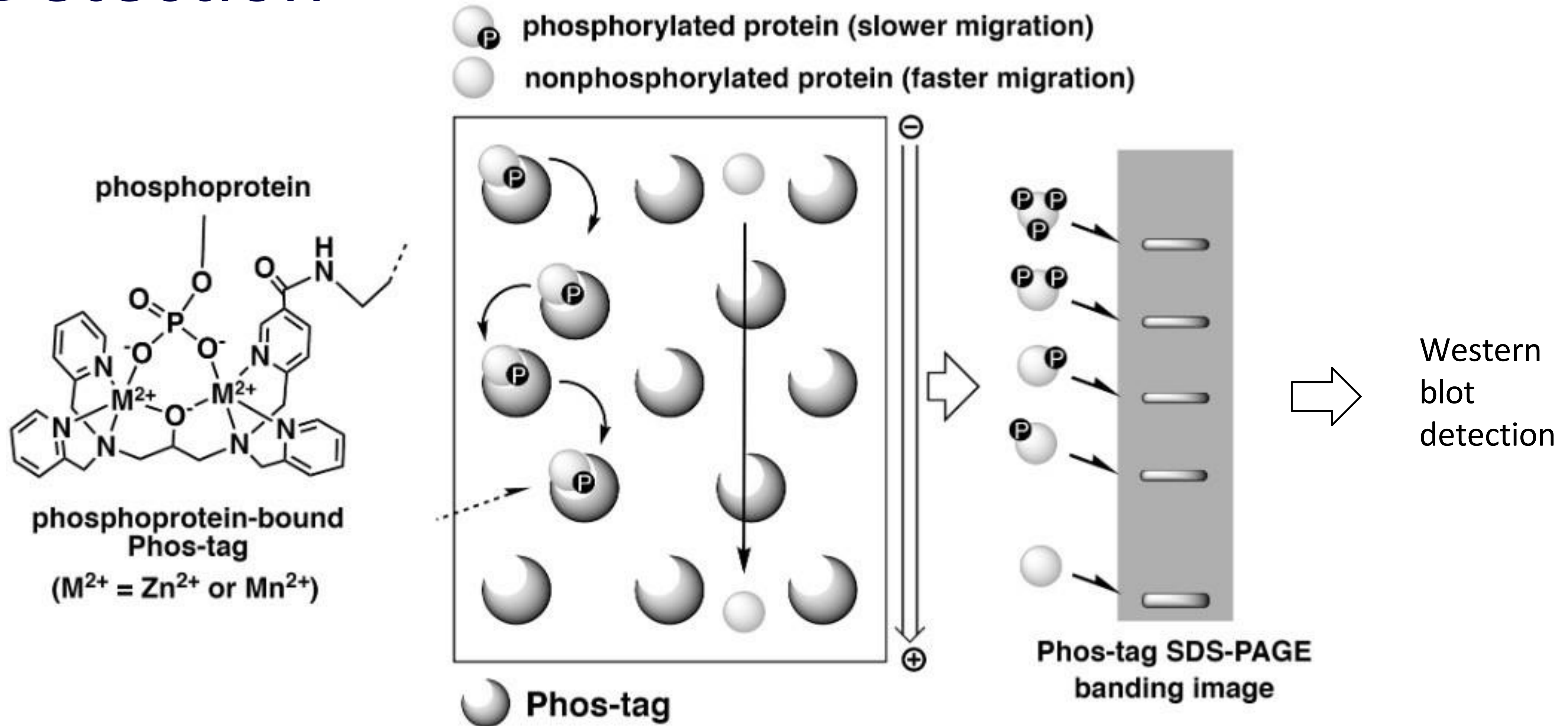
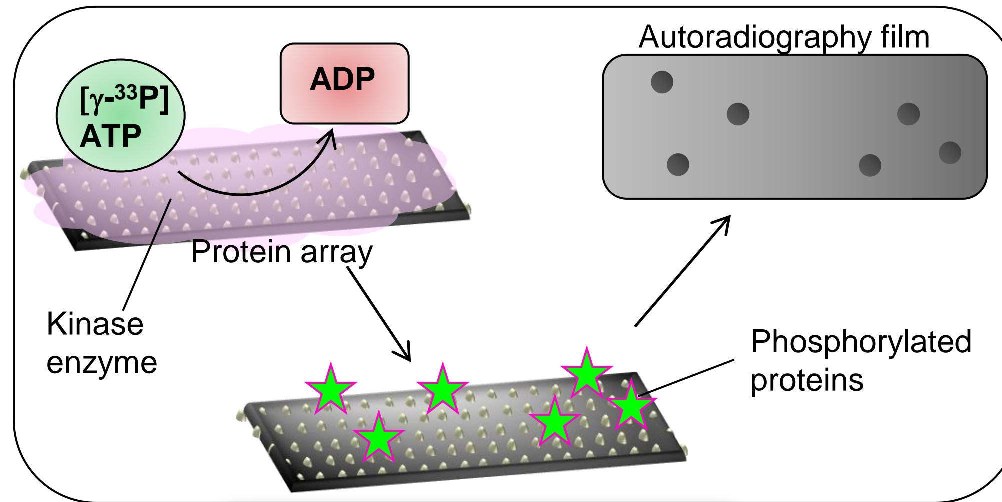
Gel-based Technique for Phosphoproteins Detection



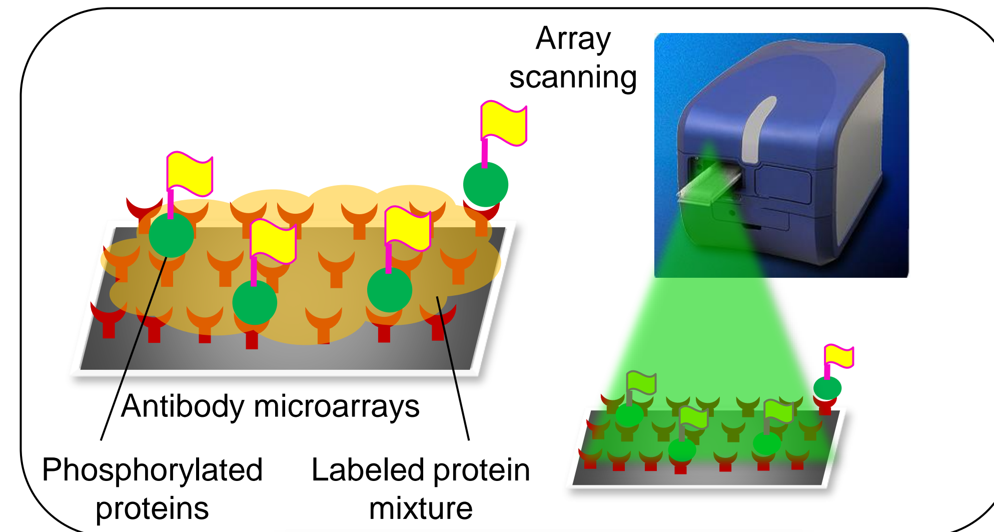
Western blot detection

Microarray-based detection techniques for Phosphorylation



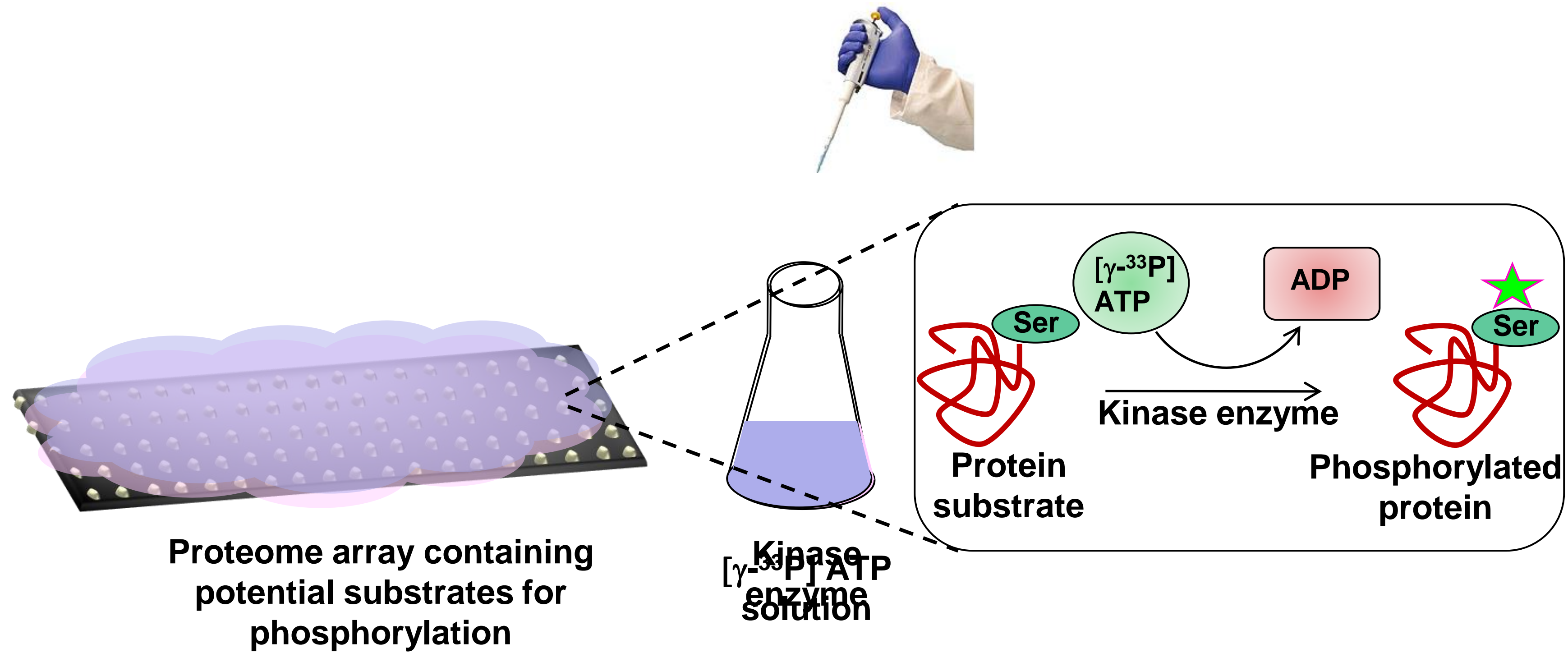
1. Protein microarrays

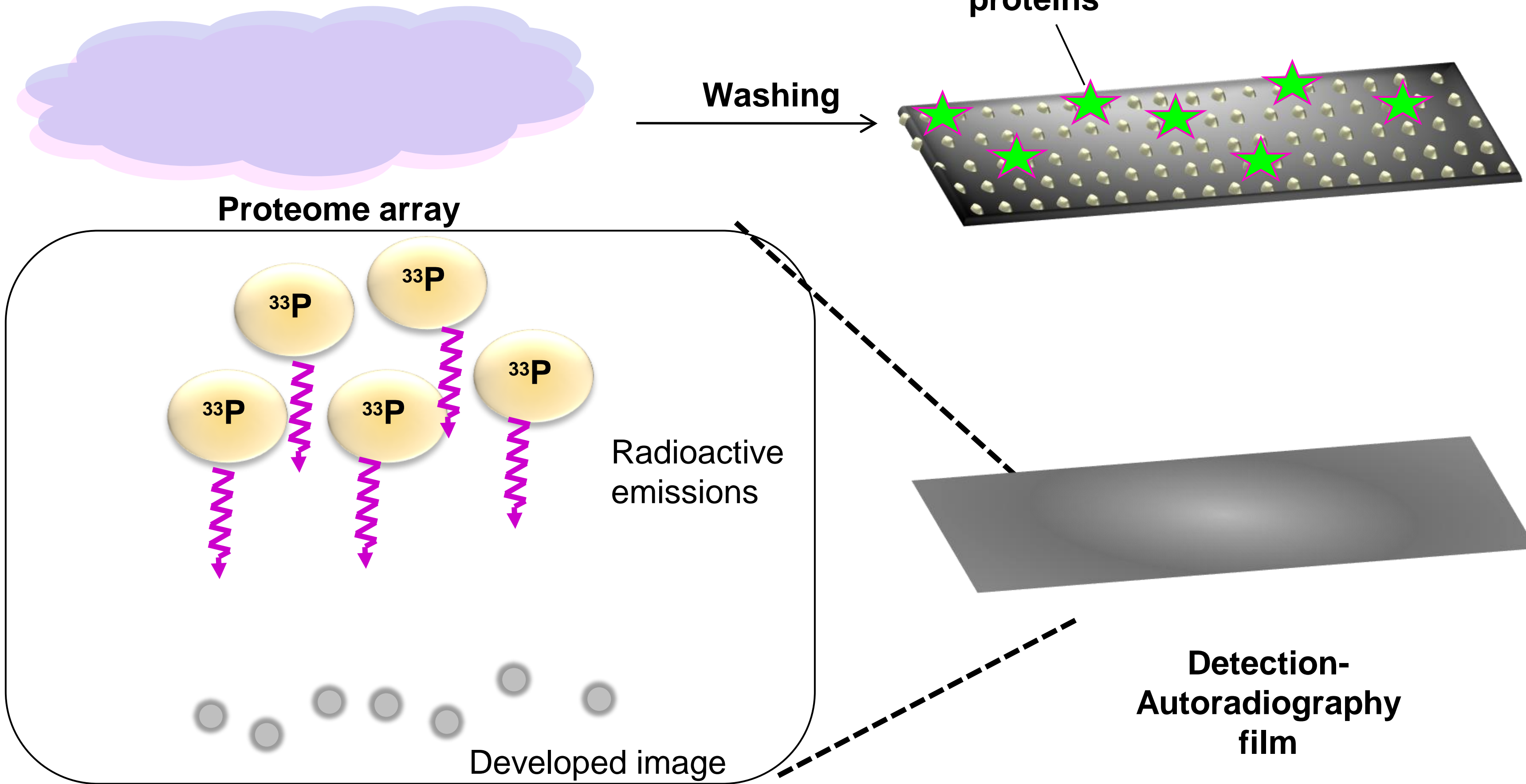
in vitro detection with known kinases



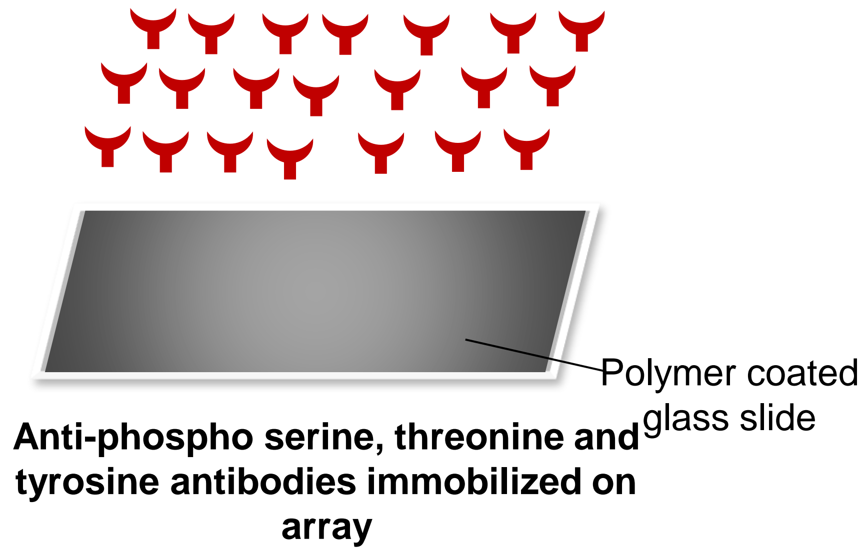
2. Antibody microarrays

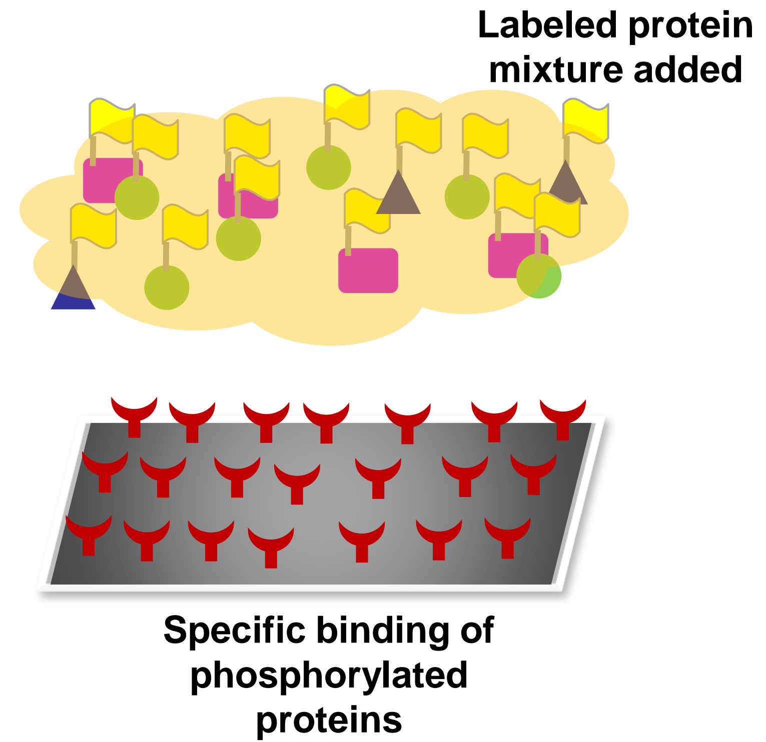
Protein microarrays

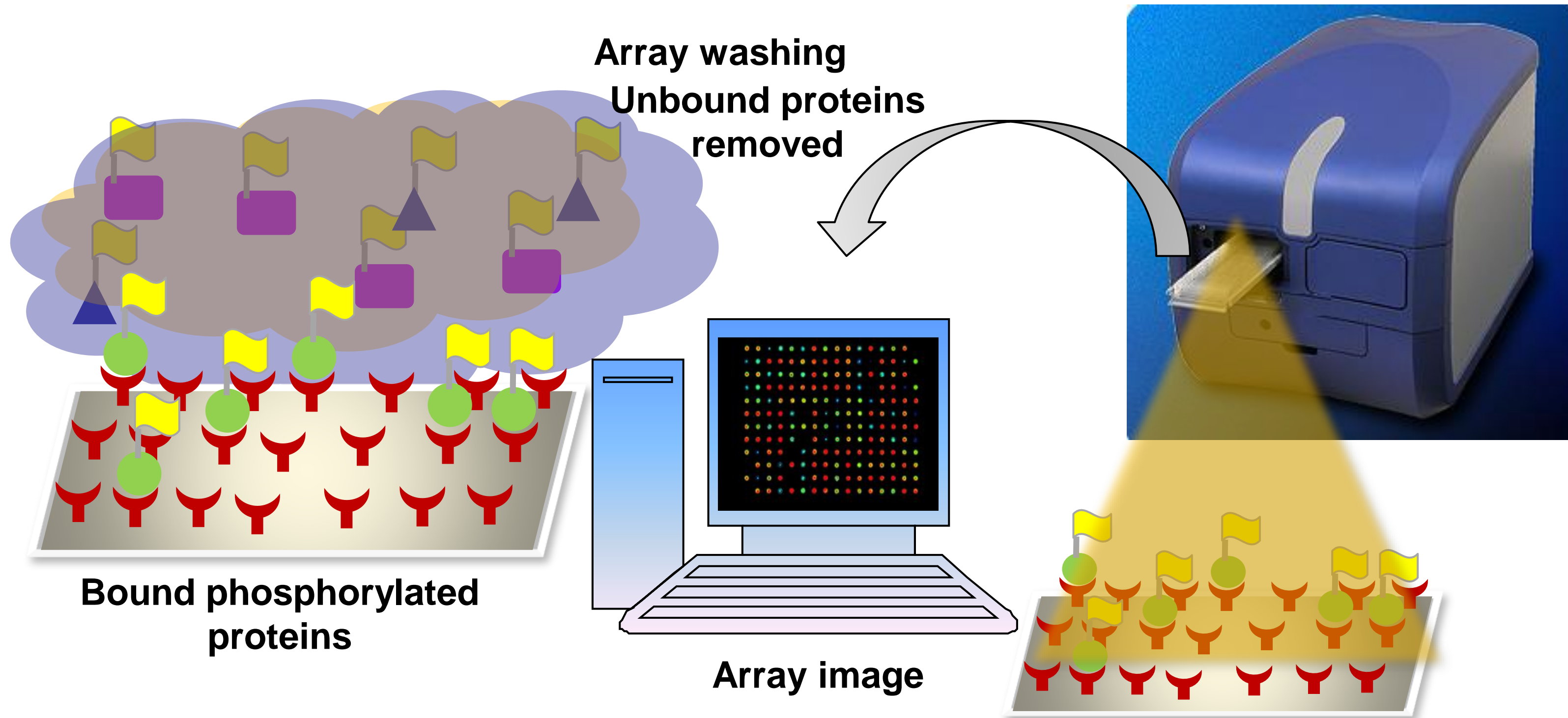



Protein microarrays
Washing
Phosphorylated proteins
Proteome array
33P
33P
33P
33P
33P
Radioactive emissions
Developed image
Detection-Autoradiography film

Antibody microarrays





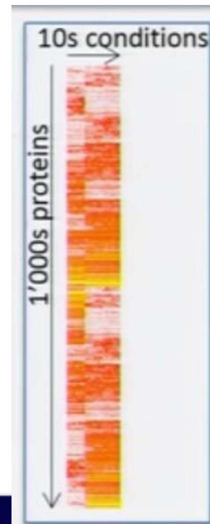

Antibody microarrays
Microarray scanner
Array washing
Unbound proteins
removed
Bound phosphorylated
proteins
Array image

LECTURE 6: TARGETED PROTEOMICS

Discovery vs Targeted Proteomics

Discovery Proteomics

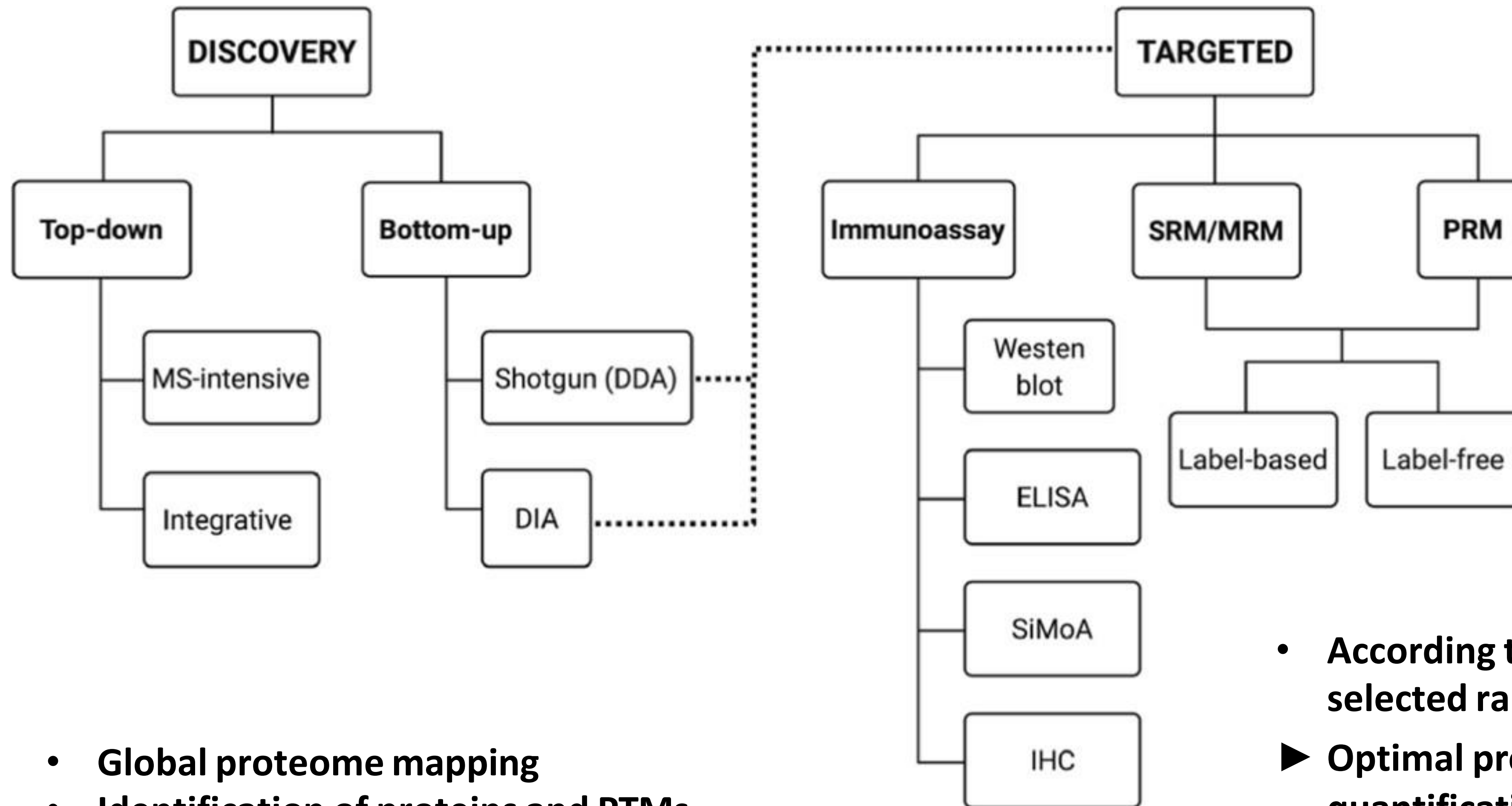
- often requires large sample quantities and multi-dimensional fractionation, which diminishes sensitivity and throughput



Targeted Proteomics(hypothesis-driven proteomics)

- limit the number of proteins that are monitored
- Optimize the chromatography instrument tuning and acquisition methods
- Achieve the highest sensitivity and throughput for hundreds or thousands of samples.

Discovery and Targeted Approaches

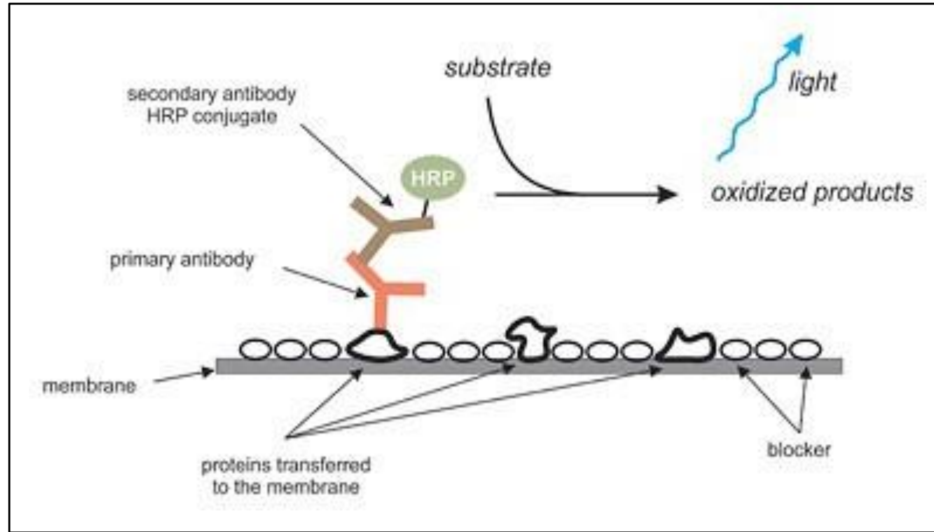


- **Global proteome mapping**
- **Identification of proteins and PTMs**

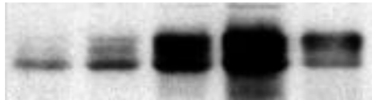
- **According to the pre-selected range of proteins**

► **Optimal protein/PTM quantification results**

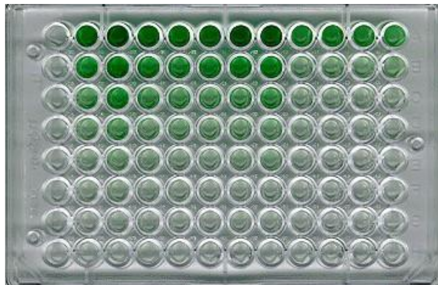
Traditional Affinity-based Approaches For Protein Quantitation

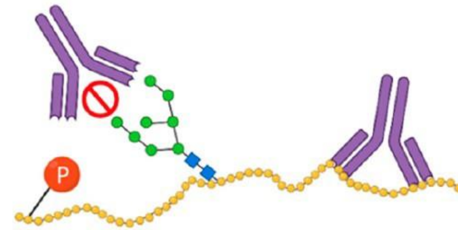


Western Blot



ELISA





- Use "high-quality" antibodies to quantify proteins (even low-level proteins)
- Drawbacks:
  - Antibodies do not exist for all proteins, vary widely in quality
  - Antibody validation may take months to years to validate
  - Lack throughput (quantitative capacity)
  - Fewer proteoforms–selective antibodies available
    - Antibodies generally identify amino acid epitopes on canonical protein sequences, so the PTMs or neighboring will be likely to block the epitope and prevent the binding/detection.
    - Proteins with high degree of sequence similarity can not be quantified.

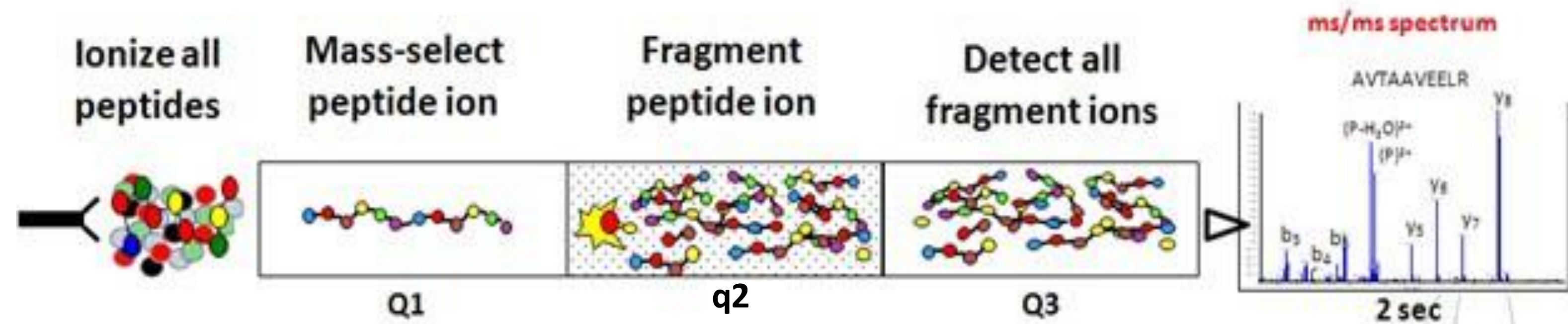
Mass Spectrometry-based Approaches for Protein Quantification in Targeted Proteomics

- Selected Reaction Monitoring (SRM)/ Multiple Reaction Monitoring (MRM)
  - Quantifies specific, predetermined ions from peptide of interest
- Parallel Reaction Monitoring (PRM)
  - Analyzes all fragment ions of the preselected peptides of interest
- Data Independent Analysis (DIA) (as known as SWATH)
  - Analyzes all peptide mass ranges within the window without pre-selection

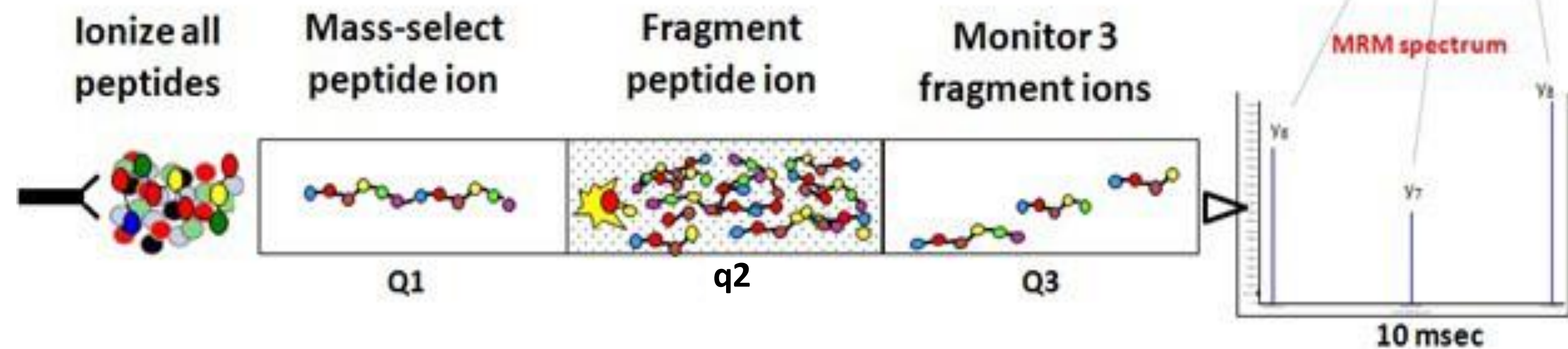
Selected Reaction Monitoring (SRM)/ Multiple Reaction Monitoring (MRM)

- Triple Quadrupole acts as ion filters
  - Precursor selected in first mass analyzer (Q1) with a narrow window (e.g., ±1 m/z)
  - Fragmentation (q2)
  - One or several of the fragments are specifically measured in the second mass analyzer (Q3)

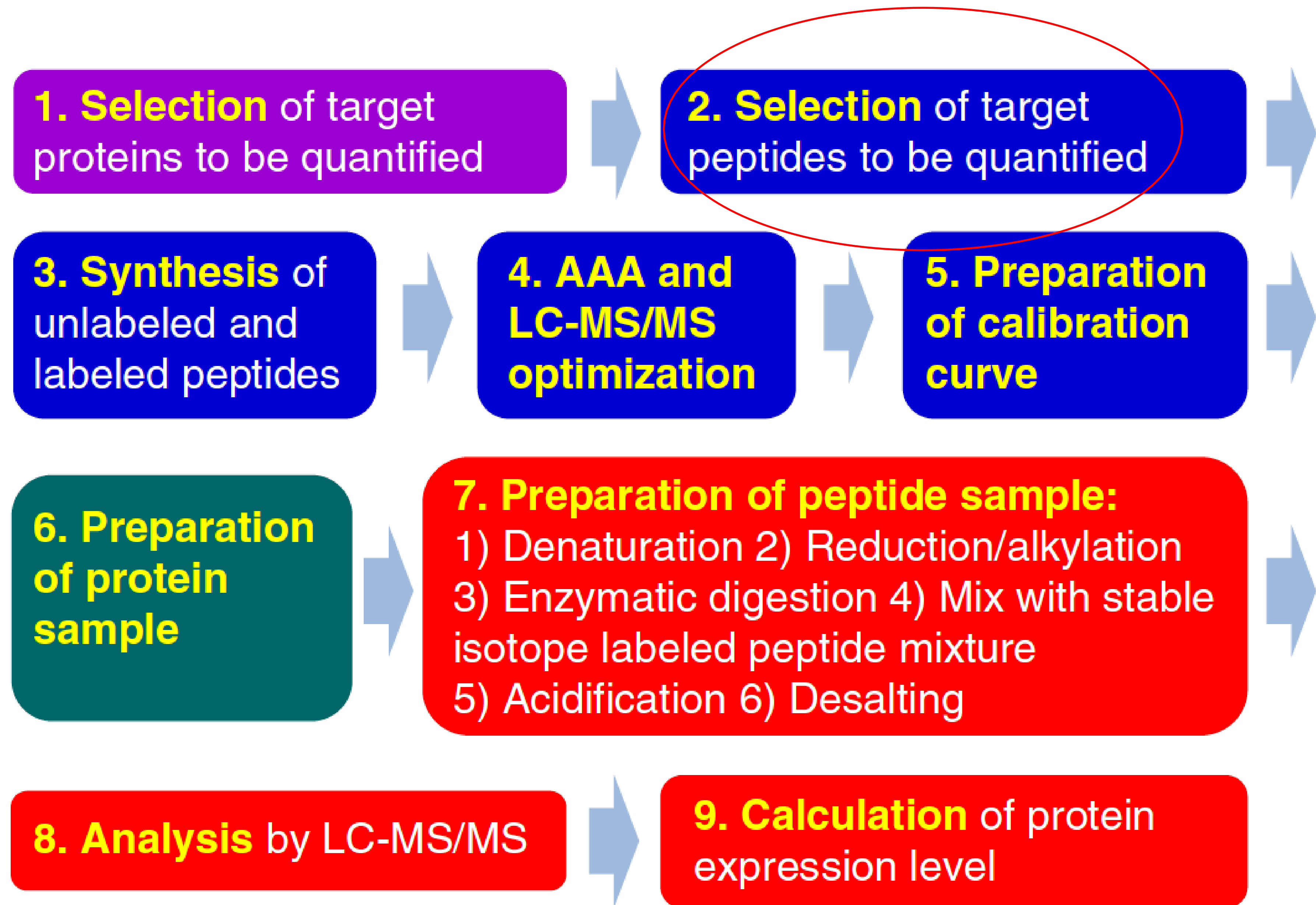
Conventional data-dependent (DDA) MS/MS Operating Mode



MRM- or SRM-MS Operating Mode



Workflow of an SRM/MRM Experiment



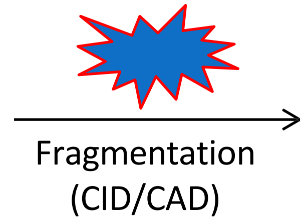
Uchida, Y., et al. "A study protocol for quantitative targeted absolute proteomics (QTAP) by LC-MS/MS: application for inter-strain differences in protein expression levels of transporters, receptors, claudin-5, and marker proteins at the blood–brain barrier in ddY, FVB, an." Fluids & Barriers of the Cns 10.1(2013):1-22.

Selection of Target Peptides

1. Selection of best **representative (proteotypic) peptides** per protein
   - Select unique peptides for a given protein to achieve maximum selectivity
   - Select best-flying peptides per protein to achieve maximal sensitivity
2. Selection of optimal **transitions** per peptide
   - **Transition**: Precursor-Fragment ion pair are used for protein identification (sequence-specific)
     - e.g., Peptide 1 (NYCGDFTSSK) and Peptide 2 (NYCGSDTFSK) has the same precursor mass but different fragmentation patterns
   - Select both Q1 and Q3 prior to run
     - Q1 doubly or triply charged peptides
     - Pick Q3 fragment ions based on discovery experiments or spectral libraries
     - Typically, 3-5 fragment ions per peptide are measured.
   - One to three unique peptides with good quantitative properties are used for protein quantification.

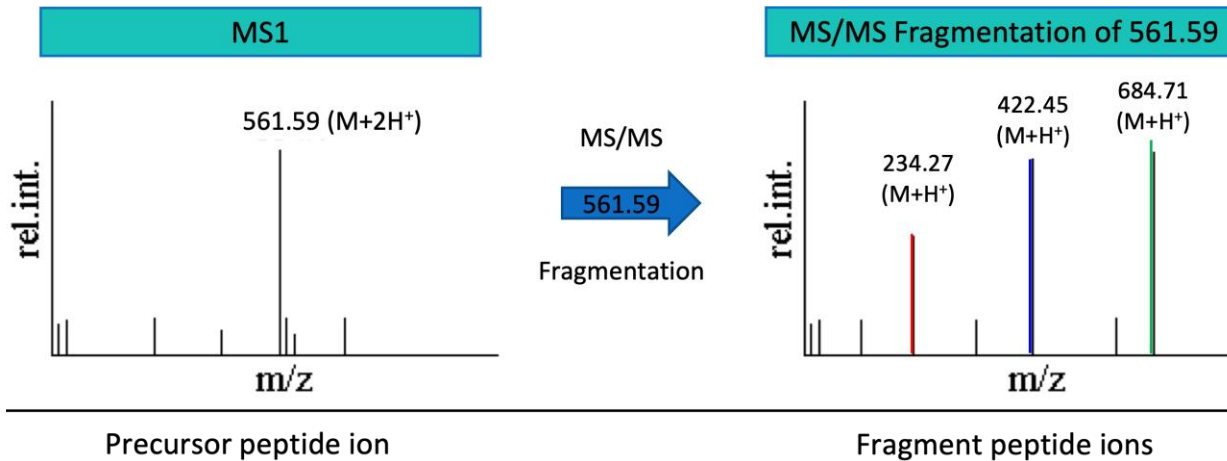
Precursor

NYCGDFTSSK
(m/z=561.59, M+2H⁺)

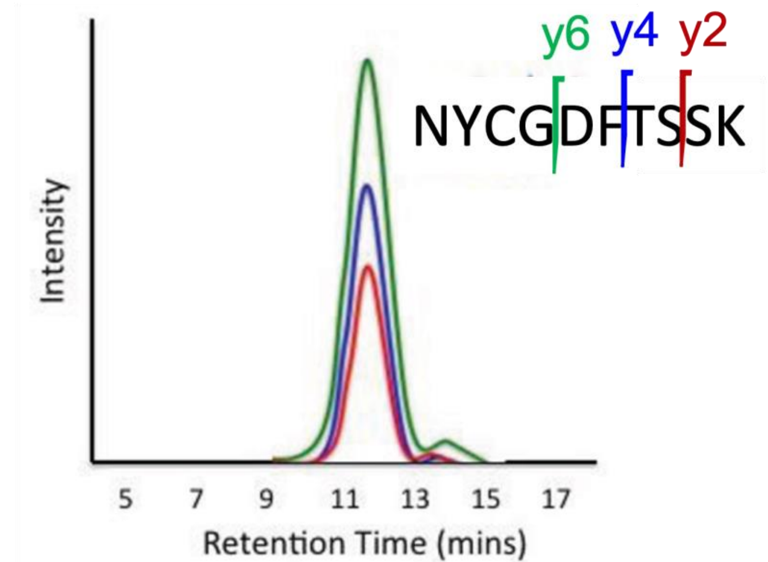


Fragments

Fragments		Fragment Ions
SK	(m/z=234.27, M+H⁺)	y2
TSSK	(m/z=422.45, M+H⁺)	y4
DFTSSK	(m/z=684.71, M+H⁺)	y6
NYCG	(m/z=456.49, M+H⁺)	b4
NYCGDF	(m/z=718.75, M+H⁺)	b6
GDFTSSK	(m/z=741.77, M+H⁺)	y7



Quantification



Transitions:

1) m/z=561.59, M+2H⁺ → m/z=234.27, M+H⁺
2) m/z=561.59, M+2H⁺ → m/z=422.45, M+H⁺
3) m/z=561.59, M+2H⁺ → m/z=684.71, M+H⁺

Selecting SRM/MRM Peptides$_1$

- A few representative peptides will be used to quantify each protein
- Need to fulfill certain characteristics
  - Have a **unique** sequence
  - Good ionization efficiency
  - Consistently observed by LC-MS methods with a good signal-to-noise ratio
  - m/z within the range of the instrument
  - No missed cleavages
  - 8-25 amino acids
  - Not too hydrophilic (poorly retained) or hydrophobic (may stick to column)

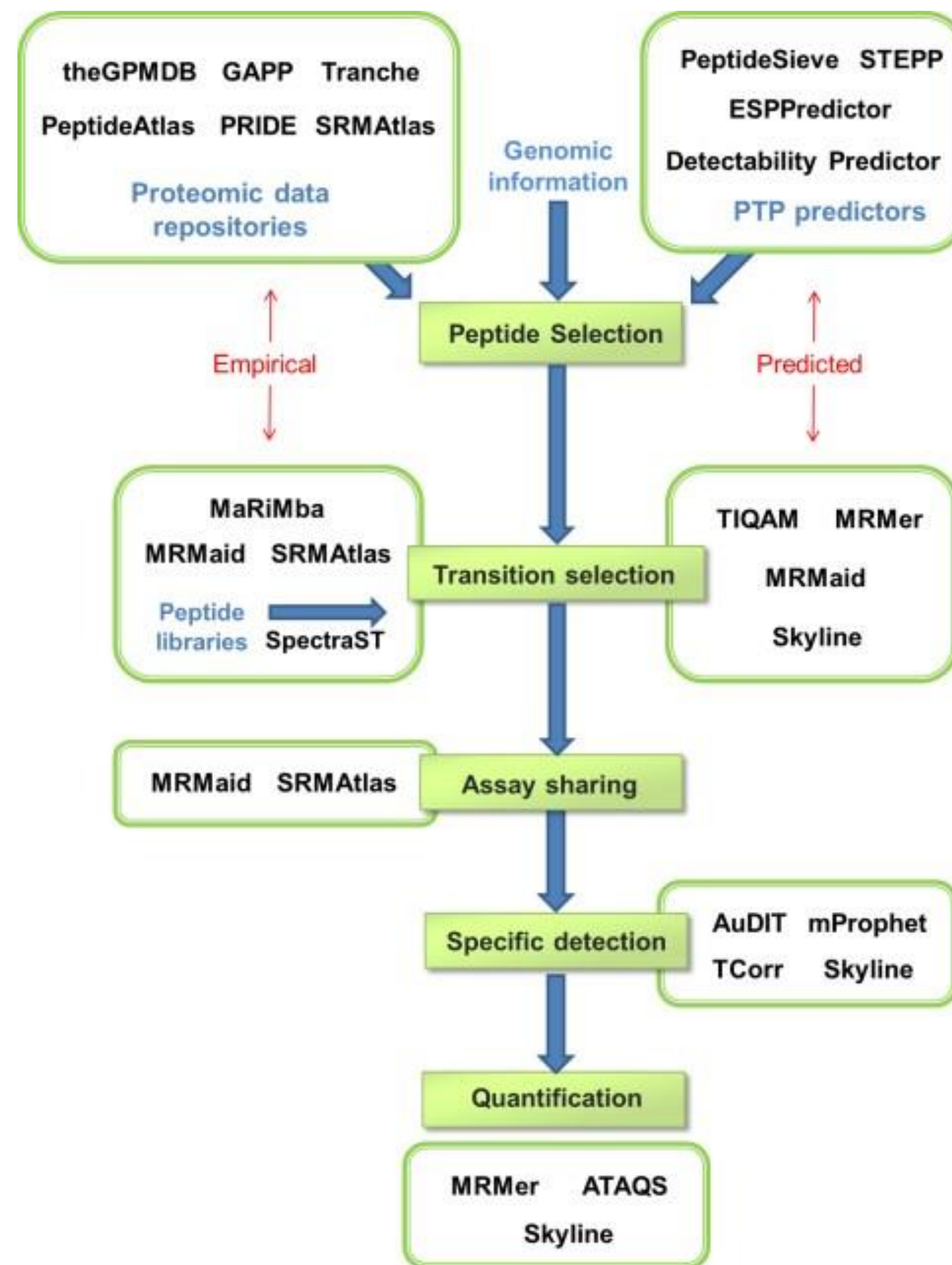
Selecting SRM/MRM Peptides$_2$

- Ensure complete proteolysis
  - Avoid missed-cleaved or partly tryptic peptides
  - Avoid tryptic peptides with two neighboring basic amino acids at either cleavage site (KK, RR, KR, RK)
  - Avoid acidic residues (D, E) close to the cleavage site
  - Avoid N-terminal proline cleavage (KP, RP)
- Avoid peptides prone to chemical modifications
  - W and M are prone to oxidation
  - N-G or Q-G are prone to deamidation
  - N-terminal cyclization of Q and E and carbamidomethylated C
- Avoid peptides carrying biological modification (PTMs)
  - Check PTM repositories (UniProt etc.) or use PTM prediction tools
  - Avoid known PTM-motif such as the N-X-S/T glycosylation motif
  - Avoid N- and C-terminal peptides as they are more prone to degradation

Open Source MRM Analysis Tools

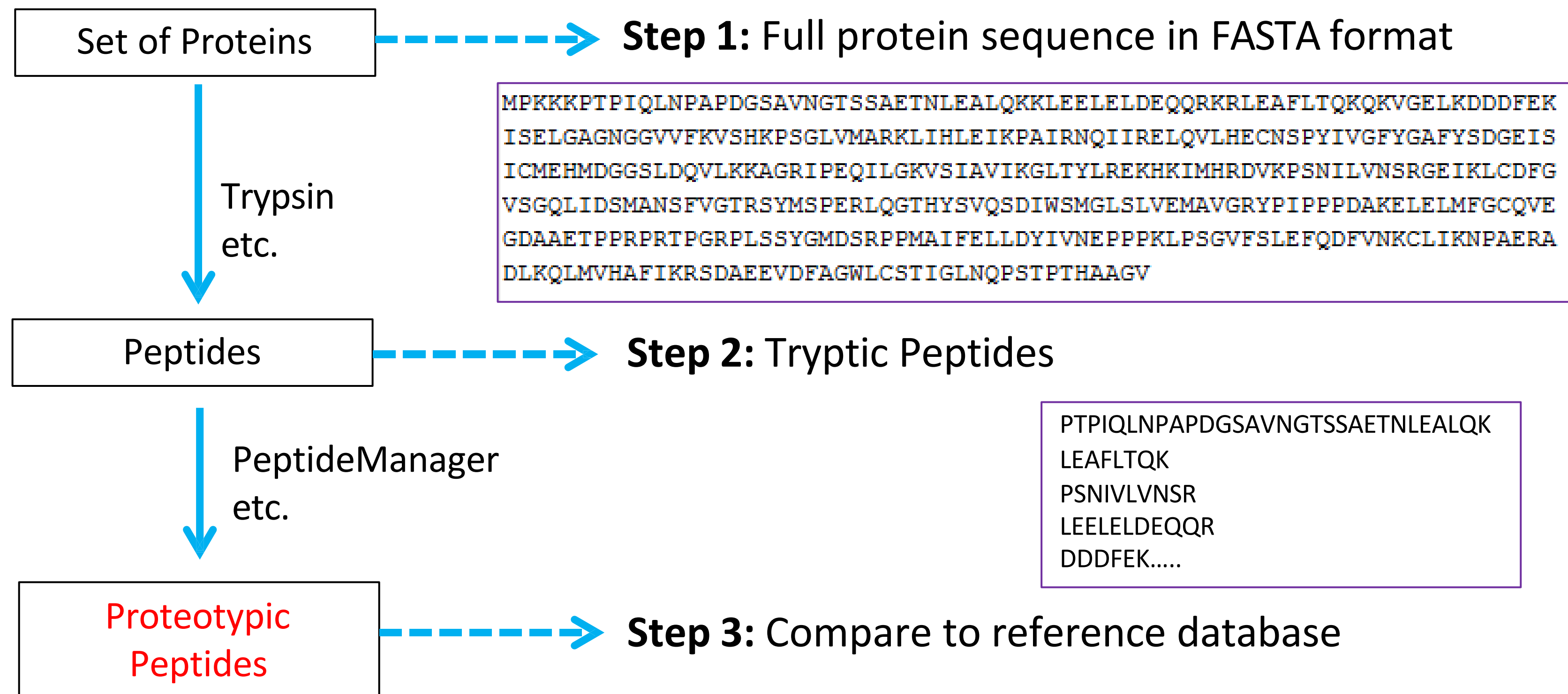
- Target peptides for each target protein are selected based on genomic information and information stored in publicly accessible proteomic data repositories or computational prediction.

- Suitable SRM transitions for each target peptide are empirically extracted from mass spectrometry data (preferably collected on triple quadrupole-type instruments), or calculated using suitable computational interfaces.



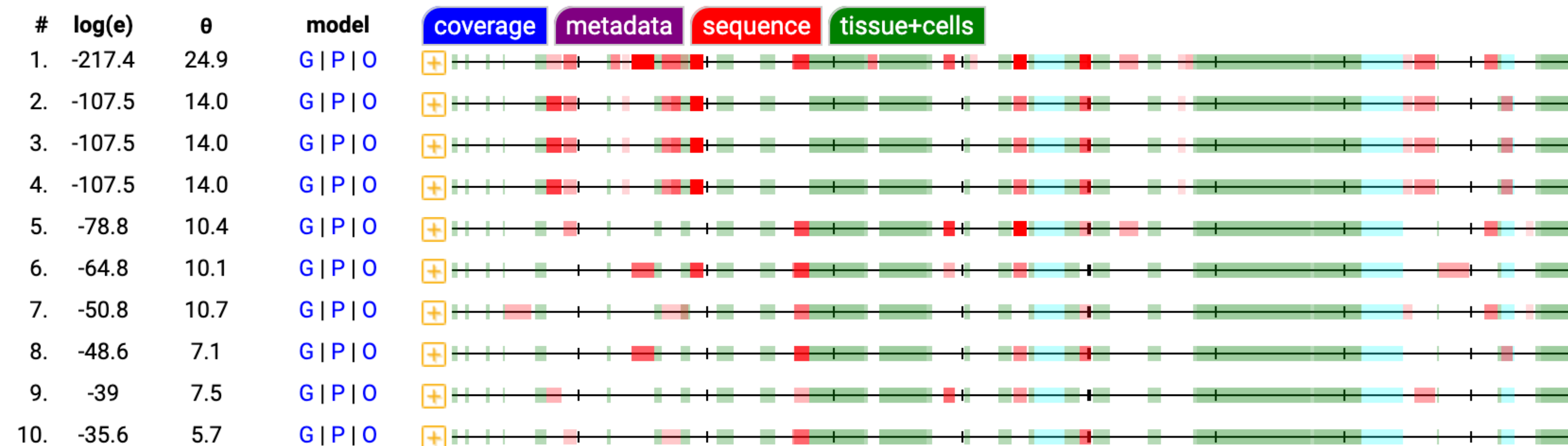
Identifying Proteotypic Peptides

Proteotypic: describes a peptide sequence that is found in only a single known protein and therefore serves to identify that protein.



GPMDB (Global Proteome Machine DataBase)

- Compares peptides to a collection of previously observed results
- Determines how many times the peptide has been observed by others
- Most proteins show very reproducible peptide patterns
- We used this site to determine **which peptides were most likely to be seen** in our own MS experimentation.



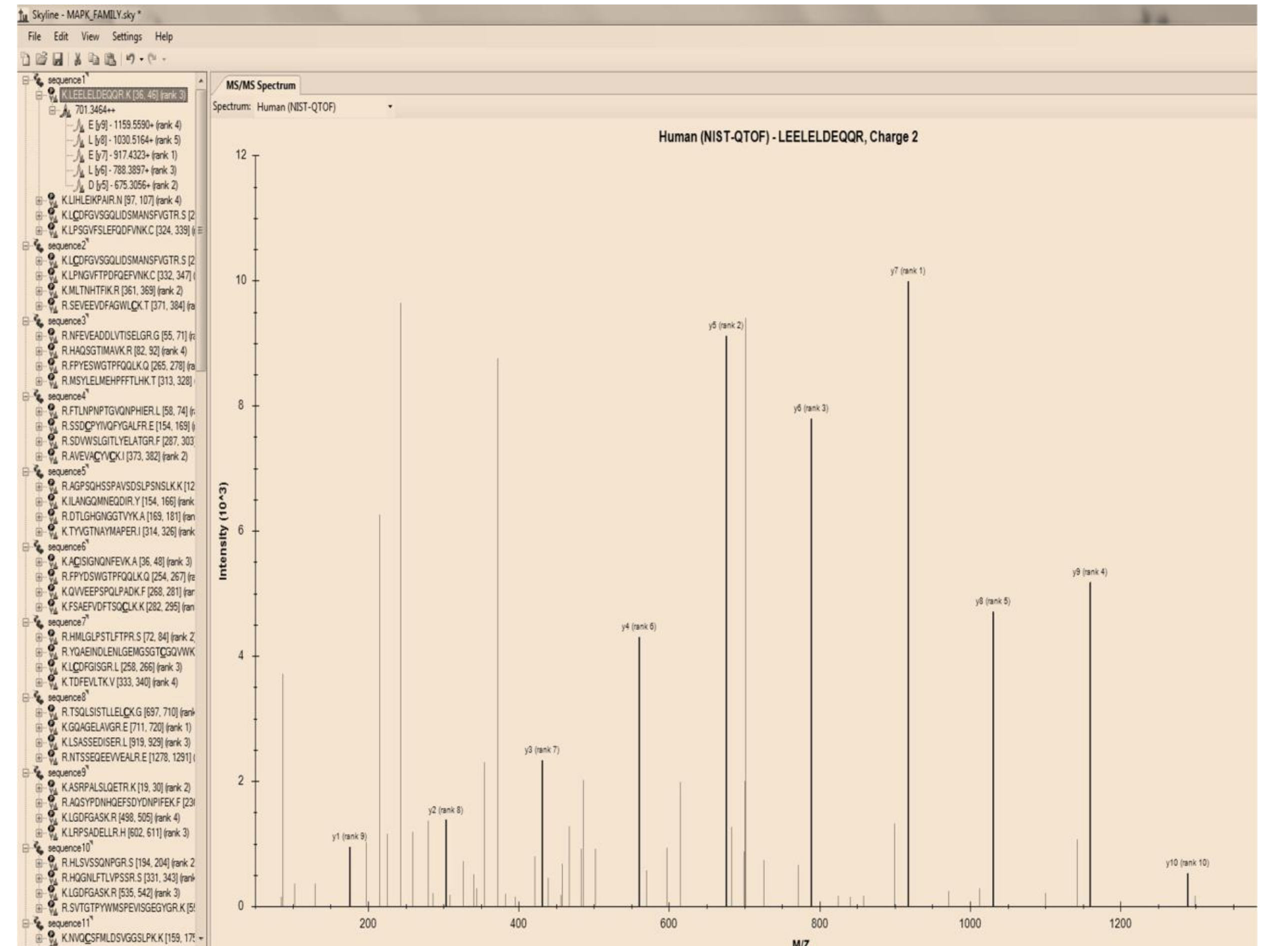
coverage | metadata | sequence | tissue+cells

#	log(e)	θ	model
1.	-217.4	24.9	G | P | O
2.	-107.5	14.0	G | P | O
3.	-107.5	14.0	G | P | O
4.	-107.5	14.0	G | P | O
5.	-78.8	10.4	G | P | O
6.	-64.8	10.1	G | P | O
7.	-50.8	10.7	G | P | O
8.	-48.6	7.1	G | P | O
9.	-39	7.5	G | P | O
10.	-35.6	5.7	G | P | O

- **black line**: indicates the full length of the protein (all sequences are the same length in this display);
- **red bars**: indicate observed peptides (**the darker the red, the more confident the observations**);
- **green bars**: indicate regions of the protein that may be difficult to observe, using standard proteomics techniques; and
- **cyan bars**: indicate peptides with the motif for N-linked carbohydrates.

Skyline

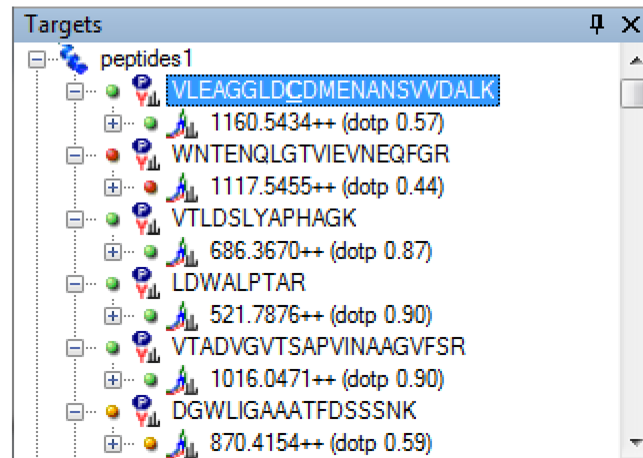
- freely-available and open source Windows client application
- Builds SRM/MRM, Parallel Reaction Monitoring (PRM), Data Independent Acquisition (DIA/SWATH) and DDA with MS1 quantitative methods
- analyzes the resulting mass spectrometer data.

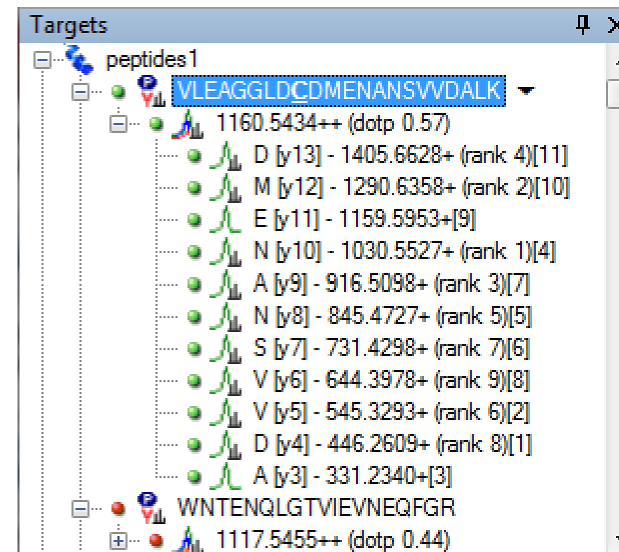


- Compares peptides to MS/MS spectral libraries
- Predicts most abundant transitions

Selecting Transitions: Skyline

- Can use it to find best transitions to pick
  - Intensity (rank)
  - Dot product (dotp, similarity to reference spectra)





Want high rank and dotp close to 1

Selecting Transitions: SRMCollider

- Input peptides of interest
- Determines the m/z values for transition pair
- Simulates a typical SRM experiment
- Predicts fragment intensities and retention time information for input peptide
- Compares the transition to all other transitions in a background proteome
- Outputs the number of predicted interferences for each transition for that peptide

SRM Collider

version 1.4
Hannes Röst 2012

Collider | Download | About | Instructions

The SRMCollider is a program that will take your input transitions and compare them to all other transitions in a given background proteome and find interferences. It will report these interferences on a per-peptide basis, allowing a researcher to identify peptides that share many transitions with the target peptide.

Please enter the peptide sequences here (see Instructions for help):

YDEDGMDCMDNER

Input peptide sequence

SSRCalc window	2	arbitrary units
Q1 mass window	0.7	Th
Q3 mass window	1.0	Th
Low mass threshold for transitions	300	Th
High mass threshold for transitions	1500	Th
Genome	Human (tryptic)	
Consider isotopes up to	3	amu
Missed Cleavages	0	
Find UIS up to order*	5	

Charge check: ☑ Check that interfering signal can actually hold charge (e.g. 2+ charge)

Modifications: ☐ oxidized Methionines ☐ deamidated Asparagines

Peptide YDEDGMDCMDNER

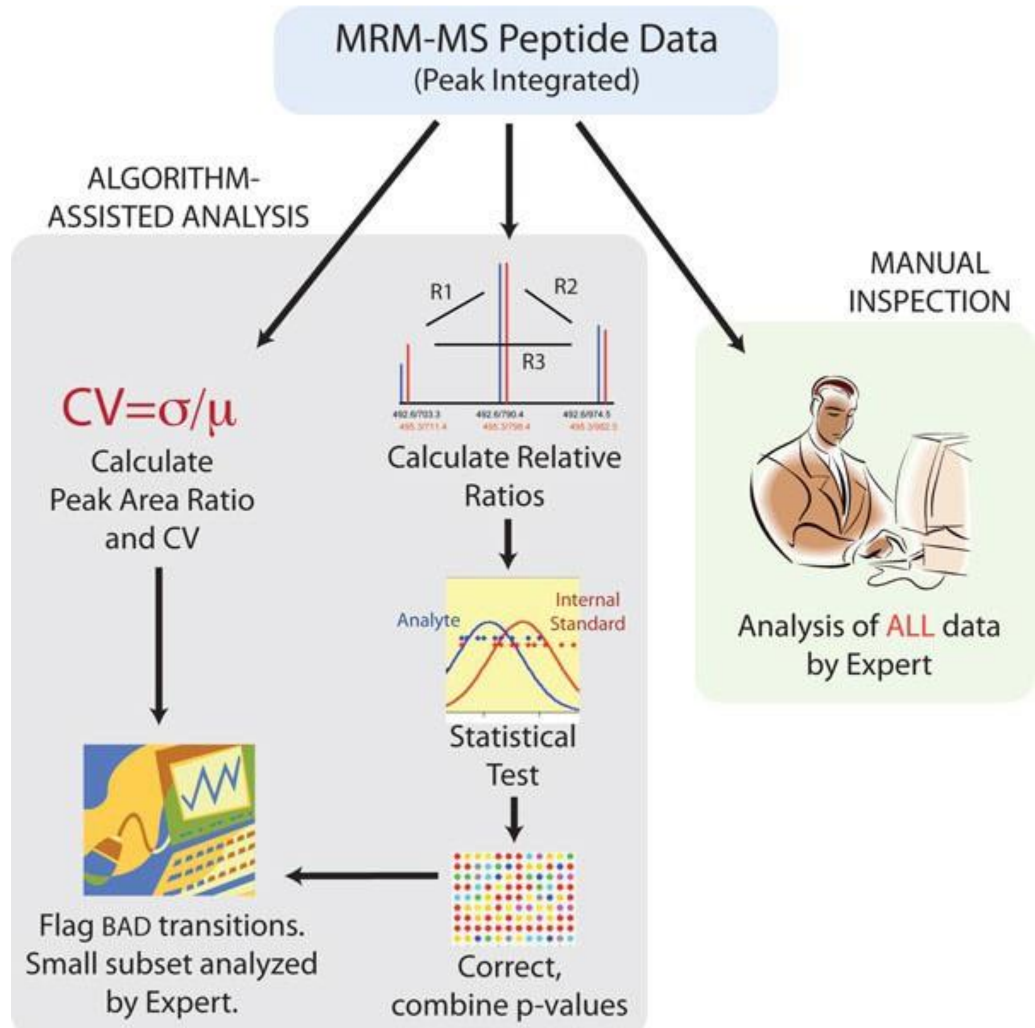
Sequence	Q1	Q1 window	SSRCalc	SSRCalc window	Interfering precursors	Background	Graph
YDEDGMDCMDNER	796.769077374	± 0.35	15.82	± 1.0	32	human	Graph

Transition Overview

Transition	Q3	Interferences	Graph
y10	1185.4	0	Graph
y9	1070.37	0	Graph
y6	767.28	0	Graph
y5	664.27	0	Graph
b3	408.14	0	Graph
b4	523.17	0	Graph
b5	580.19	0	Graph
b6	711.23	0	Graph
b7	826.26	0	Graph
b10	1175.33	0	Graph
y11	1314.44	1	Graph
y8	1013.35	1	Graph
y7	882.31	1	Graph
y4	533.23	1	Graph
y3	418.21	1	Graph
y2	304.16	1	Graph
b8	929.27	1	Graph
b9	1060.31	1	Graph
y12	1429.47	2	Graph
b11	1289.38	3	Graph
b12	1418.42	3	Graph

Choose peptides that have at least one transition with zero interferences

Validating Transitions: AuDIT

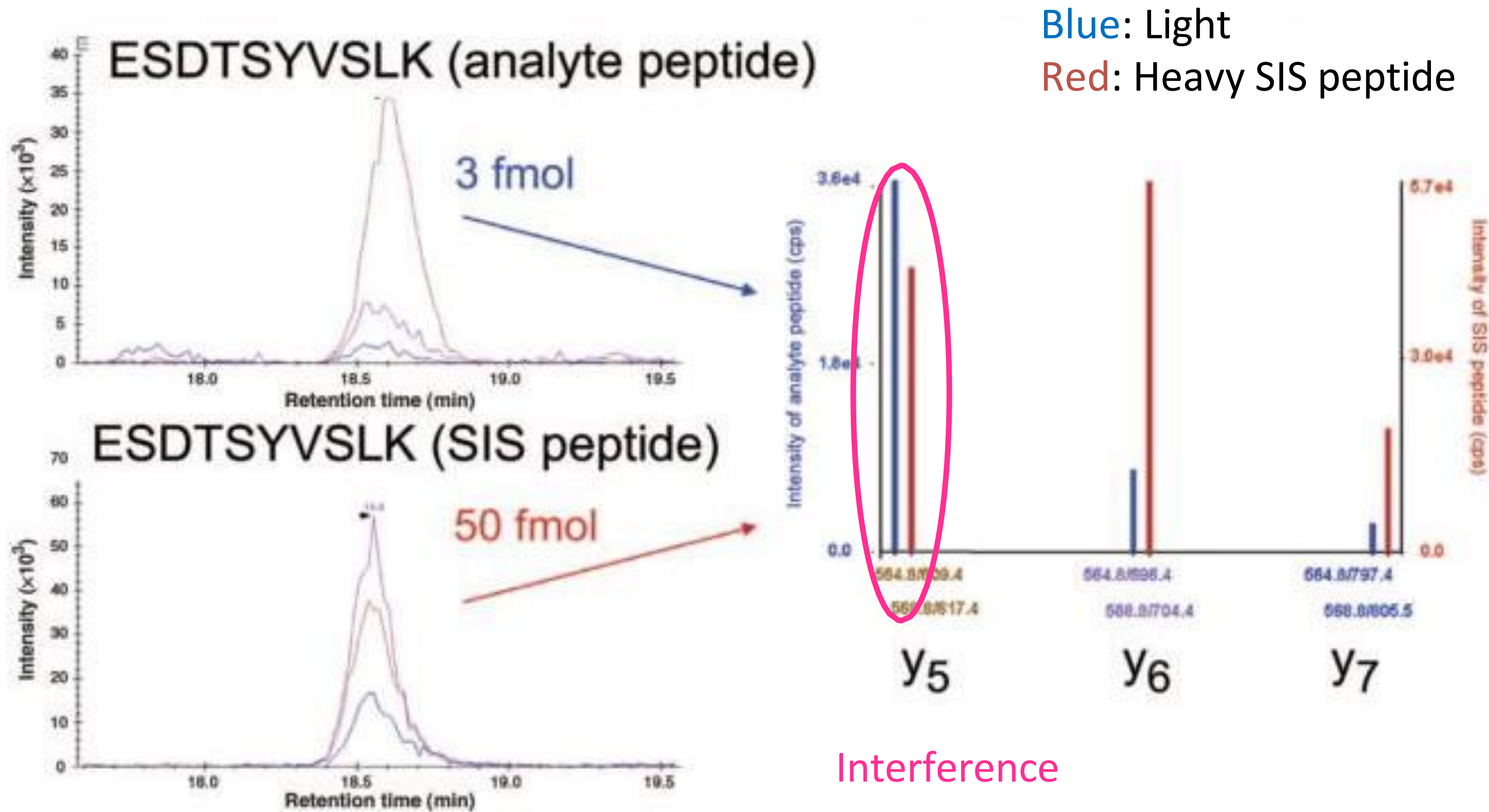


- AuDIT: Automated Detection of Inaccurate and imprecise Transitions
- The **orthogonal modalities** allows a transition with either a significant P value or an unacceptable CV to be marked as “bad” as a whole and thus be deemed unacceptable for quantification.
    1. Apply t-test to determine if relative ratios of analytes are different from relative ratios of SIS (Stable Isotope Standard)
    2. CV measurements of replicate samples (either technical replicate injections of the same sample or process replicates). Usually, CV > 20% is deemed problematic.

Validating Transitions: AuDIT

XICs for 3 transitions of analyte peptide and SIS

Blue: Light
Red: Heavy SIS peptide



Interference

Relative product ions should have a constant relationship

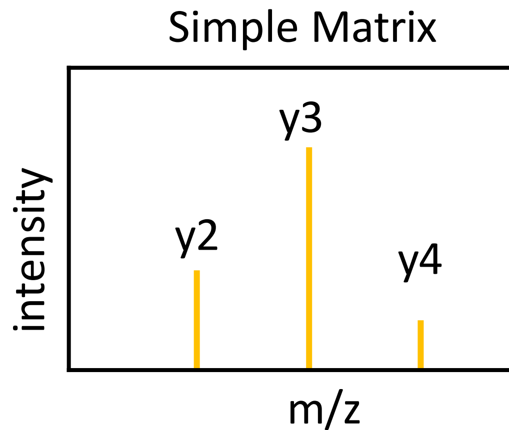
Clin Chem. 2010 February ; 56(2): 291–305. doi:10.1373/clinchem.2009.138420.

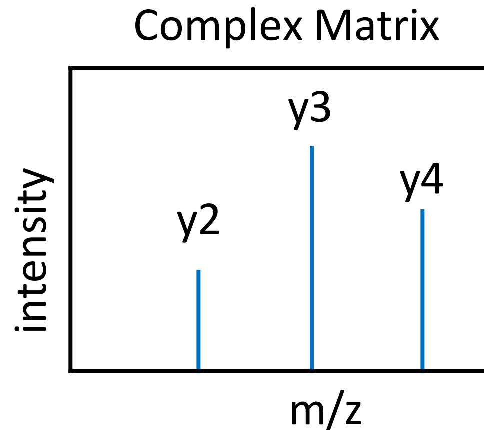
Finding Interference: Simple vs. Complex Matrix

Complex = millions of peptides

- MRM/SRM are most useful when quantifying protein in a complex matrix
  - Tumor lysate
  - Plasma/Serum
- Simple matrix (buffer) should have no interference – use this as *reference*
- Compare the transitions in complex to those in simple matrix
- Transitions in complex should have the same relative intensities of transitions
- Ratio close to 1 indicates low interference

Simple Example of Complex vs. Simple





intensity

Transition	Simple	Complex
y2	200	200
y3	400	400
y4	100	300

Ratio of Transitions

	y2	y3	y4
y2	y2/y2	y2/y3	y2/y4
y3	y3/y2	y3/y3	y3/y4
y4	y4/y2	y4/y3	y4/y4

Simple Matrix

	y2	y3	y4
y2	1	0.5	**2**
y3	2	1	**4**
y4	**0.5**	**0.25**	1

Complex Matrix

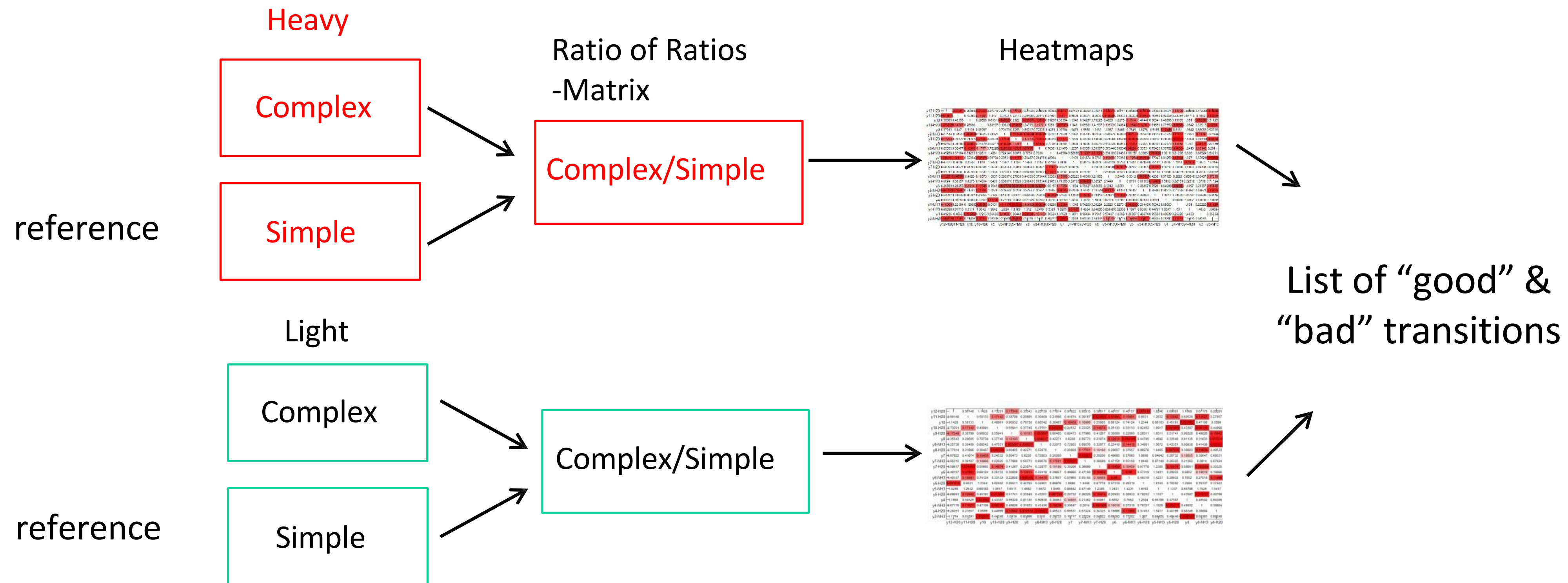
	y2	y3	y4
y2	1	0.5	**0.67**
y3	2	1	**1.33**
y4	**1.5**	**0.75**	1

Transition Ratio Matrix
Complex/Simple

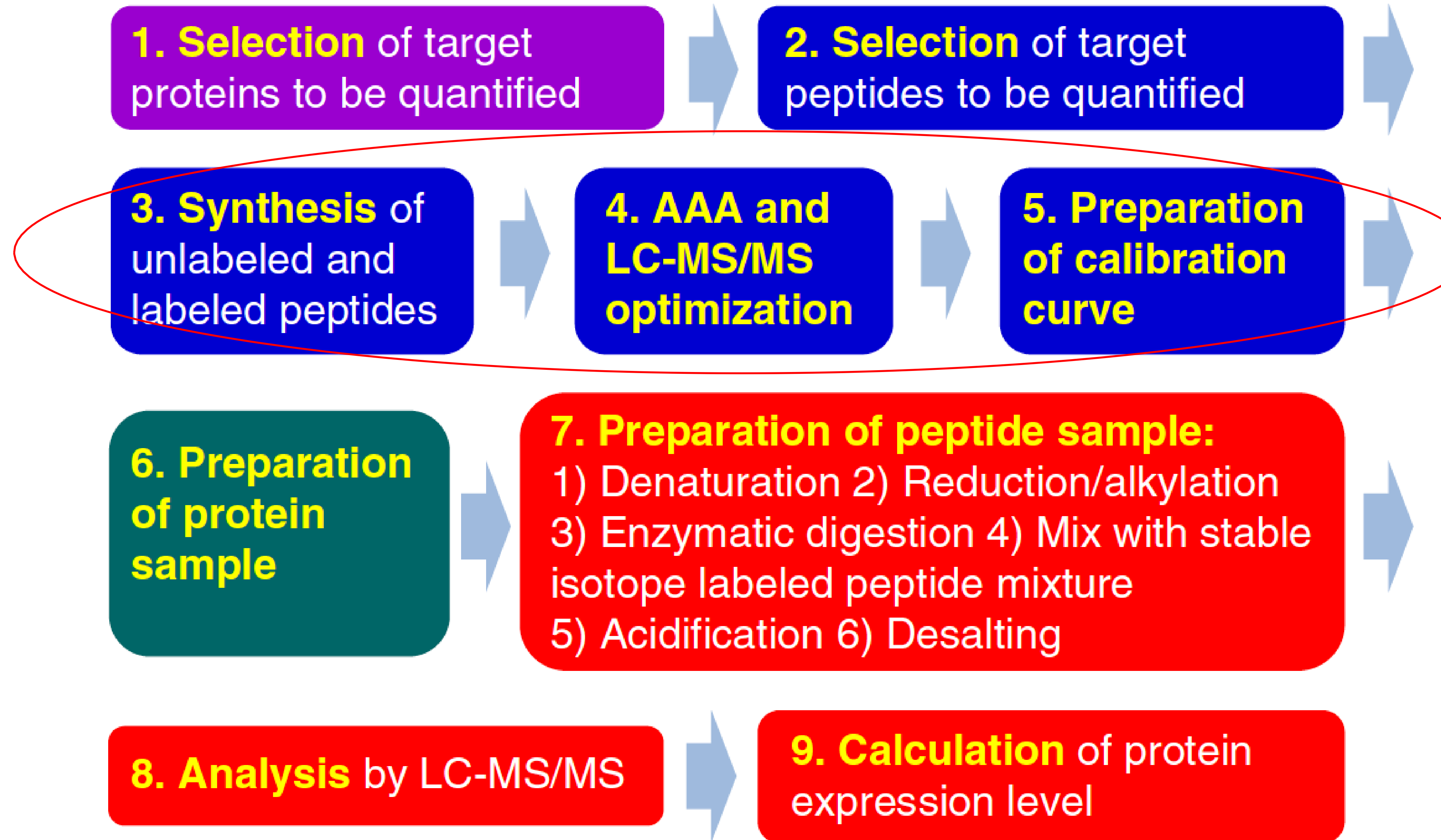
	y2	y3	y4
y2	1	1	**.335**
y3	1	1	**.333**
y4	**3**	**3**	1

y2 and y3 are “good” transitions with no interference

Finding Interference: Simple vs. Complex Matrix



Workflow of an SRM/MRM Experiment



Uchida, Y., et al. "A study protocol for quantitative targeted absolute proteomics (QTAP) by LC-MS/MS: application for inter-strain differences in protein expression levels of transporters, receptors, claudin-5, and marker proteins at the blood–brain barrier in ddY, FVB, an." Fluids & Barriers of the Cns 10.1(2013):1-22.

Absolute Quantification

- Use known quantity of isotopically labeled reference protein or $^{13}C/^{15}N$ labeled peptide analogs that are chemically identical to the target peptide but with mass difference.
- Compare signals for the analyte (light) to the stable isotope standard (SIS, heavy) to calculate the concentration.

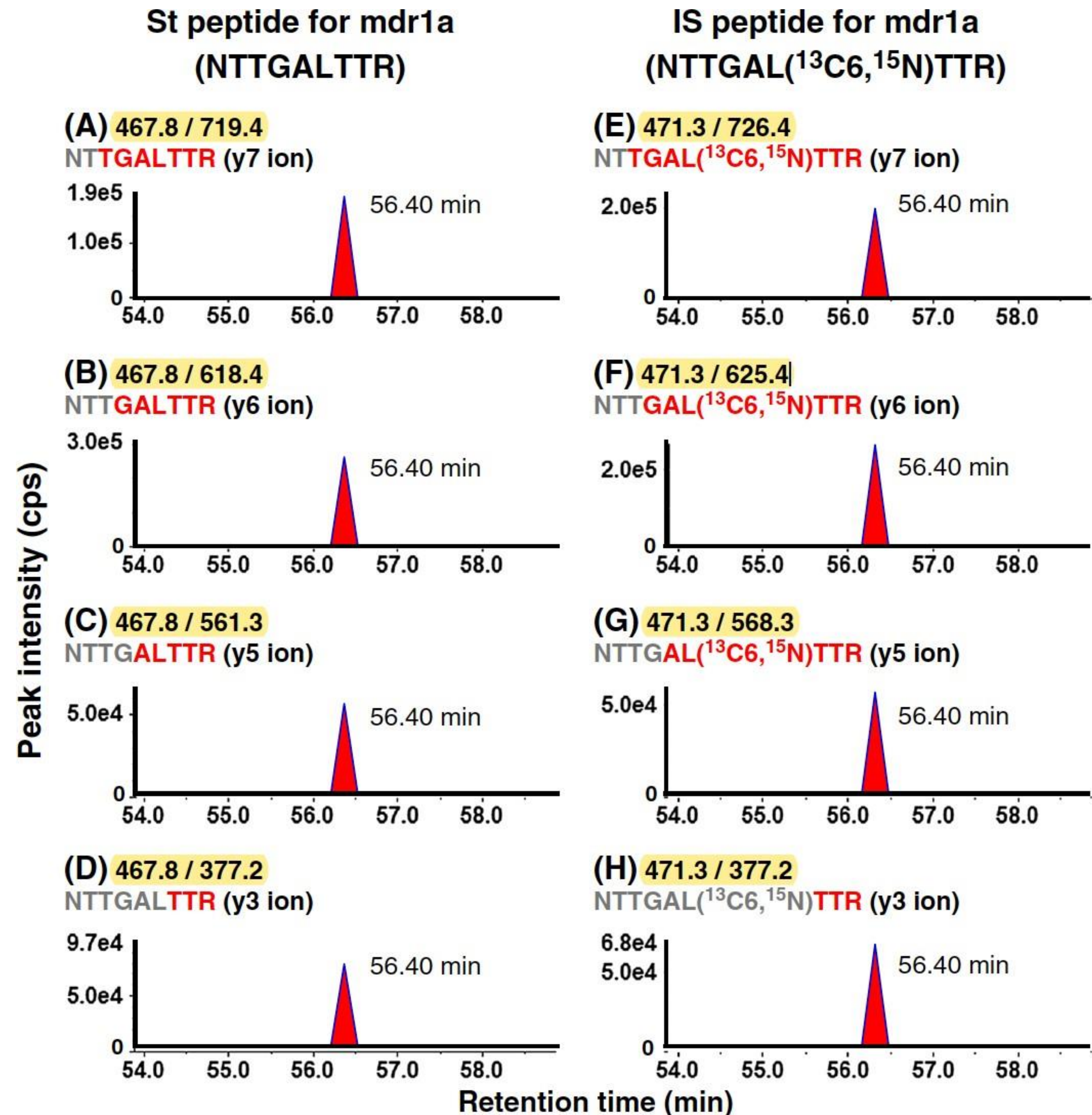
- Peak area ratio (PAR) = $\frac{analyte(light) peak\ area}{SIS\ (heavy)\ peak\ area}$

  -Use at least 3 transitions

  -Have to make sure no interference in those transitions

1 Precursor ion with 4 fragment ions for both analyte (left) and IS (right)

37 different proteins can be simultaneously quantified in a single analysis using the currently available maximum of 300 SRM/MRM transitions (multiplexed SRM/MRM analysis).



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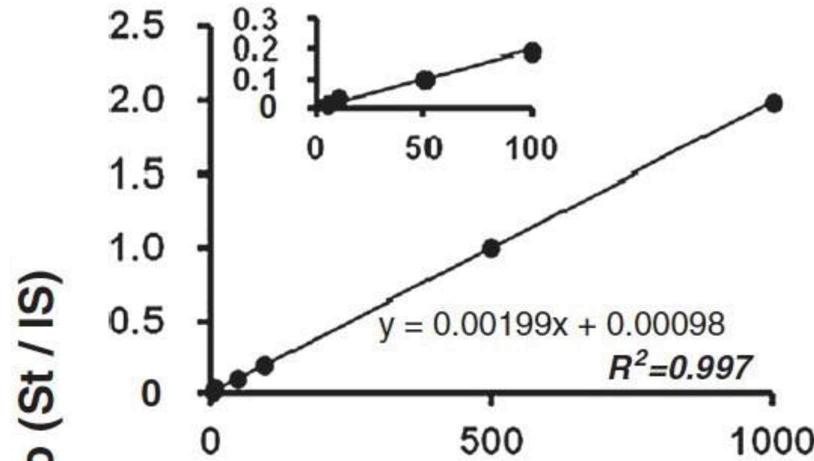
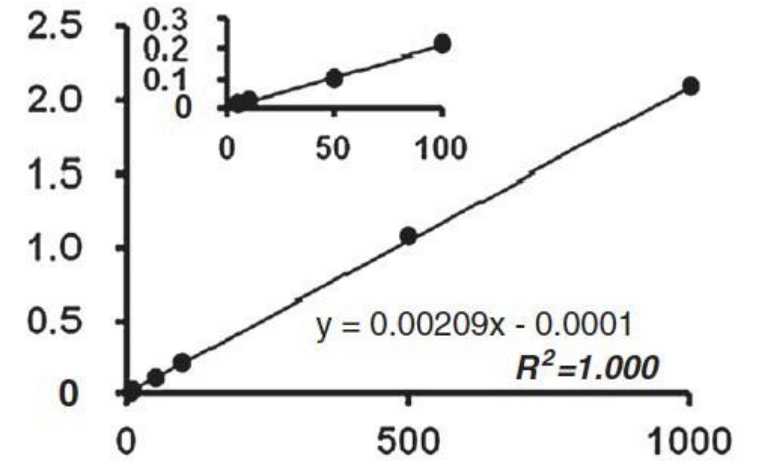
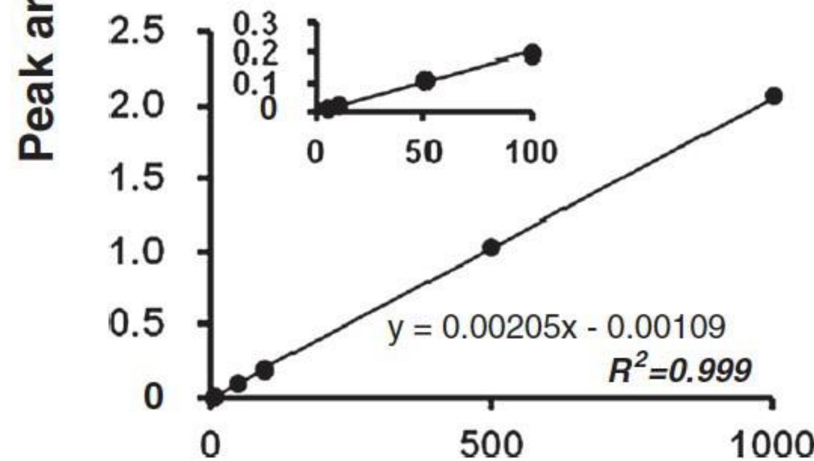
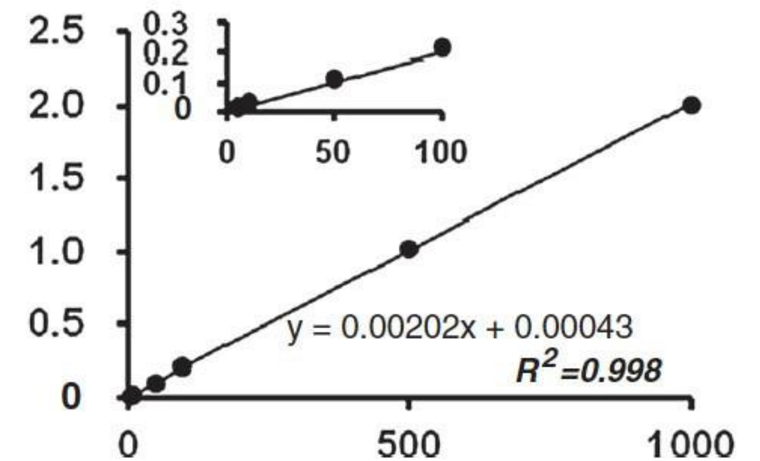
Typical calibration curve for absolute quantification









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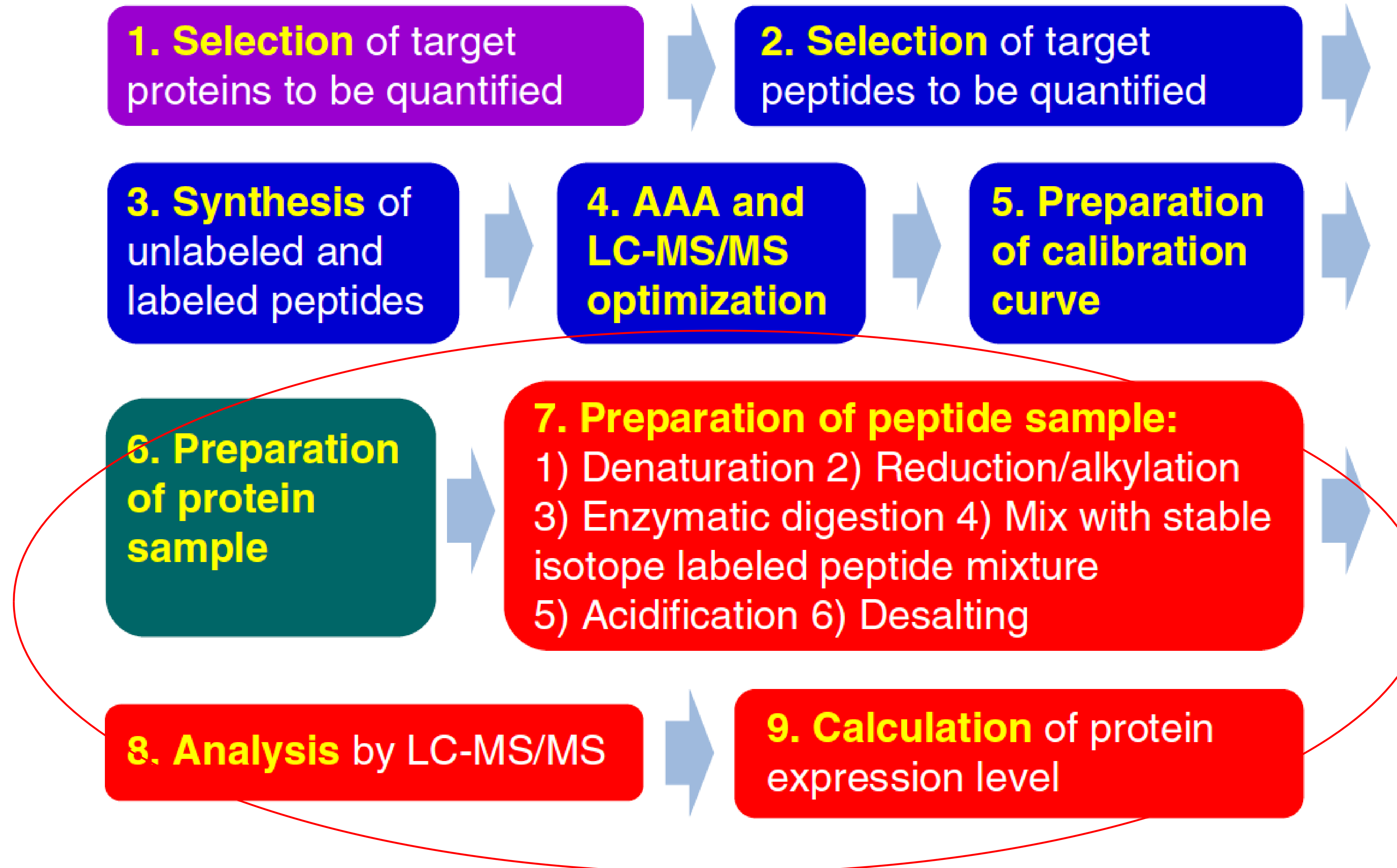
Workflow of an SRM/MRM Experiment



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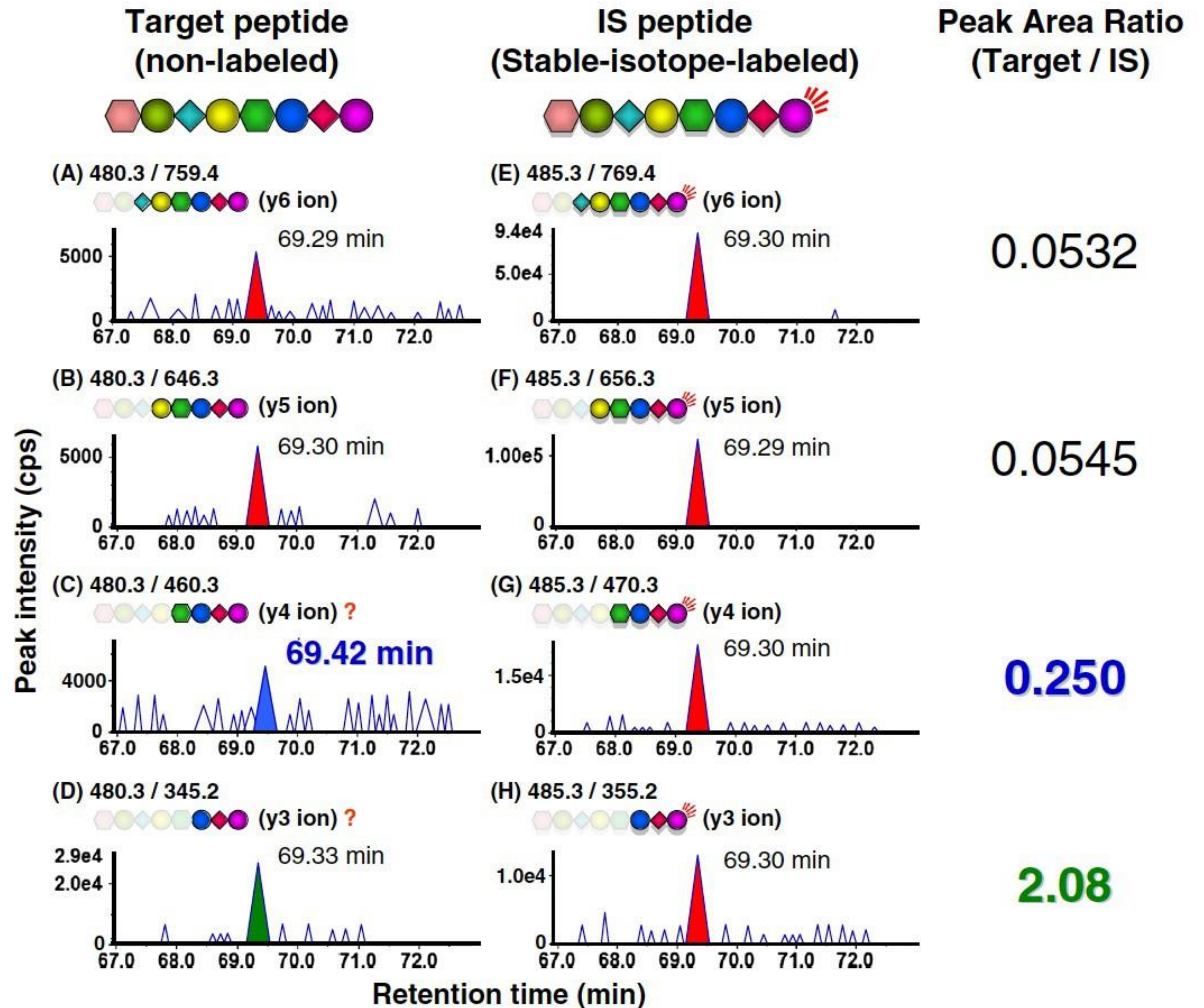
An example of ambiguous SRM/MRM chromatograms of a biological sample



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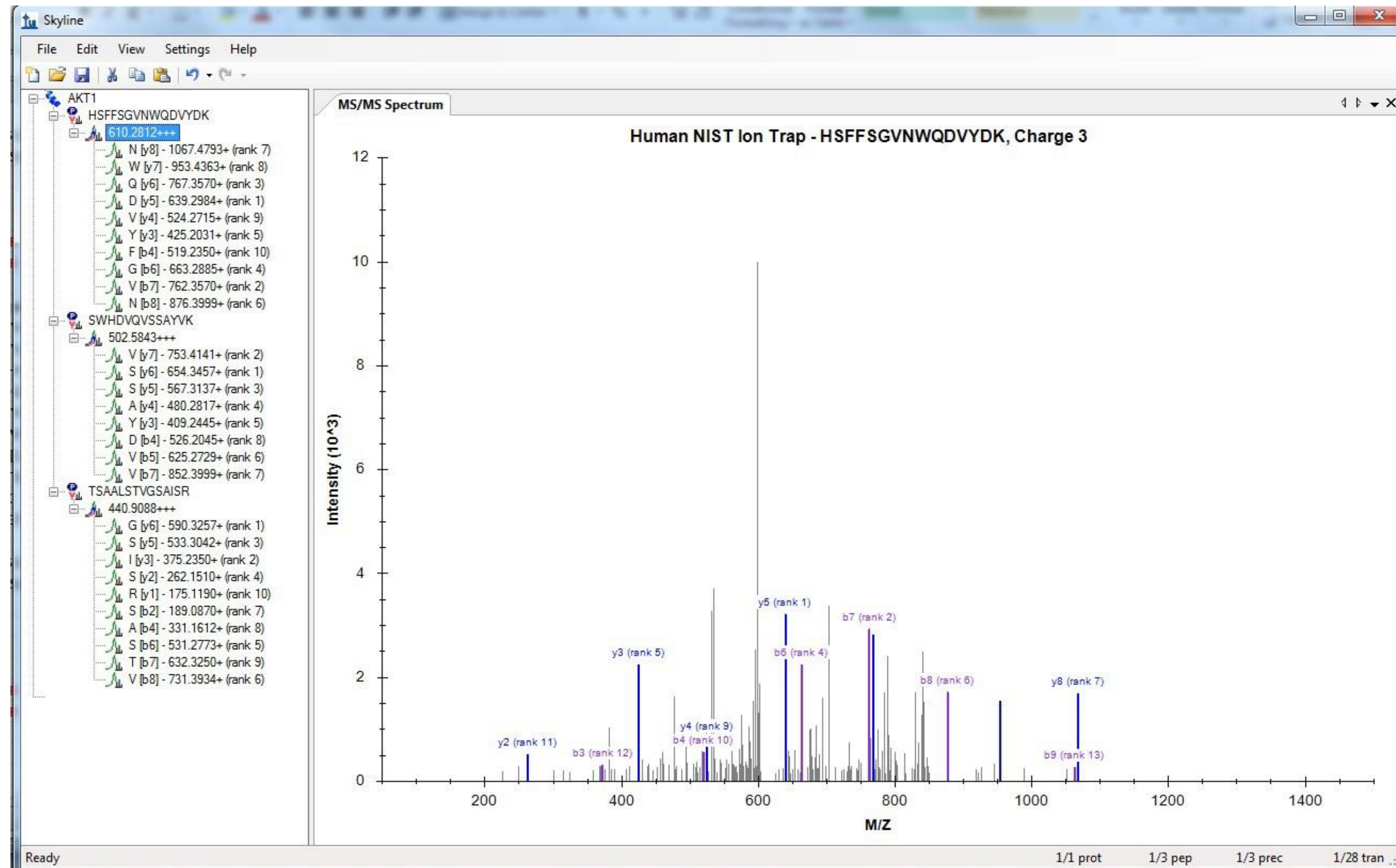
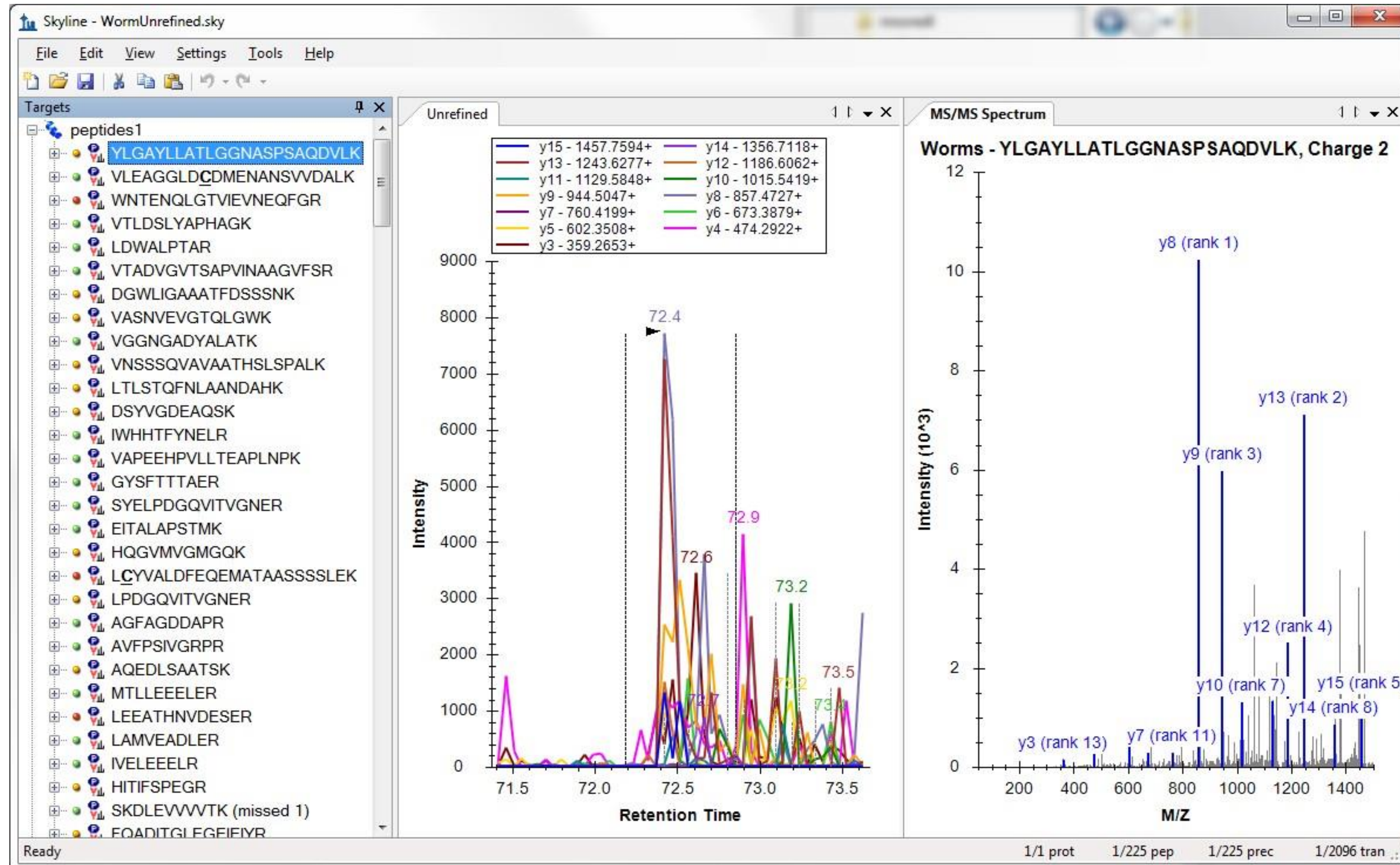
Step 1: SKYLINE for creating targeted MS/MS methods

Skyline digests proteins, and fragments peptides, and uses spectral library to find transition intensity



Step 2: Skyline for MRM - Method Building



Input all peptides of interest

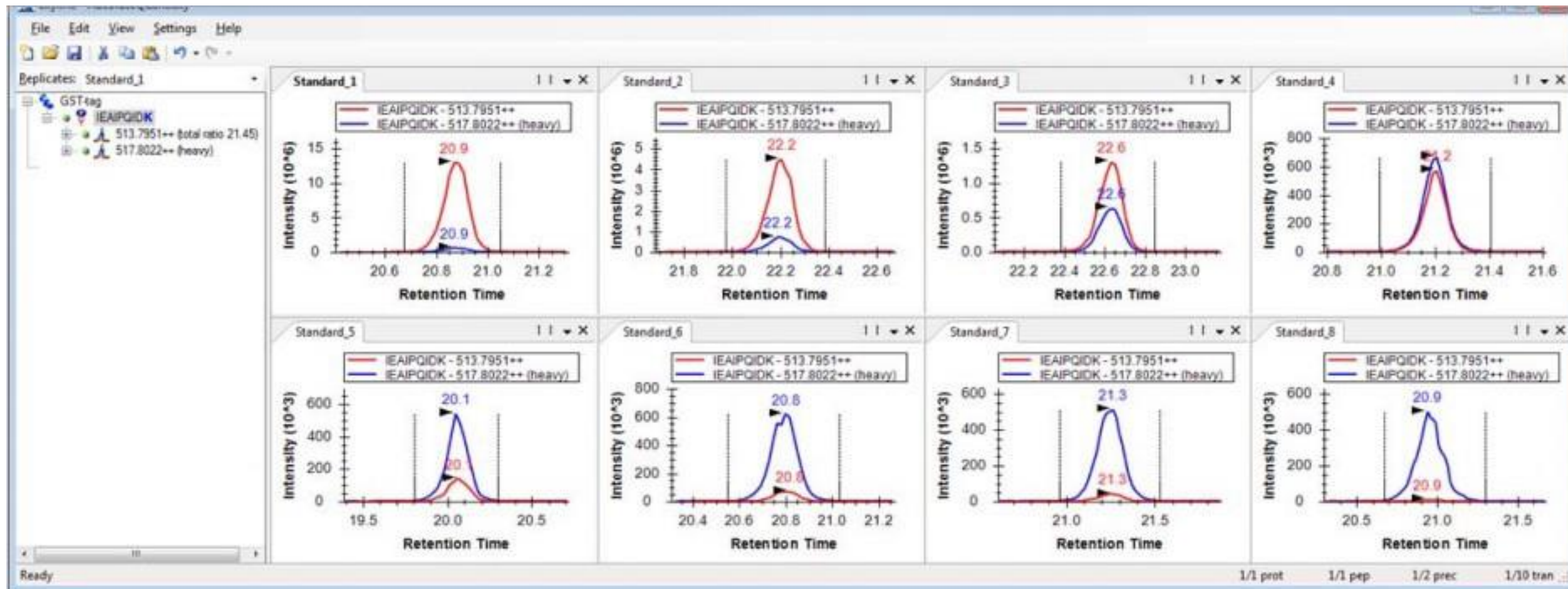
Shows graphs of MS/MS spectra from spectral library

Step 3: Skyline for MRM - Method Building

- Helps generate proteotypic peptide lists using MS/MS spectral libraries
- Find which peptides can be measured in specific matrix
- Find best transitions to measure for a peptide
- Creates transition lists and vendor-specific instrument methods for MRM experiments

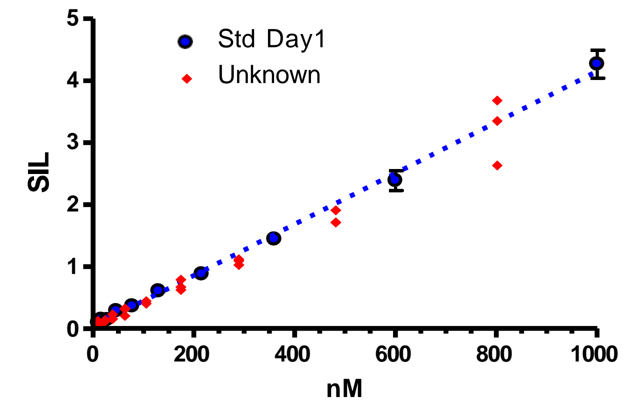
Step 4a: Skyline for MRM - Quantification

- Import raw files into skyline
- Pick peptide of interest
- Check standard peaks



Step 4b: Skyline for MRM - Quantification

- Use the heavy standard PAR (Peak-Area-Ratio) to make calibration curve
- Determine sample quantity based on curve



Advantages of SRM/MRM

- Rapid: faster than antibody-based methods – Relative or Absolute Quantitation
- Ideal for monitoring abundance of a set of predefined target proteins or PTMs over many samples.
- Wide dynamic quantification range (4-5 orders of magnitude) and high sensitivity
- High degree of reproducibility
- Well supported by public resources
- Efficient reduction of noise
- Rapid switch between transitions (<2ms)
- High sensitivity, multiplexing

Disadvantages of SRM/MRM

- Low resolution
- You need to know what to look for in advance!
- Prior knowledge about target proteins and PTMs required (SRM assay)
  - Need to know charge state, retention time and relative product ion intensities before experimentation
  - Peptide candidates need to be unique

- Limited multiplexing capacity that can be measured at once (ca. 1000 transitions per run)
  - Physical limit to the number of transitions can get around this by using time-scheduled SRM, monitor transitions for a peptide in small window near retention time

Applications of SRM/MRM

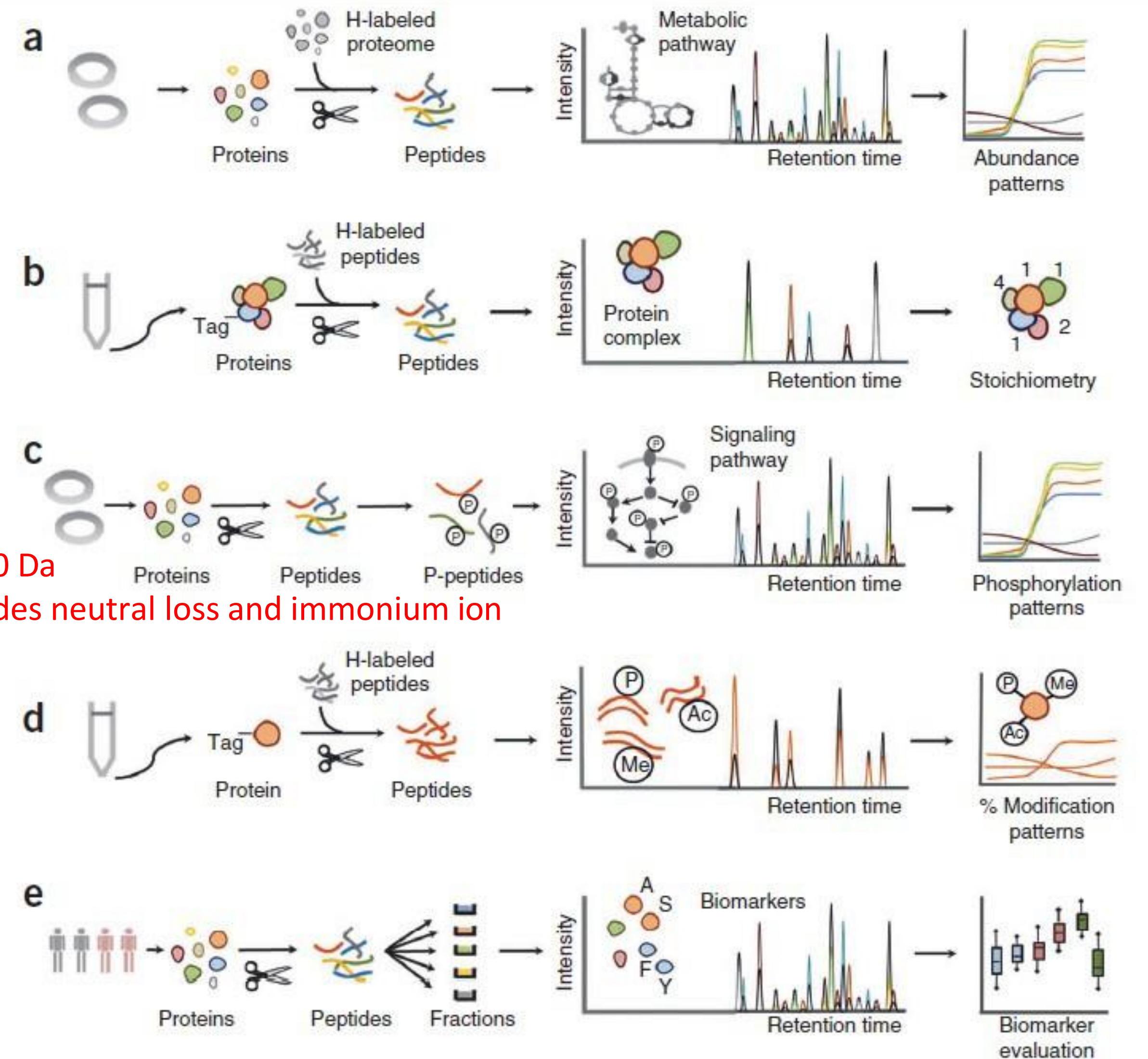
Metabolic pathway analysis

Protein complex subunit stoichiometry

Phosphorylation (known or hypothesized) Q1: n x 80 Da
Q3: includes neutral loss and immonium ion

Modifications within protein

Biomarkers: protein indicator correlating to a disease state

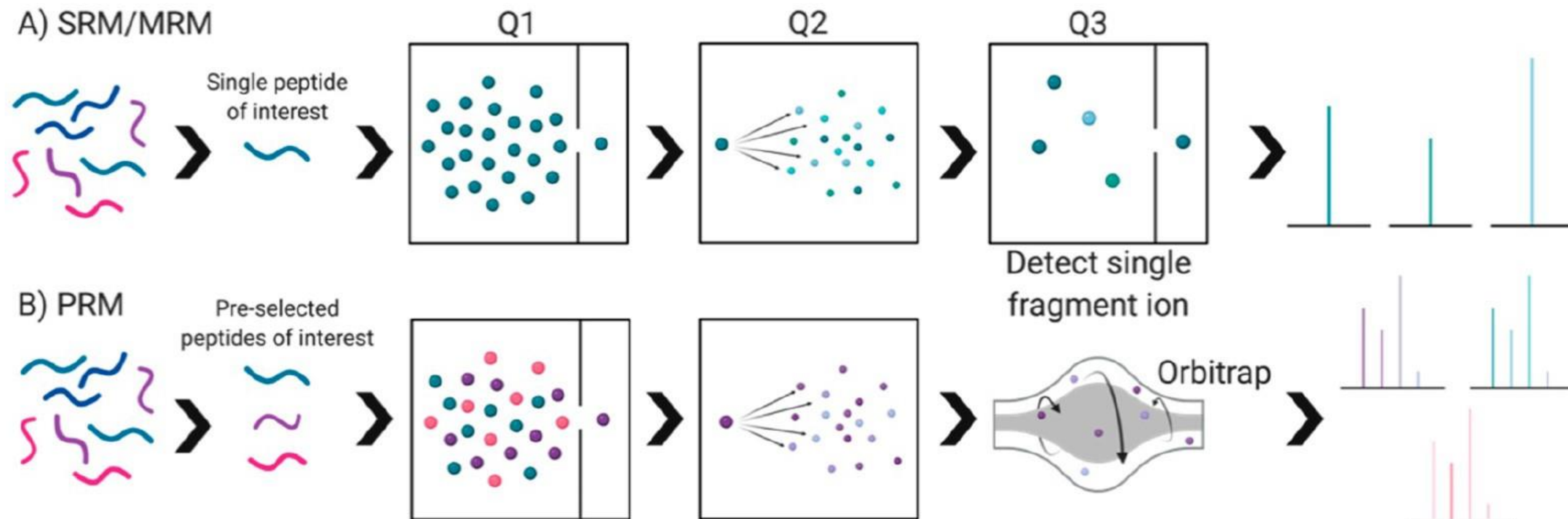


Recommended reading: **Tutorial: best practices and considerations for mass-spectrometry-based protein biomarker discovery and validation.** *Nature Protocols* **volume 16**, pages3737–3760 (2021)

Parallel Reaction Monitoring (PRM)

- Quadrupole Orbitrap or Quadrupole TOF
  - Simplified method design
  - Produce **full** MS2 spectra for each precursor
  - Flexible data analysis
  - Don't have to choose fragment ions beforehand

Q1: Filter mode with a narrow isolation window
Q2: Fragmentation
Q3: Analyze **all fragment ions** simultaneously with Screening mode

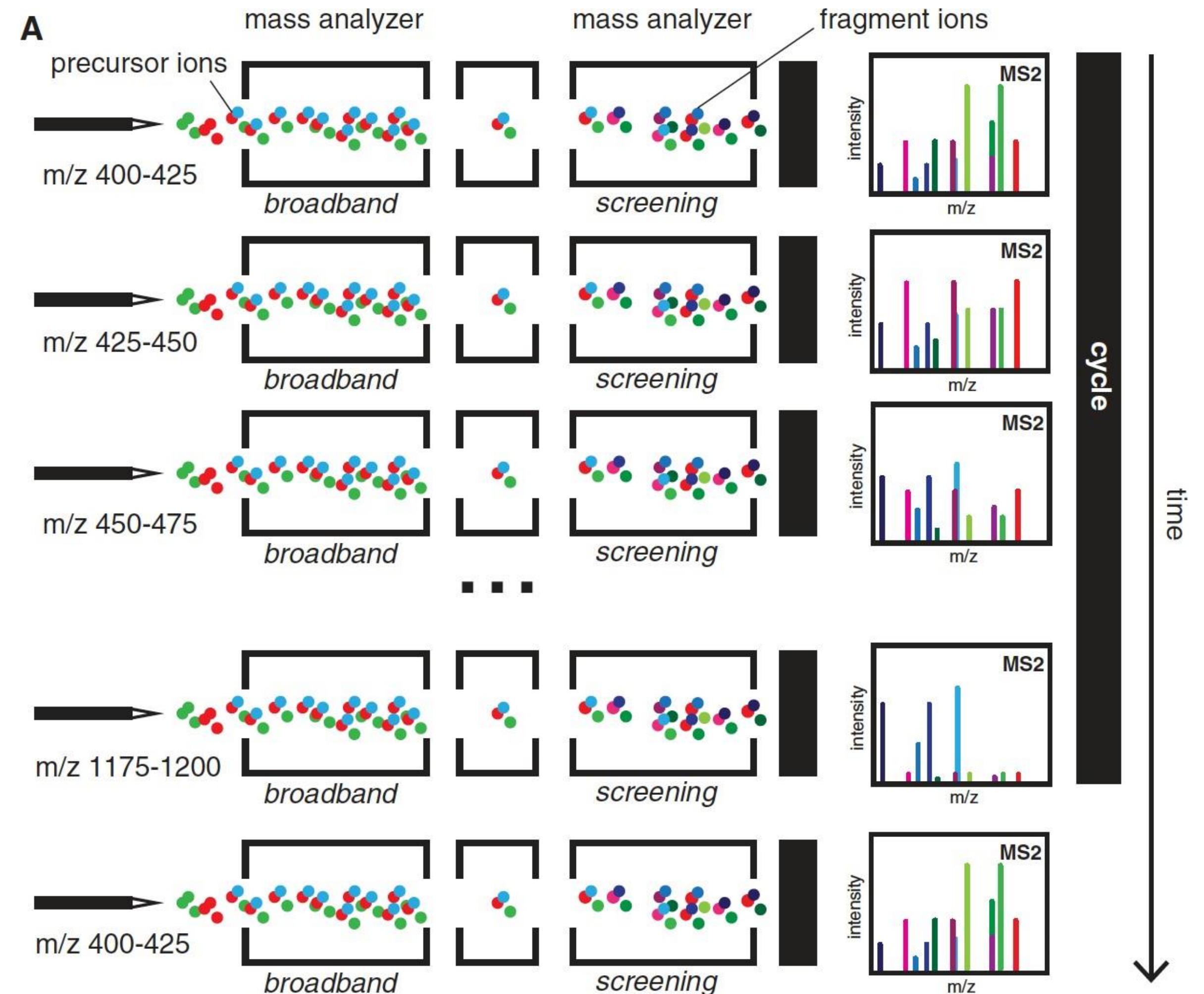


Advantages of PRM

- PRM has flexibility in selecting the fragment ions, because it retrieves the chromatographic peaks of those fragment ions that are most suitable for peptide identification and quantification
- High resolution and high mass accuracy, which results in an increased selectivity as compared to triple quadrupole instruments.
- PRM exhibits a high dynamic range, high signal-to-noise, and excellent sensitivity and specificity for peptide and protein quantification.
- PRM only acquires information for the pre-selected peptides of interest.
- PRM provides both identification and quantification information.

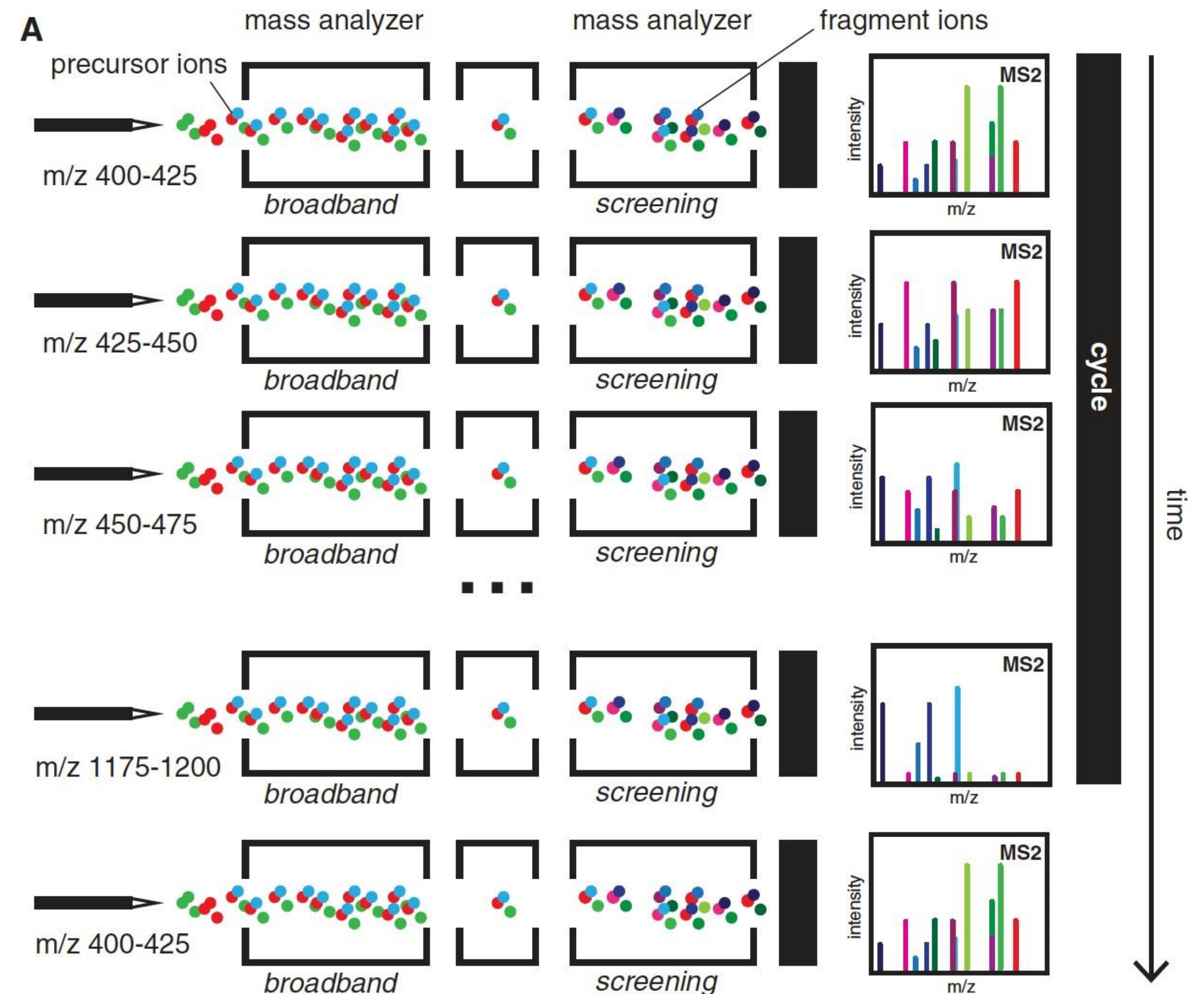
Data-Independent Acquisition (DIA)/SWATH

- **MS2 data-independent acquisition methods** are usually implemented in quadrupole-orbital ion traps, and quadrupole TOF hybrid mass analyzers.
- These methods rely on the use of one or several broadband isolation windows that isolate all peptide ions in a sample within each cycle.
  - 32 consecutive 25 Da precursor isolation windows



Data-Independent Acquisition (DIA)/SWATH

- All peptide ions within a window are simultaneously fragmented, and the resulting fragment ion maps can be subjected to targeted data analysis to identify the coeluting fragment ion groups from the peptides of interest.
- Generate data for all peptides and therefore, the targets of interest can be selected post-acquisition.
- Can only be considered targeted as long as there is a hypothesis to be tested.
    - different protein isoforms, splicing variants, and specific modified peptides



Classification in Proteomics

- According to the information they can provide:
  - Relative quantification
    - peptide abundance ratio ➔ relative protein abundances calculation
  - Absolute quantification
    - Comparing ion intensity of a peptide with the ion intensity of an **identical chemically synthesized heavy isotope labeled peptide** spiked in with *known concentration* as an internal standard.
- According to the underlying methodology:
  - Label based proteomics
  - Label free proteomics
- According to the pre-selected range of proteins
  - Discovery proteomics
  - Targeted proteomics