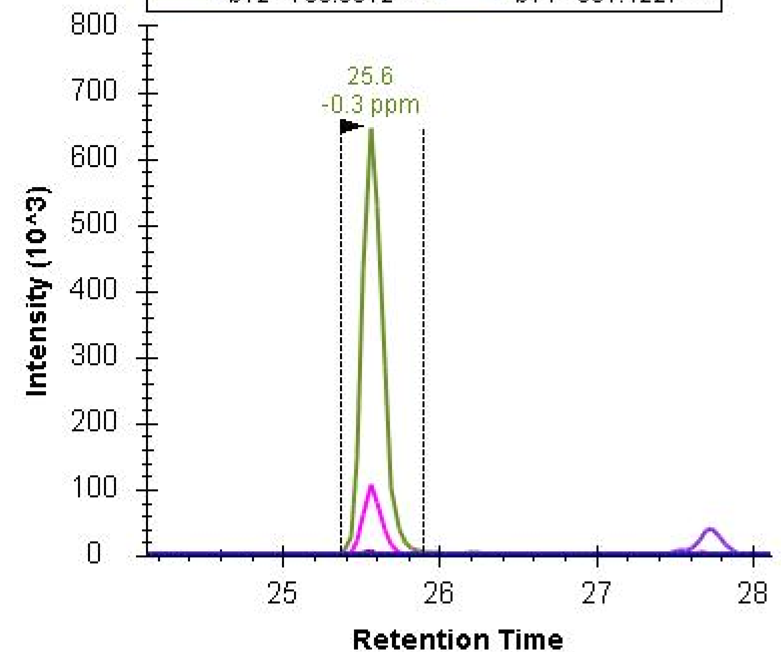


Peptide verification

Making sure that the integrated signal corresponds to the targeted peptide

1. Co-elution of concurrent transitions

2. Sequence information / coverage



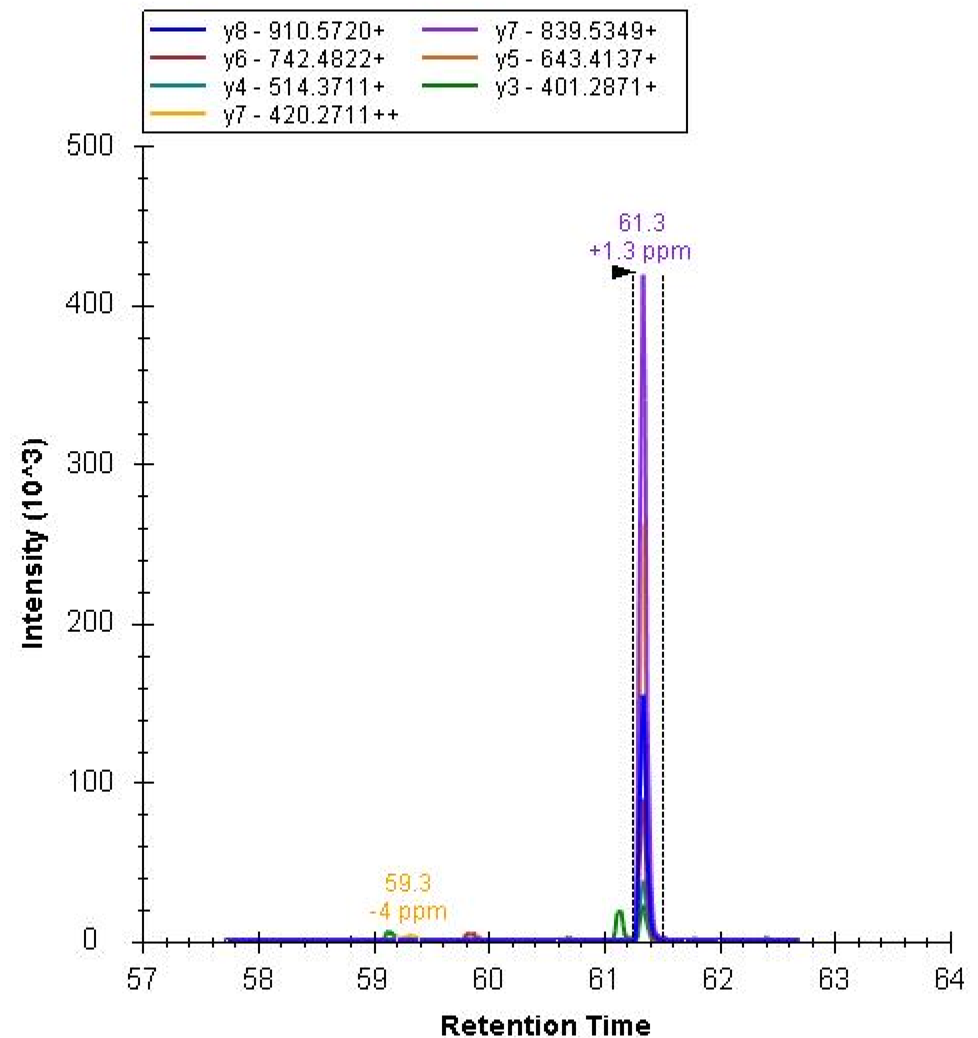
Peptide verification

Making sure that the integrated signal corresponds to the targeted peptide

1. Co-elution of concurrent transitions

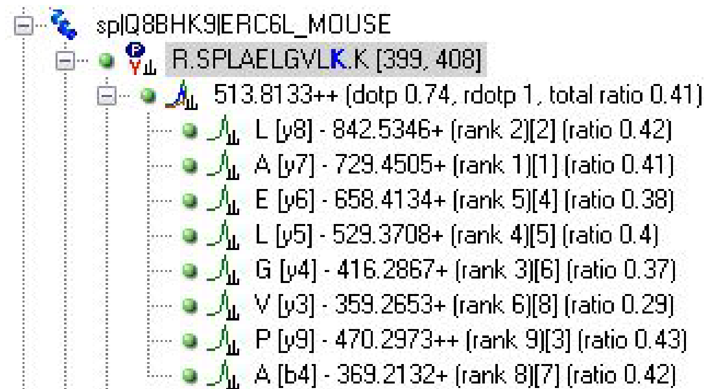
2. Sequence information / coverage

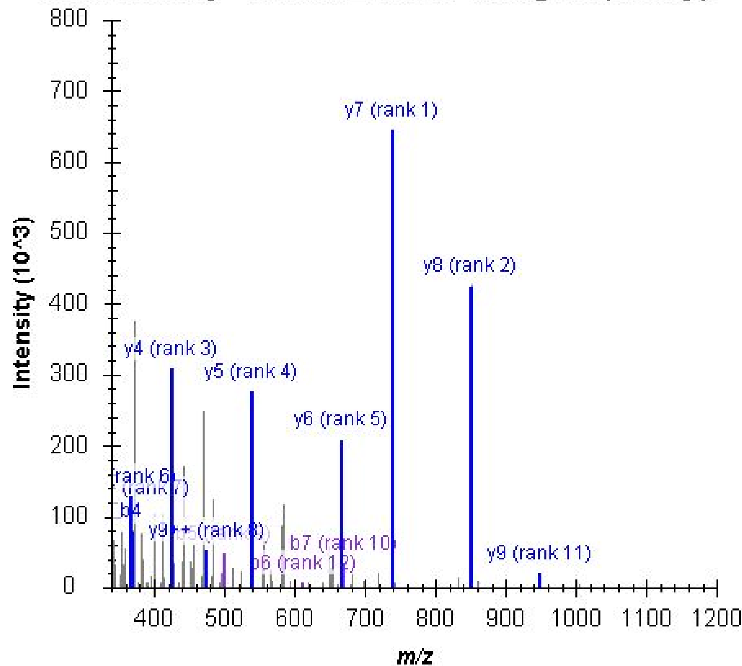
3. Reference MS2 spectra (libraries)

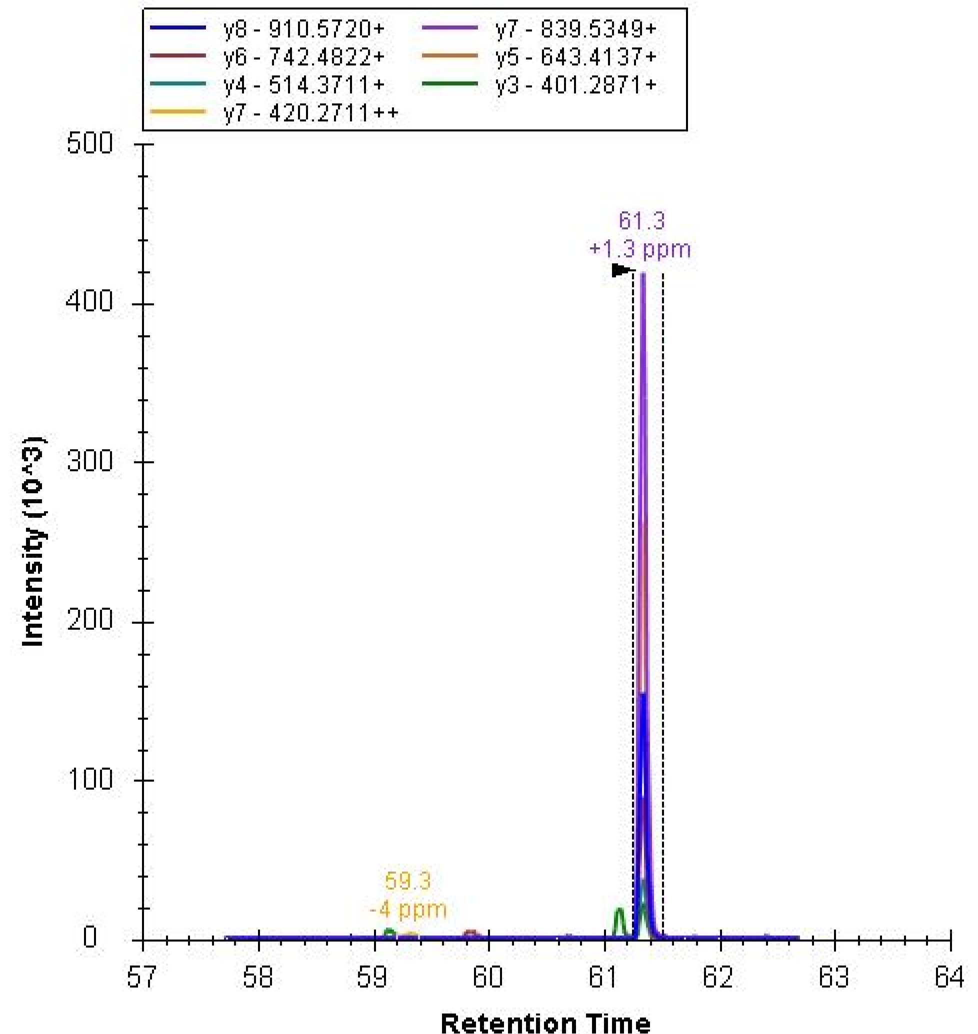


Peptide verification

Making sure that the integrated signal corresponds to the targeted peptide



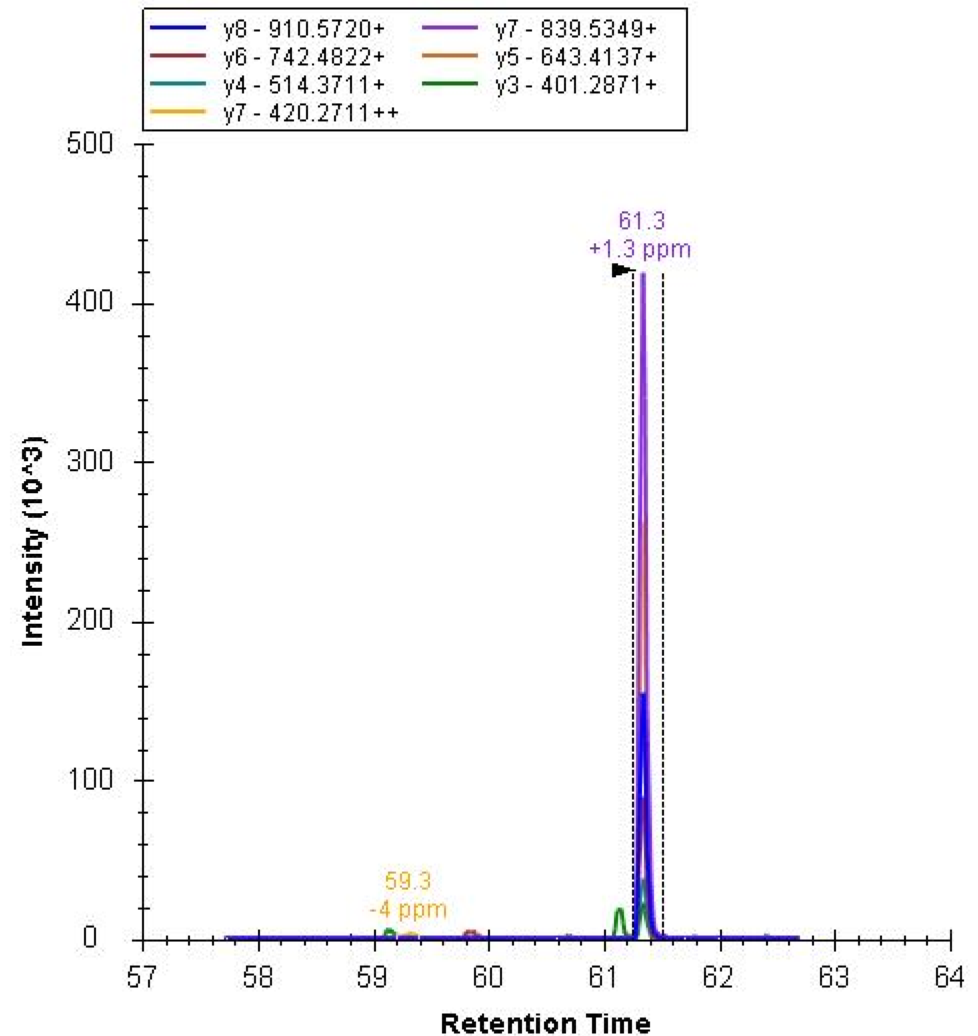




Peptide verification

Making sure that the integrated signal corresponds to the targeted peptide

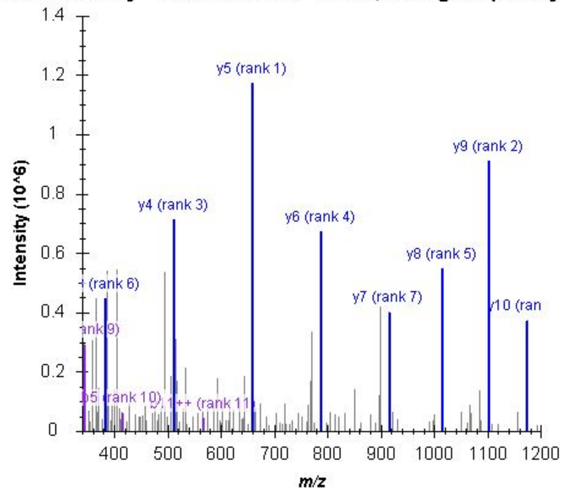
1. Co-elution of concurrent transitions
2. Sequence information / coverage
3. Reference MS2 spectra (libraries)
4. Retention time information

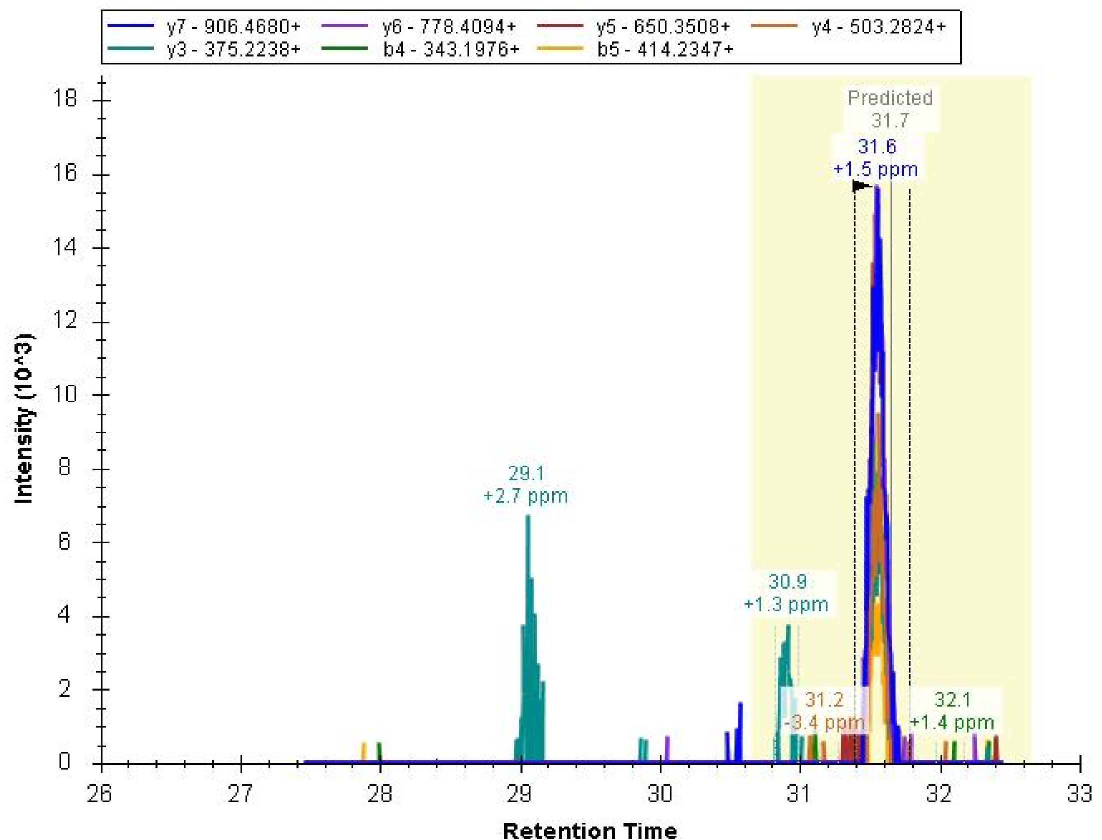


Centre de Regulació Genòmica

Peptide verification

Making sure that the integrated signal corresponds to the targeted peptide

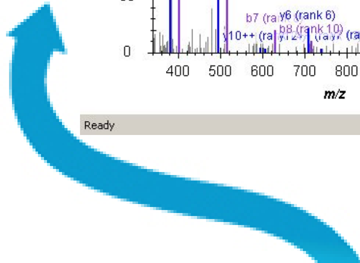




Centre de Regulació Genòmica

Peptide verification

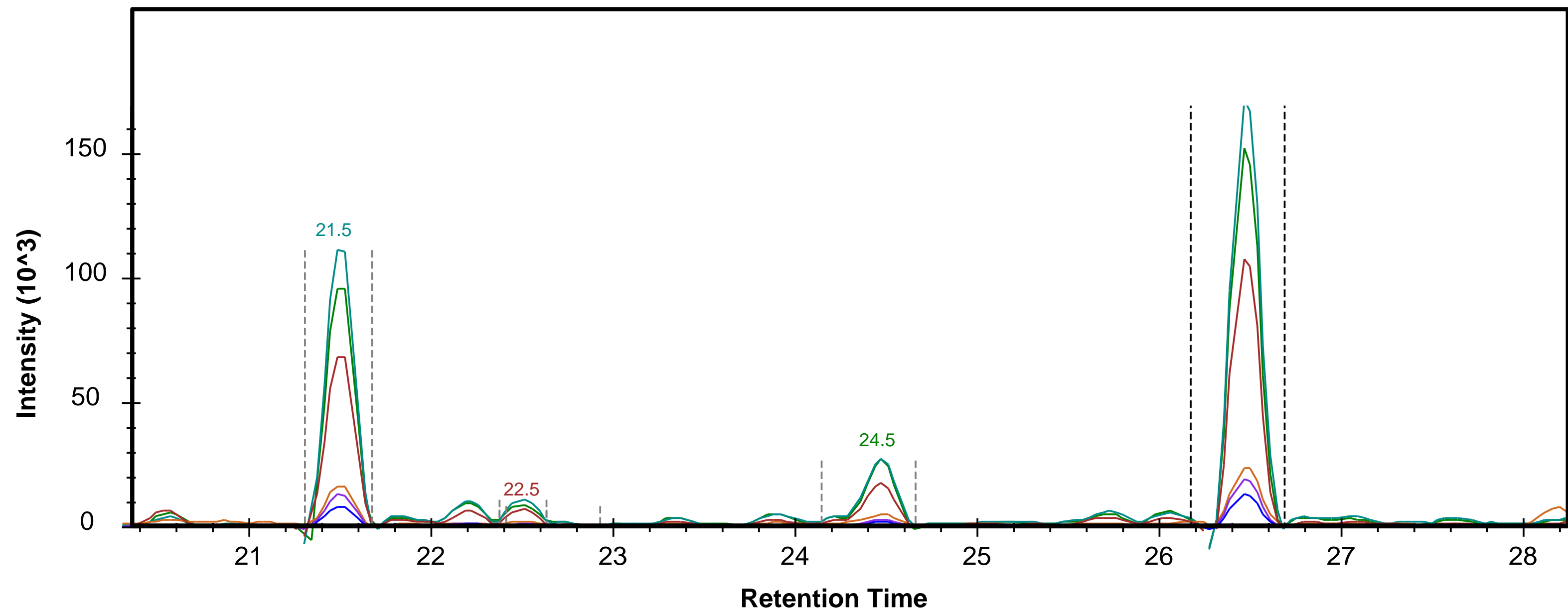
Making sure that the integrated signal corresponds to the targeted peptide



Centre de Regulació Genòmica

Peptide verification

Making sure that the integrated signal corresponds to the targeted peptide



CRG Centre de Regulació Genòmica

Peptide verification

Making sure that the integrated signal corresponds to the targeted peptide

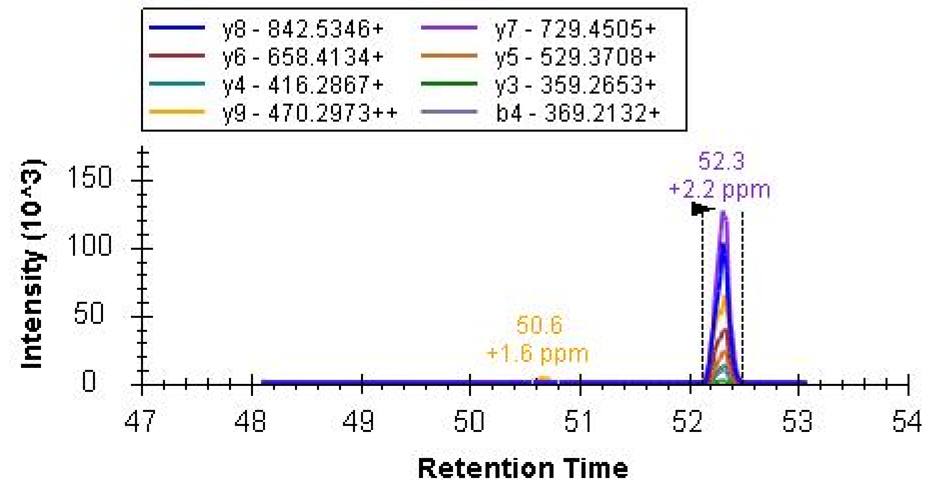
1. Co-elution of concurrent transitions

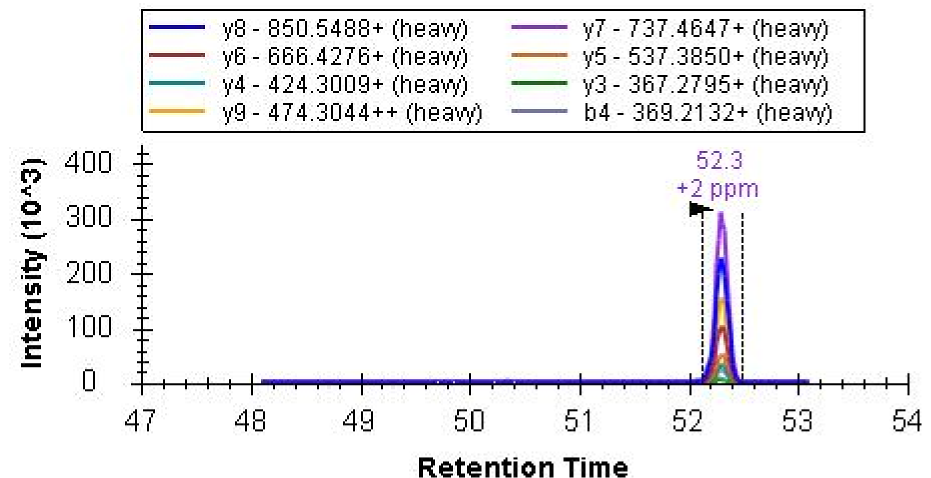
2. Sequence information / coverage

3. Reference MS2 spectra (libraries)

4. Retention time information

5. Reference internal standard

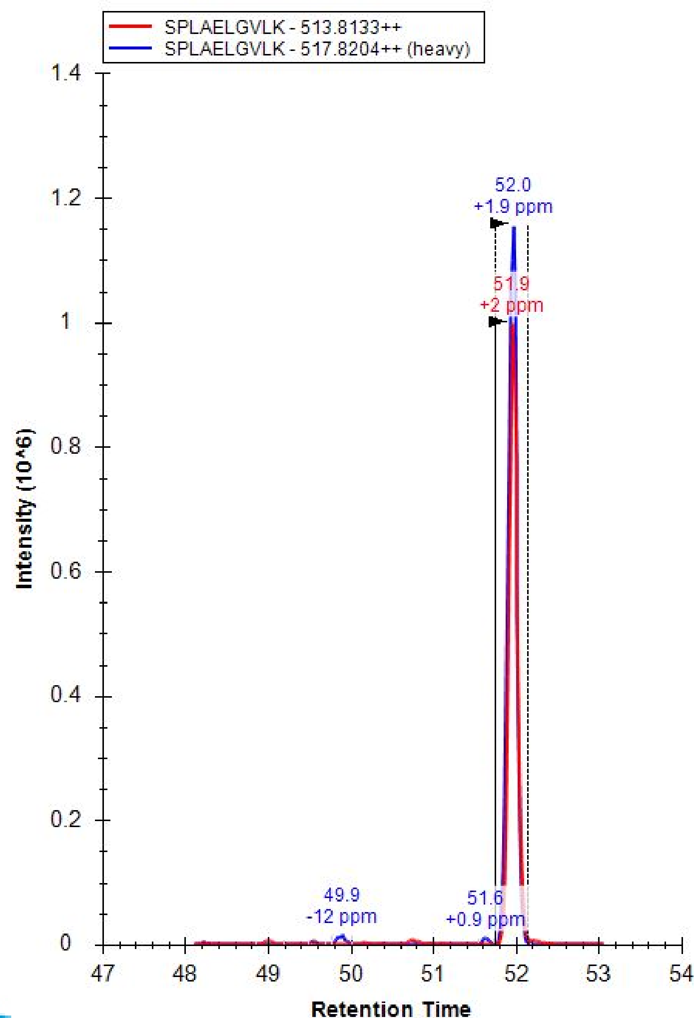


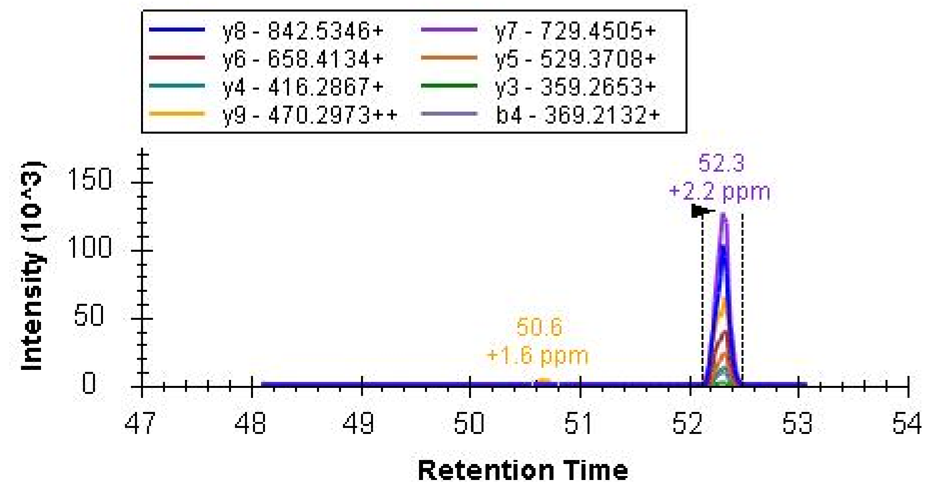


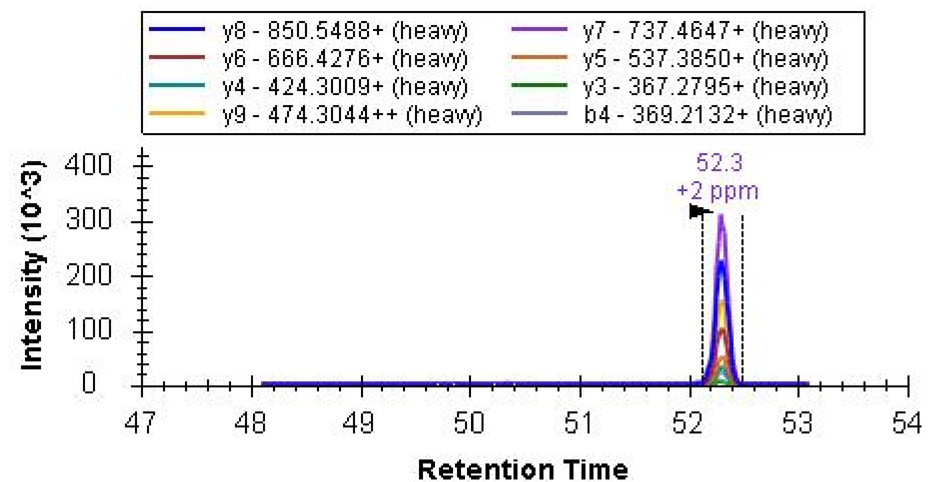
CRG Centre de Regulació Genòmica

Peptide verification

Making sure that the integrated signal corresponds to the targeted peptide







CRG Centre de Regulació Genòmica

Peptide quantification

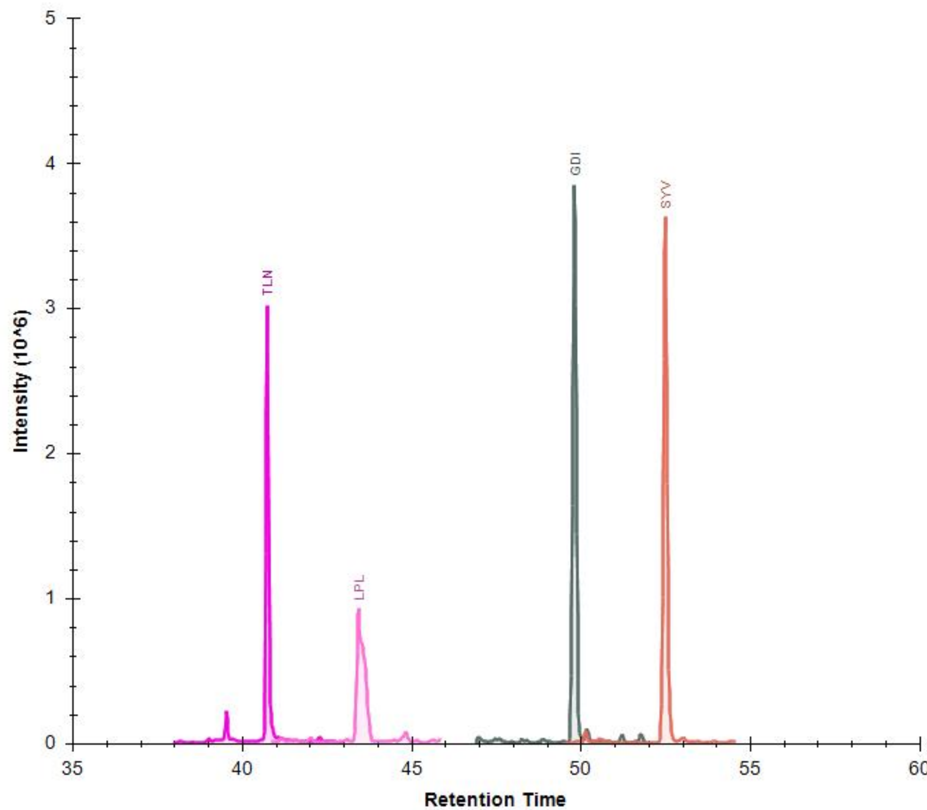
Aim for a peptide quantification with high accuracy and precision

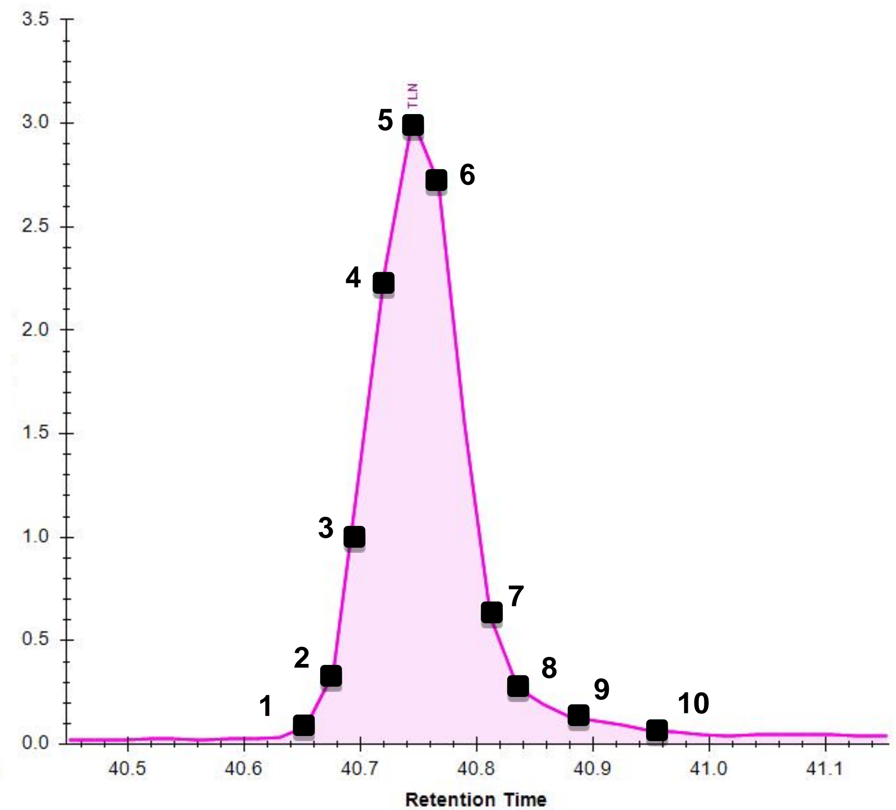
CRG Centre de Regulació Genòmica

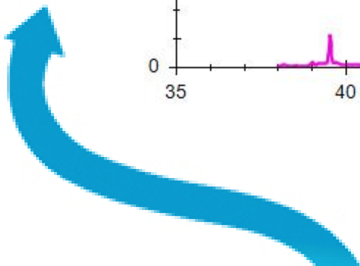
Peptide quantification

Aim for a peptide quantification with high accuracy and precision

<u>8-10 points</u> across chromatographic peak to define elution profile





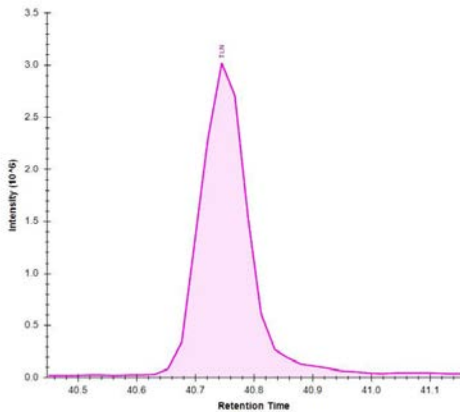


CRG Centre de Regulació Genòmica

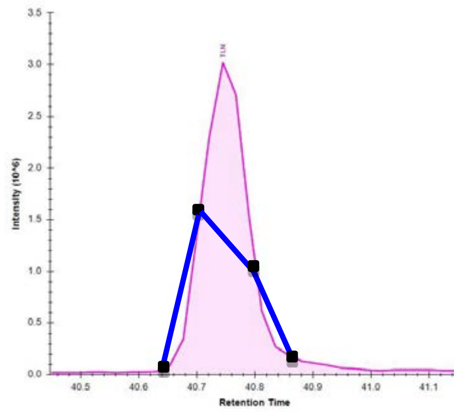
Peptide quantification

Aim for a peptide quantification with high accuracy and precision

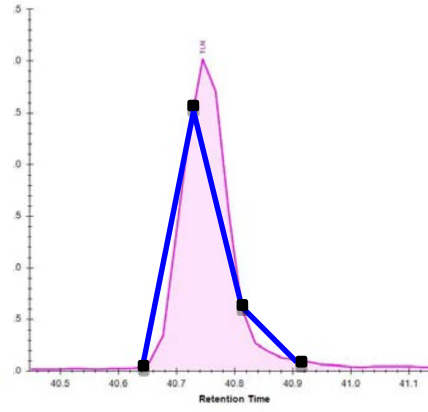
Theoretical

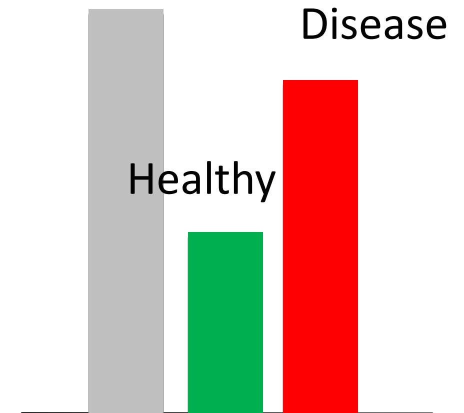


Healthy



Disease





4 points across chromatography peak

Centre de Regulació Genòmica

Peptide quantification

Aim for a peptide quantification with high accuracy and precision

Calibration strategies with internal standards

1. Single-point calibration
1. External calibration curve
1. Reverse calibration curve

Webinar #13 (2016)

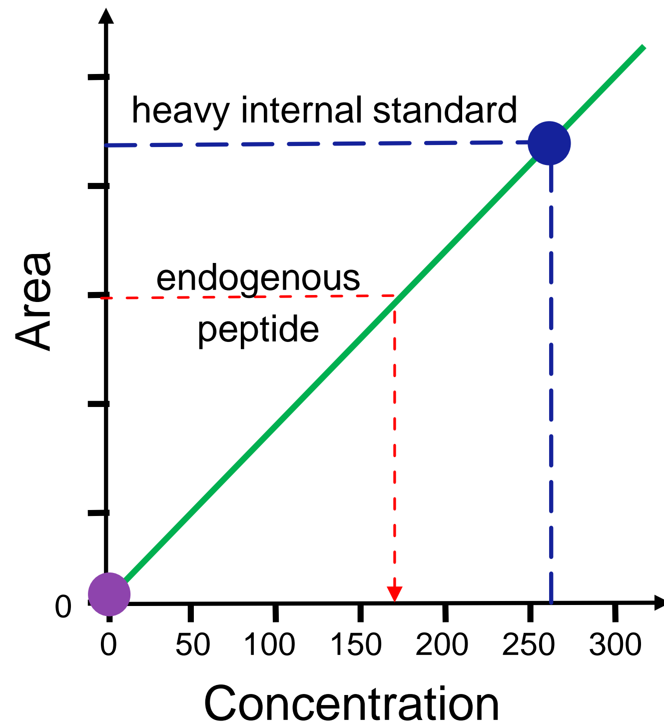
Calibrated Quantification with Skyline
Chris Shuford

CRG Centre de Regulació Genòmica

Peptide quantification

Aim for a peptide quantification with high accuracy and precision

Single-point calibration



We establish the calibration line with two points

- The response of the heavy internal standard
- The zero — that is, zero response for zero concentration.

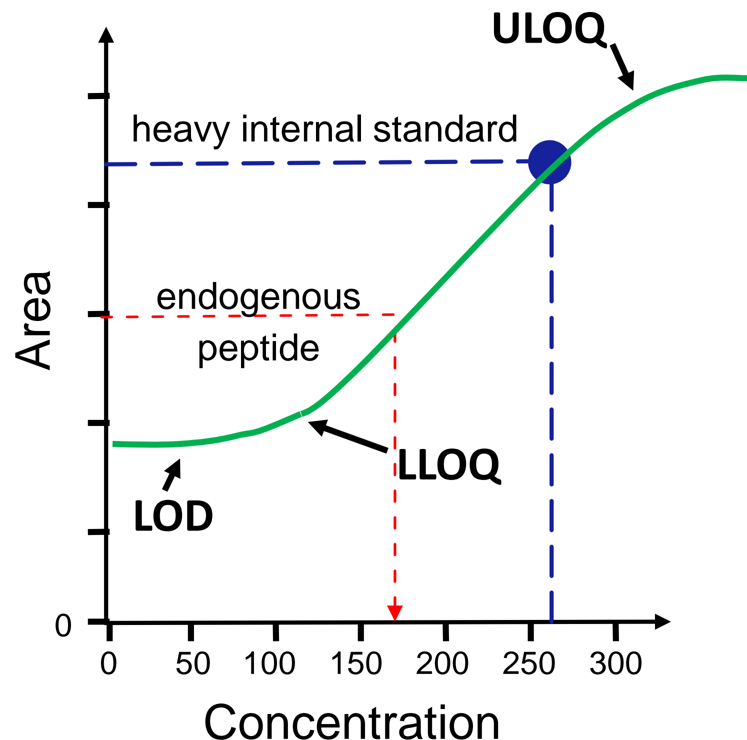
We assume a linear range response

Centre de Regulació Genòmica

Peptide quantification

Aim for a peptide quantification with high accuracy and precision

Single-point calibration



We establish the calibration line with two points

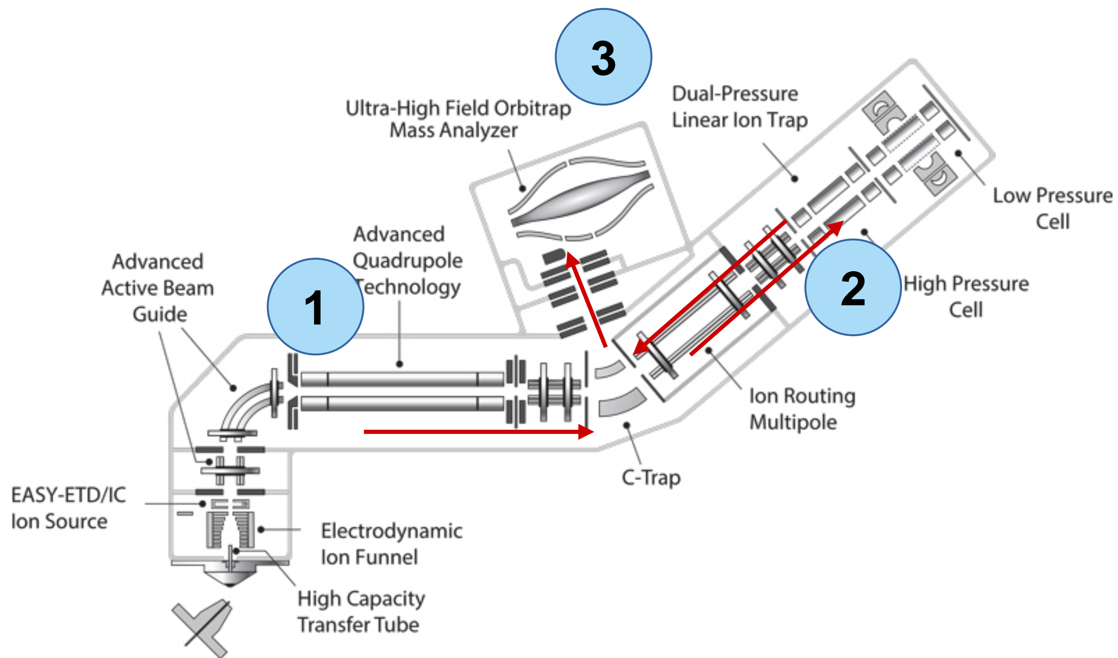
- The response of the heavy internal standard
- The zero — that is, zero response for zero concentration.

We assume a linear range response

Centre de Regulació Genòmica

Sensitivity and selectivity: a double boost for PRM

PRM in the Orbitrap Fusion Lumos

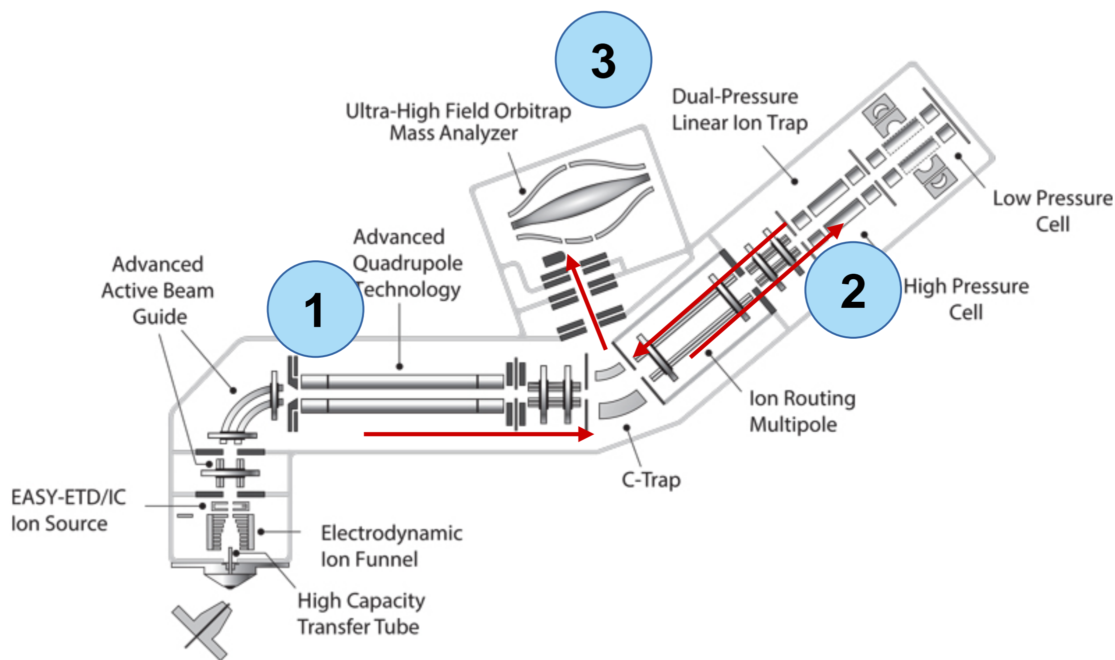


1. Precursor selection in quadrupole
   - Isolation window
2. Precursor fragmentation in collision cell
   - Fill time
   - Collision energy
3. Fragment ion detection in the Orbitrap
   - Resolving power

Centre de Regulació Genòmica

Sensitivity and selectivity: a double boost for PRM

PRM in the Orbitrap Fusion Lumos



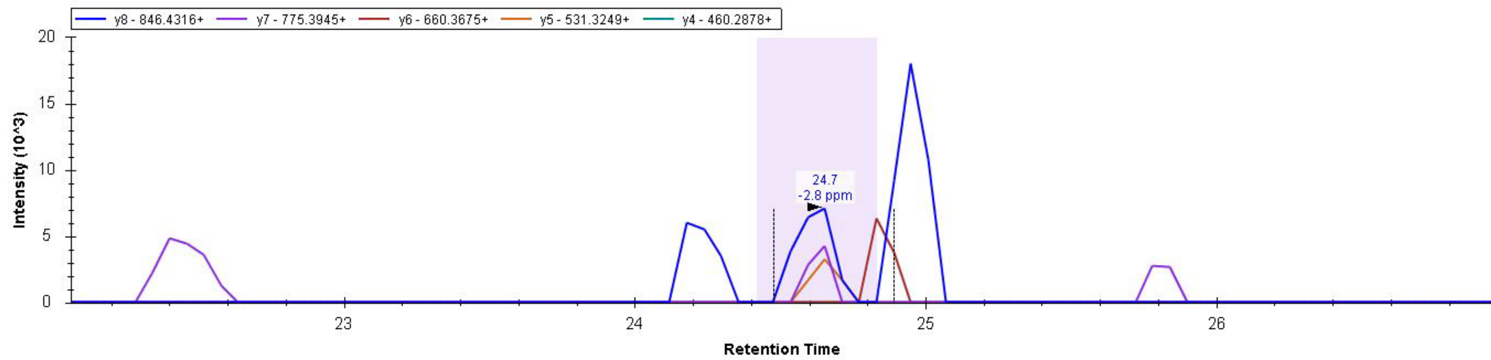
1. Precursor selection in quadrupole
   - Isolation window
2. Precursor fragmentation in collision cell
   - **Fill time** → SENSITIVITY
   - Collision energy
3. Fragment ion detection in the Orbitrap
   - **Resolving power** → SELECTIVITY

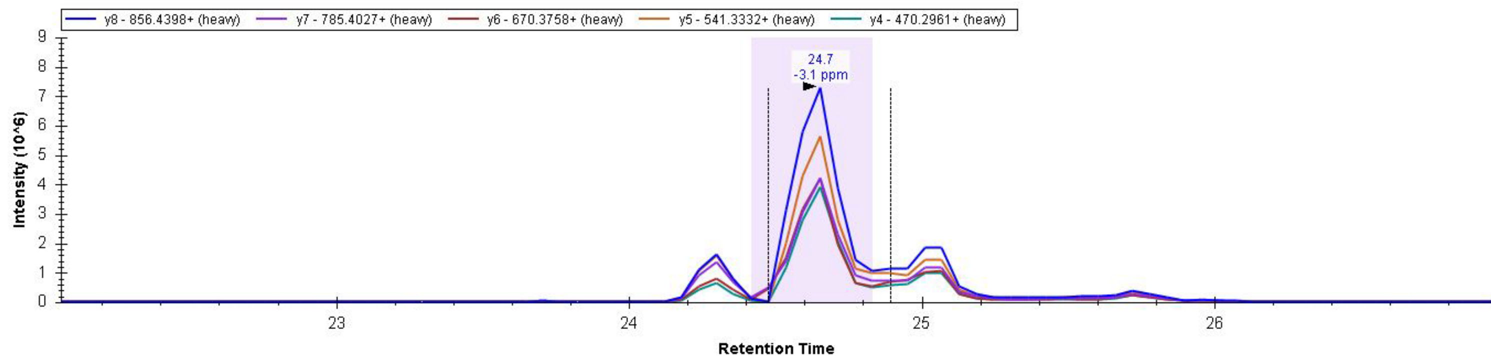
Centre de Regulació Genòmica

Sensitivity and selectivity: a double boost for PRM

PRM in the Orbitrap Fusion Lumos

Fill time = 60 ms Res Power = 30K



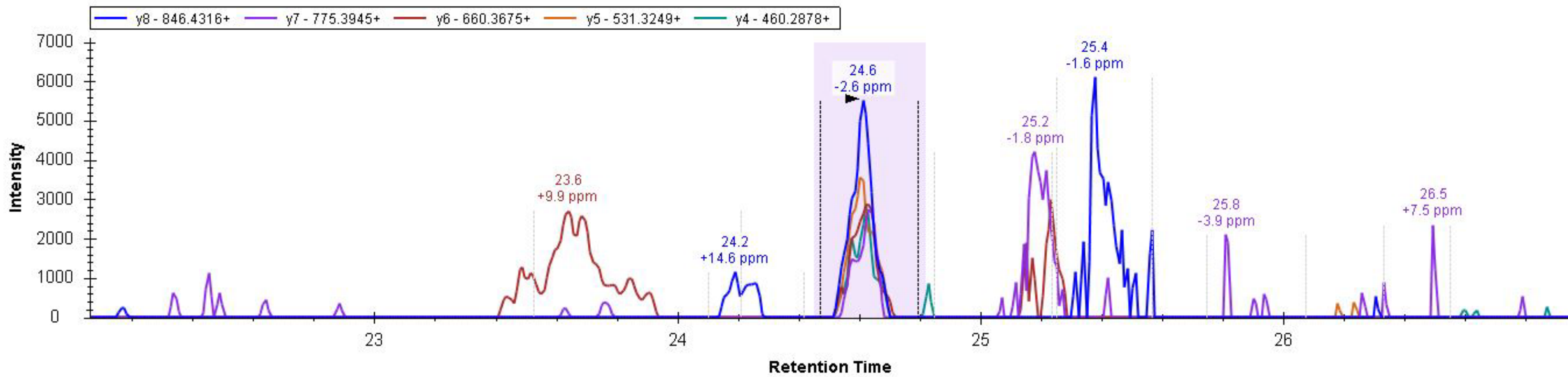


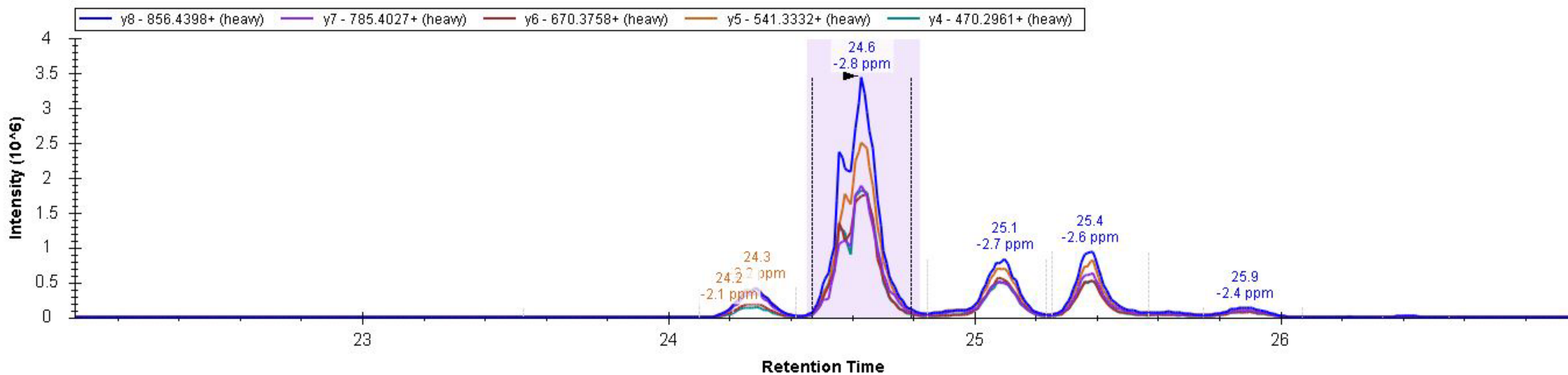
CRG
Centre de Regulació Genòmica

Sensitivity and selectivity: a double boost for PRM

PRM in the Orbitrap Fusion Lumos

Fill time = 250 ms Res Power = 120K





CRG
Centre
de Regulació
Genòmica

Sensitivity and selectivity: a double boost for PRM

PRM in the Orbitrap Fusion Lumos

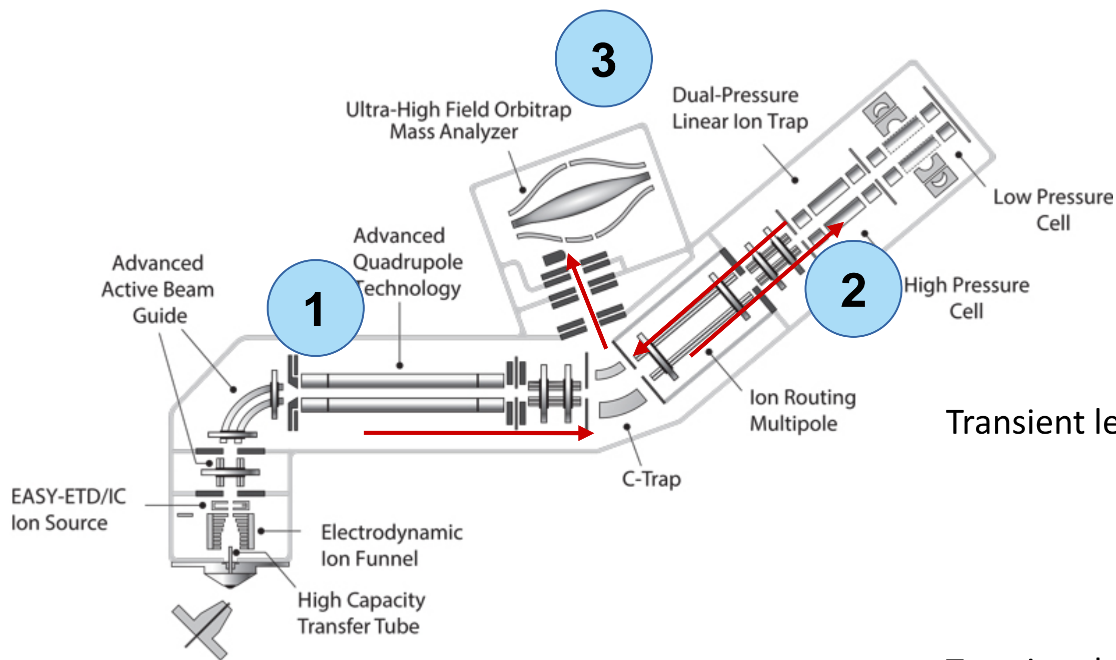
3
Ultra-High Field Orbitrap Mass Analyzer
Dual-Pressure Linear Ion Trap
Low Pressure Cell
Advanced Quadrupole Technology
Advanced Active Beam Guide
1
2
High Pressure Cell
Ion Routing Multipole
C-Trap
EASY-ETD/IC Ion Source
Electrodynamic Ion Funnel
High Capacity Transfer Tube

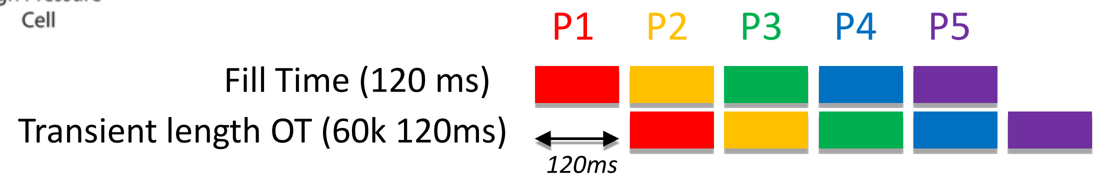
	P1	P2	P3	P4	P5	
Fill Time (120 ms)	P1	P2	P3	P4	P5	
Transient length OT (60k 120ms)	← 120ms →	P1	P2	P3	P4	P5

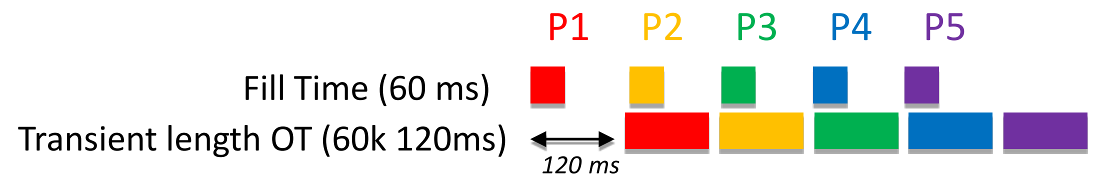
Centre de Regulació Genòmica

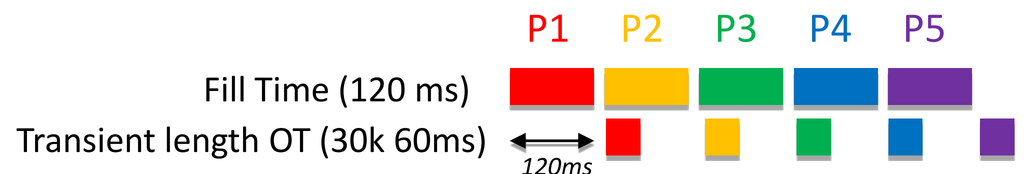
Sensitivity and selectivity: a double boost for PRM

PRM in the Orbitrap Fusion Lumos









CRG Centre de Regulació Genòmica

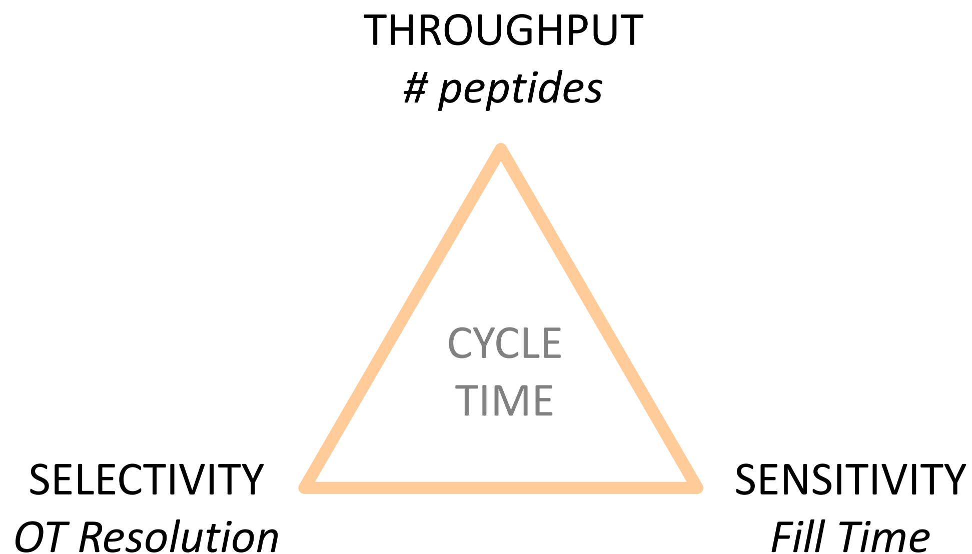
Sensitivity and selectivity: a double boost for PRM

PRM in the Orbitrap Fusion Lumos

Res. at m/z 200	Transient length [ms]	Approx. scan speed [Hz]	"Free" fill time [ms]
15,000	32	na	22
30,000	64	15	54
60,000	128	7.5	118
120,000	256	4	246
240,000	512	2	502
450,000	1024	<1	1014

Sensitivity and selectivity: a double boost for PRM

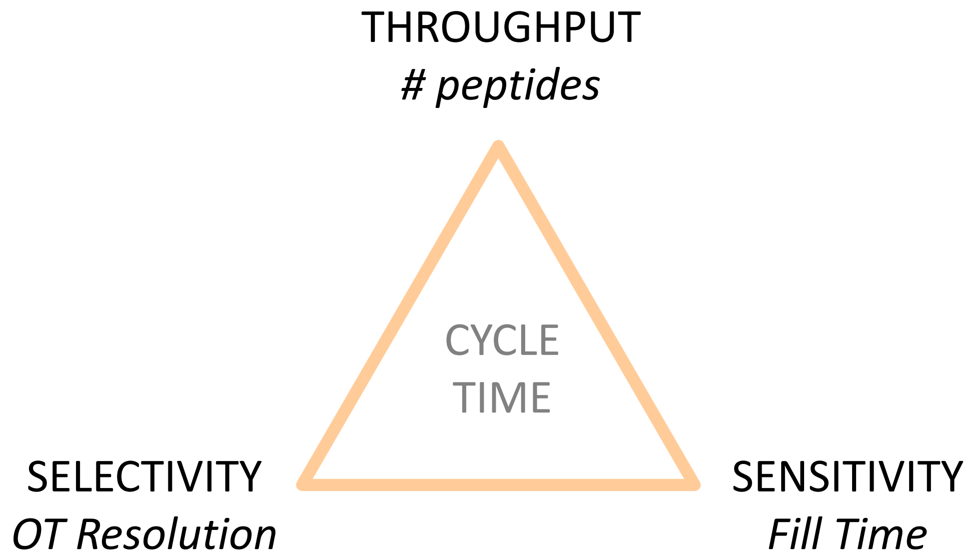
PRM in the Orbitrap Fusion Lumos

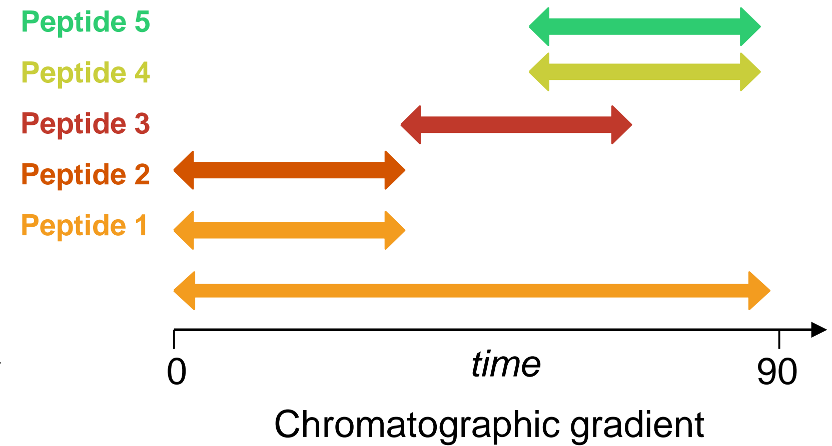


Centre de Regulació Genòmica

Sensitivity and selectivity: a double boost for PRM

PRM in the Orbitrap Fusion Lumos





CRG
Centre de Regulació Genòmica

Orbitrap Fusion Lumos Method Editor for PRM

STD-FL-PRM-90min-R_webinar.meth - Thermo Xcalibur Instrument Setup

Method Editor | Global Parameters | Scan Parameters | Summary

Method Timeline

Method Duration (min): 110

Experiment 1 — Time Range: 0-110 min

tMS² OT HCD

Scans: MS, MSⁿ
Filters: Precursor Selection Range, MIPS, Intensity, Charge State, Dynamic Exclusion, Targeted Inclusion, Targeted Exclusion, Apex Detection
Triggers: Targeted Mass Trigger, Targeted Loss Trigger
Alternate Precursor Sorts: Sort by Charge

Targeted MSⁿ Scan Properties

Property	Value
MSⁿ Level (n)	2
Multiplex Ions	
Isolation Mode	Quadrupole
Isolation Window (m/z)	1.4
Activation Type	HCD
HCD Collision Energy (%)	30
Stepped Collision Energy	
Detector Type	Orbitrap
Orbitrap Resolution	Defined in Table
Mass Range	Normal
Scan Range (m/z)	340-950
RF Lens (%)	30
AGC Target	5.0e4
Inject Ions for All Available Parallelizable Time	
Maximum Injection Time (ms)	Defined in Table
Microscans	1
Data Type	Centroid
Polarity	Positive
Source Fragmentation	

Ready

CRG
Centre de Regulació Genòmica

Targeted MS^n Scan Properties — Show Favorites

Property	Value
MS^n Level (n)	2
Multiplex Ions	☐
Isolation Mode	Quadrupole
Isolation Window (m/z)	1.4
Activation Type	HCD
HCD Collision Energy (%)	30
Stepped Collision Energy	☐
Detector Type	Orbitrap
Orbitrap Resolution	Defined in Table
Mass Range	Normal
Scan Range (m/z)	340-950
RF Lens (%)	30
AGC Target	5.0e4
Inject Ions for All Available Parallelizable Time	☐
Maximum Injection Time (ms)	Defined in Table
Microscans	1
Data Type	Centroid
Polarity	Positive
Source Fragmentation	☐

CRG Centre de Regulació Genòmica

Orbitrap Fusion Lumos Method Editor for PRM

Mass List Table — Import Export

	Compound	Formula	Adduct	m/z	z	t start (min)	t stop (min)	Orbitrap Resolution	Maximum Injection Time (ms)
1	IPGIIIAASAVR_light			590.8742	2	71.3	81.3	60000	118
2	IPGIIIAASAVR_heavy			595.8784	2	71.3	81.3	60000	118
3	FGLTTSR_light			391.2138	2	28.52	38.52	60000	118
4	FGLTTSR_heavy			396.2179	2	28.52	38.52	60000	118
5	LAALPNVYEVISK_light			708.9085	2	80.26	90.26	60000	118
6	LAALPNVYEVISK_heavy			712.9156	2	80.26	90.26	60000	118
7	ASGQAFELILSPR_light			694.8803	2	75.58	85.58	30000	54
8	ASGQAFELILSPR_heavy			699.8844	2	75.58	85.58	30000	54
9	ESVPEFPLSPPK_light			663.8506	2	68.26	78.26	30000	54
10	ESVPEFPLSPPK_heavy			667.8577	2	68.26	78.26	30000	54
11	SHEAEVLK_light			456.7429	2	4.98	14.98	60000	118
12	SHEAEVLK_heavy			460.75	2	4.98	14.98	60000	118
13	VADYIPQLAK_light			559.3162	2	56.85	66.85	60000	118
14	VADYIPQLAK_heavy			563.3233	2	56.85	66.85	60000	118
15	YAIAVNDLGTEYVHR_light			574.2933	3	59.83	69.83	120000	246
16	YAIAVNDLGTEYVHR_heavy			577.6294	3	59.83	69.83	120000	246
17	VLSPEAVR_light			435.7558	2	26.21	36.21	120000	246
18	VLSPEAVR_heavy			440.7599	2	26.21	36.21	120000	246
19	VLKPIQLTDPGK_light			436.9344	3	40.72	50.72	60000	118
20	VLKPIQLTDPGK_heavy			439.6058	3	40.72	50.72	60000	118

More PRM Webinars

Webinar #3 (2013)

PRM Targeted Proteomics Using Full-Scan MS and Skyline
Bruno Domon

Webinar #9 (2015)

Research-grade targeted proteomics assay development: PRM for PTM studies with Skyline.
Jacob D. Jaffe

Other webinars
https://skyline.ms/wiki/home/software/Skyline/page.view?name=webinars

Centre de Regulació Genòmica

Hands-on tutorial

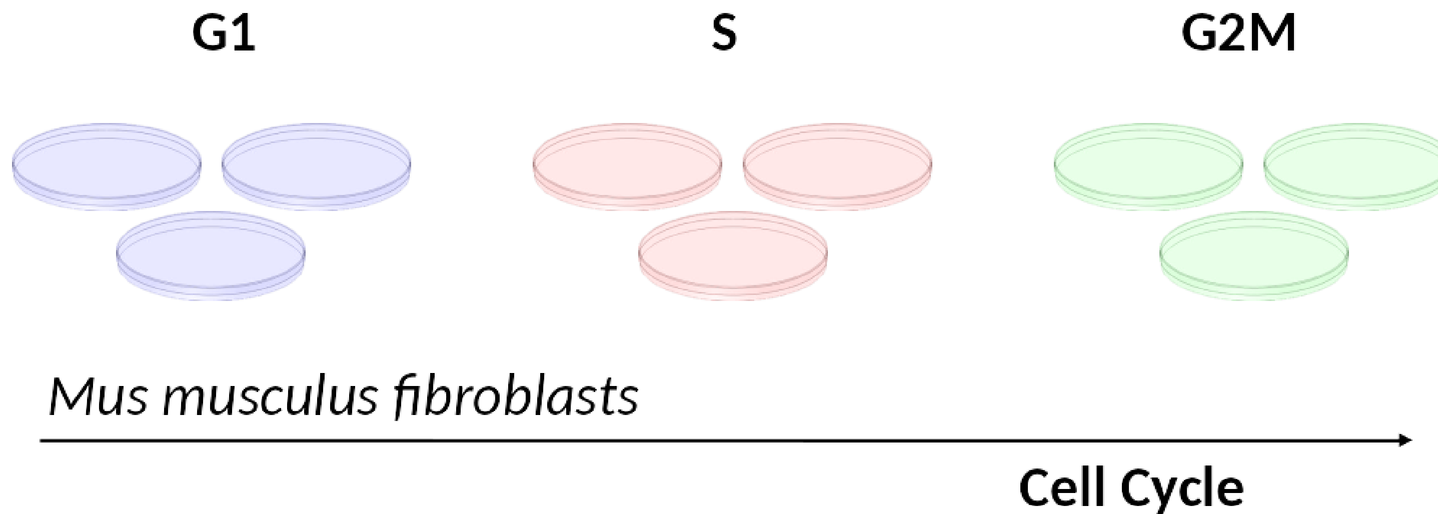
PRM method development and step-by-step analysis of Lumos PRM data

CRG Centre de Regulació Genòmica

Hands-on tutorial

PRM method development and step-by-step analysis of Lumos PRM data

31 peptides from 19 proteins related to the cell cycle of mouse fibroblasts



CRG
Centre de Regulació Genòmica

Hands-on tutorial

PRM method development and step-by-step analysis of Lumos PRM data

1. Setting up a Skyline document for PRM acquisition

2. Prepare and export the PRM method

3. PRM data analysis

Centre de Regulació Genòmica

Hands-on tutorial

PRM method development and step-by-step analysis of Lumos PRM data

EMBO Practical Course on Targeted Proteomics
Barcelona, 11-16 November 2018

Registration Opens March 2018

Other courses
https://skyline.ms/project/home/software/Skyline/events/begin.view?

Learn More

- Webinar #18: TBD (coming soon)

- Weeklong Courses 2018
  - Buck Institute, Novato, CA – April 2-6
  - NEU, Boston – April 30 – May 11
  - ETH, Zurich – July 2-6
  - U. of Wa., Seattle – July 30 – August 3
  - New! Duke, Durham, NC – September 17-21
  - **CRG, Barcelona – November 12-16**
  - Shanghai – October 22-26

- Workshops and Conferences 2018
  - MSACL, Palm Springs – January 20&21
  - pre-Lorne, Melbourne – January 29-31
  - IIT Bombay, Mumbai – February
  - US HUPO, Minneapolis – March 10&11
  - ASMS, San Diego – June 2&3
  - *User Group Meeting at ASMS, San Diego – June 3*
  - CNPEM, Campinas, Brazil – November 7-9

Listings updated in **Join Us** section of Skyline homepage:
https://skyline.ms/Skyline.url

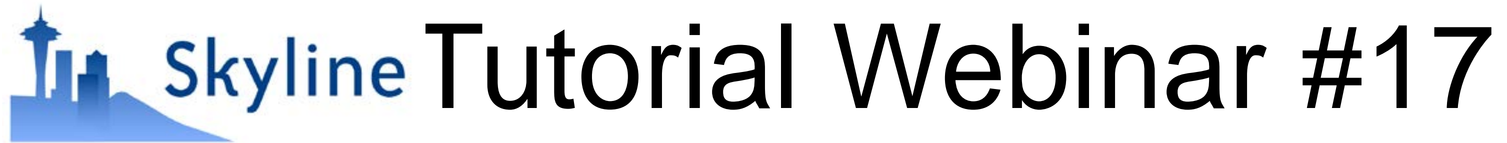
Questions?

- Ask any questions at the following form:

https://skyline.ms/QA4Skyline.url

- Take the post-webinar survey:

https://skyline.ms/survey4webinar.url



This ends this Skyline Tutorial Webinar.

Please give us feedback on the webinar at the following survey:

https://skyline.ms/survey4webinar.url

A recording of today’s meeting will be available shortly at the Skyline website.

We look forward to seeing you at a future Skyline Tutorial Webinar.