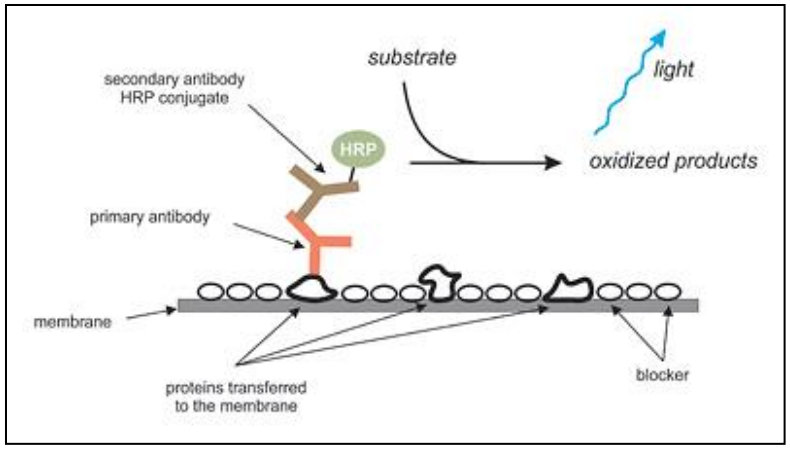
Protein Quantitation II: Multiple Reaction Monitoring

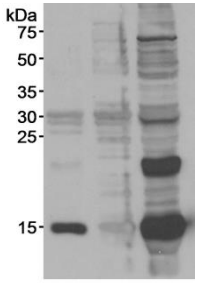
Kelly Ruggles
kelly@fenyolab.org
New York University

Traditional Affinity-based proteomics

Use antibodies to quantify proteins

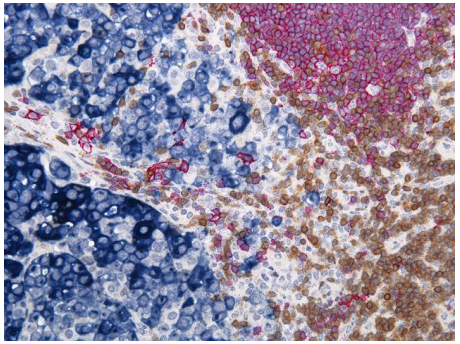


Western Blot



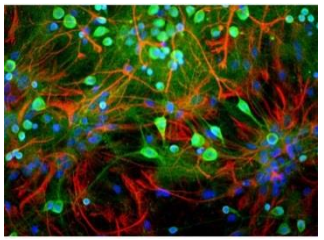
ELISA

Immunohistochemistry



RPPA

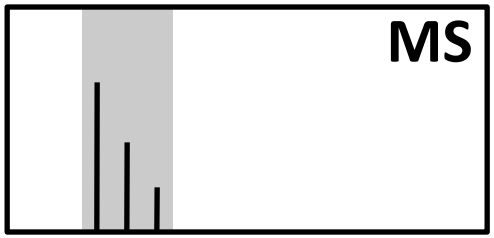
Immunofluorescence



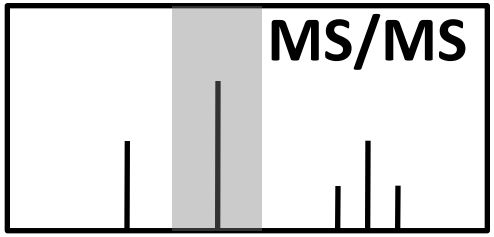
Mass Spectrometry based proteomic quantitation

Shotgun proteomics ← **LC-MS** → **Targeted MS**

1. Records M/Z



2. Selects peptides based on abundance and fragments



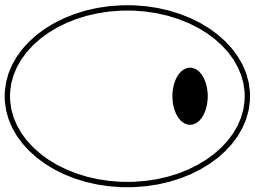
3. Protein database search for peptide identification

Data Dependent Acquisition (DDA)

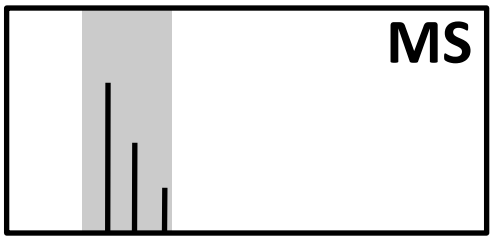
Digestion

Fractionation

Lysis



1. Select precursor ion



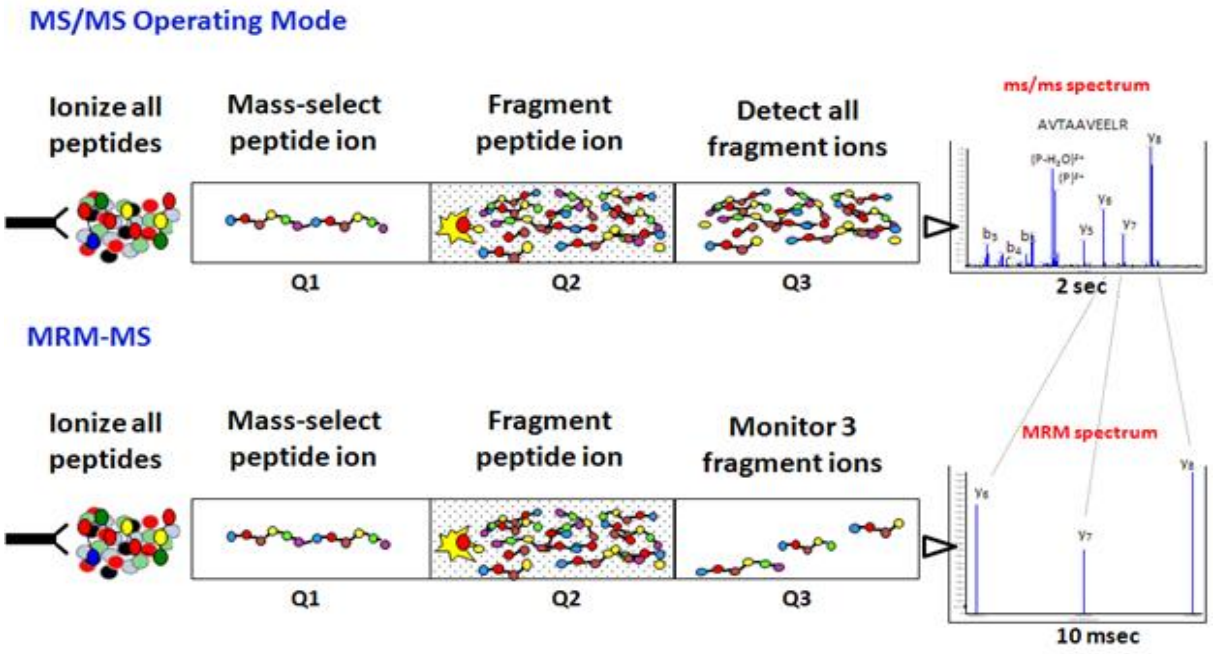
2. Precursor fragmentation

3. Use Precursor-Fragment pairs for identification

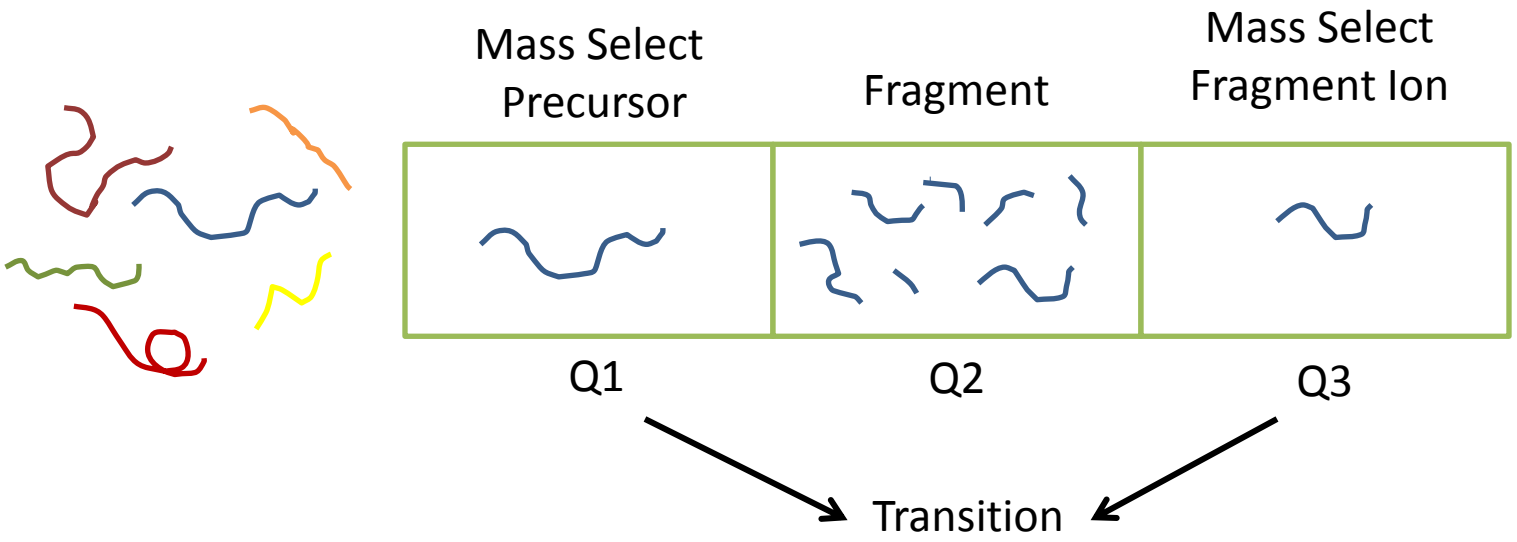
Uses predefined set of peptides

Multiple Reaction Monitoring (MRM)

- Triple Quadrupole acts as ion filters
- Precursor selected in first mass analyzer (Q1)
- Fragmented by collision activated dissociation (Q2)
- One or several of the fragments are specifically measured in the second mass analyzer (Q3)



Peptide Identification with MRM

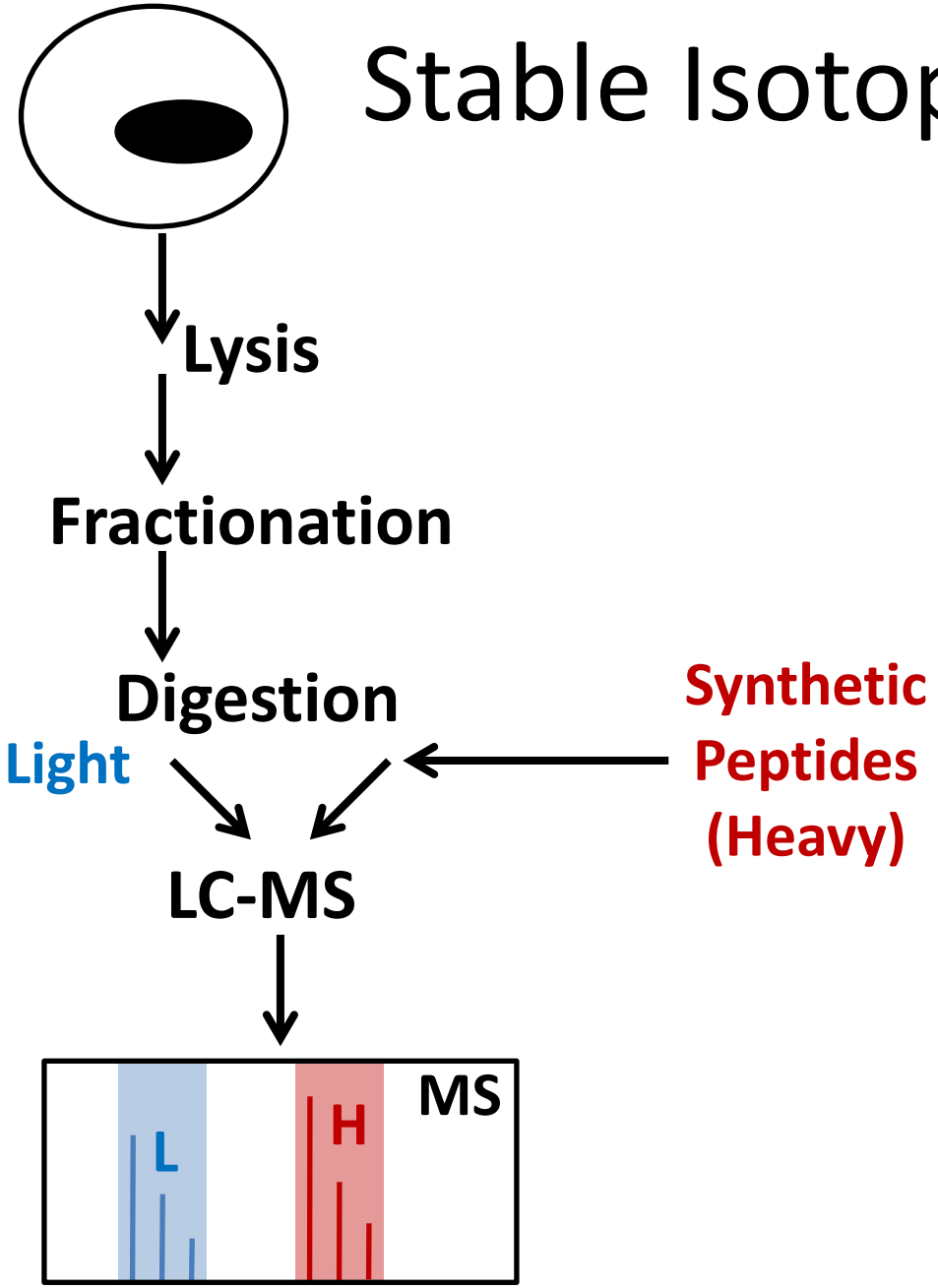


- Transition: Precursor-Fragment ion pair are used for protein identification
- Select both Q1 and Q3 prior to run
  - Pick Q3 fragment ions based on discovery experiments, spectral libraries
  - Q1 doubly or triply charged peptides
- Use the 3 most intense transitions for quantitation

Label-free quantification

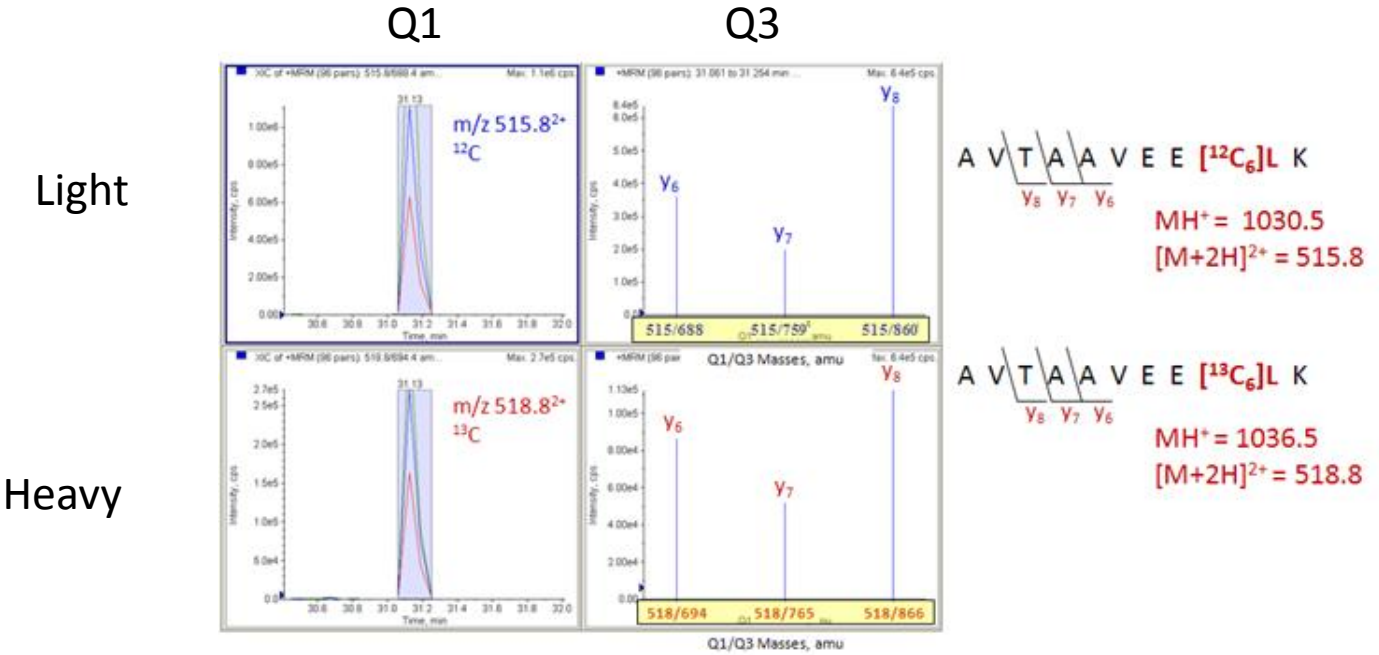
- Usually use 3 or more precursor-product ion pairs (transitions) for quantitation
- Relies on direct evaluation of MS signal intensities of naturally occurring peptides in a sample.
- Simple and straightforward
- Low precision
- Several peptides for each protein should be quantified to avoid false quantification

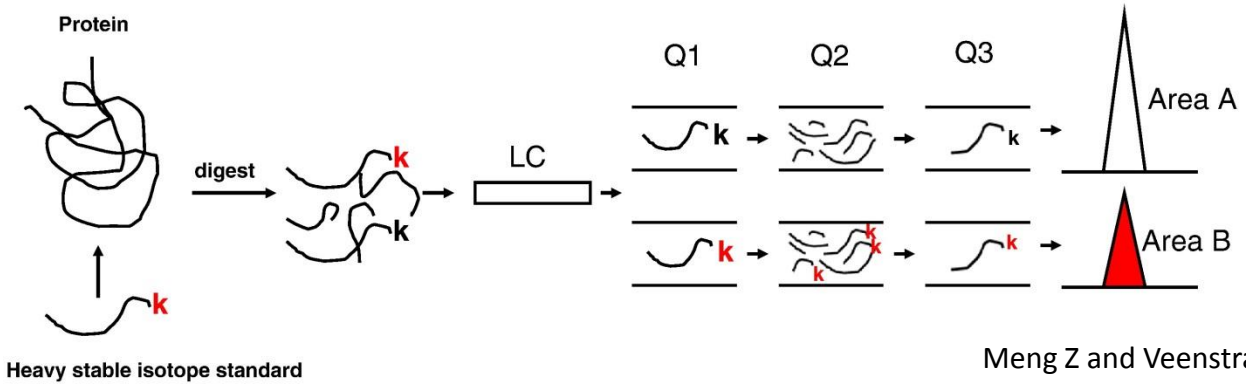
Stable Isotope Dilution (SID)



- Use isotopically labeled reference protein
- 13C and/or 15N labeled peptide analogs
- Chemically identical to the target peptide but with mass difference
- Add known quantity of heavy standard
- Compare signals for the light to the heavy reference to determine for precise quantification

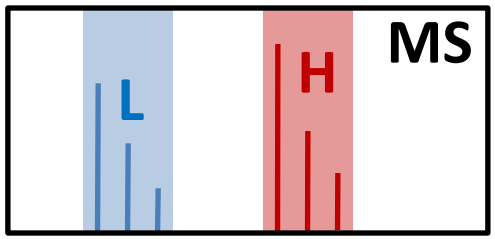
Fragment Ion Detection and Protein Quantitation





Meng Z and Veenstra TD, 2011

Quantification Details



SIS: Stable Isotope Standard
PAR: Peak Area Ratio

$$\text{PAR} = \frac{\text{Light (Analyte) Peak Area}}{\text{Heavy (SIS) Peak Area}}$$

Analyte concentration= PAR*SIS peptide concentration

-Use at least 3 transitions

-Have to make sure these transitions do not have interferences

Strengths of MRM

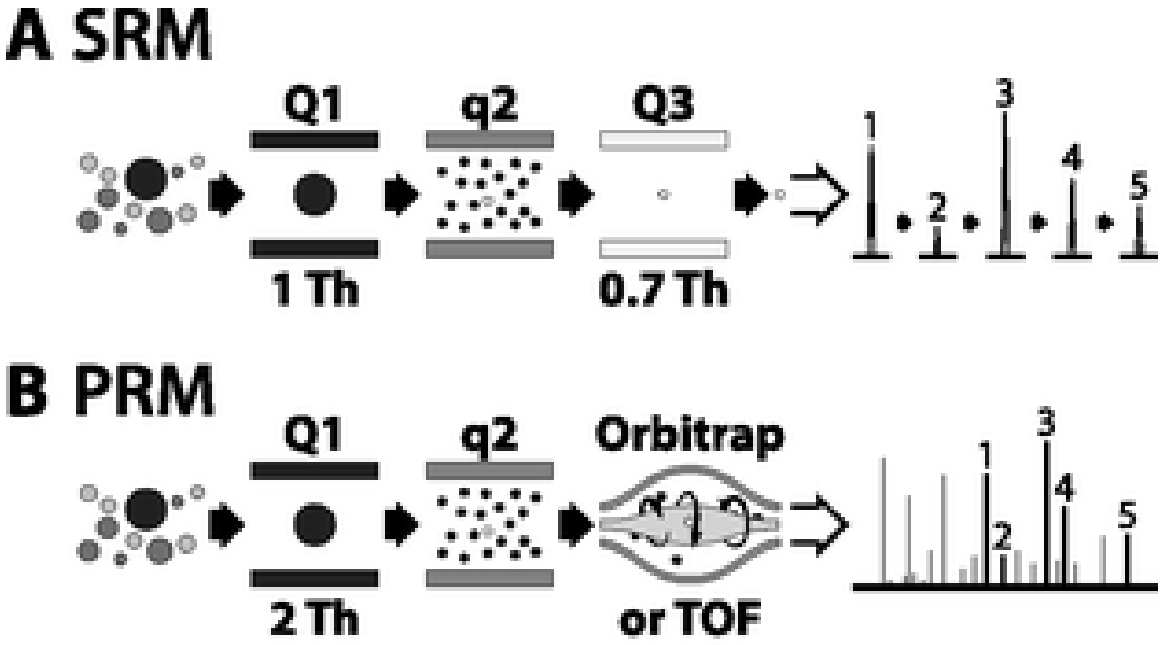
- Can detect multiple transitions on the order of 10msec per transition
- Can analyze many peptides (100s) per assay and the monitoring of many transitions per peptide
- High sensitivity
- High reproducibility
- Detects low level analytes even in complex matrix
- Golden standard for quantitation!

Weaknesses of MRM

- Focuses on defined set of peptide candidates
  - Need to know charge state, retention time and relative product ion intensities before experimentation
- Physical limit to the number of transitions that can be measured at once
  - Can get around this by using time-scheduled MRM, monitor transitions for a peptide in small window near retention time

Parallel Reaction Monitoring (PRM)

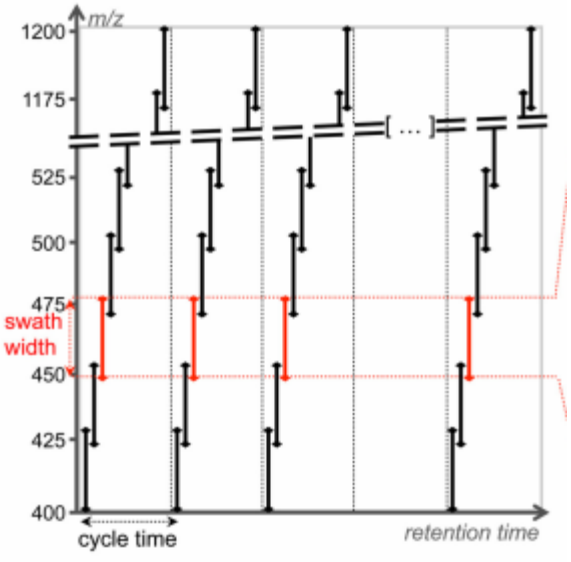
- Q3 is substituted with a high resolution mass analyzer to detect all target product ions
- Generates high resolution, full scan MS/MS data
- All transitions can be used to confirm peptide ID
- Don’t have to choose ions beforehand



Peterson et al., 2012

SWATH-MS: Data Collection

- Data acquired on quadrupole-quadrupole TOF high resolution instrument cycling through 32-consecutive 25-Da precursor isolation windows (swaths).
- Generates fragment ion spectra for all precursor ions within a user defined precursor retention time and m/z
- Records the fragment ion spectra as complex fragment ion maps



32 discrete precursor isolation windows of 25–Da width across the 400-1200 m/z range

Gillet et al., 2012

Applications of MRM

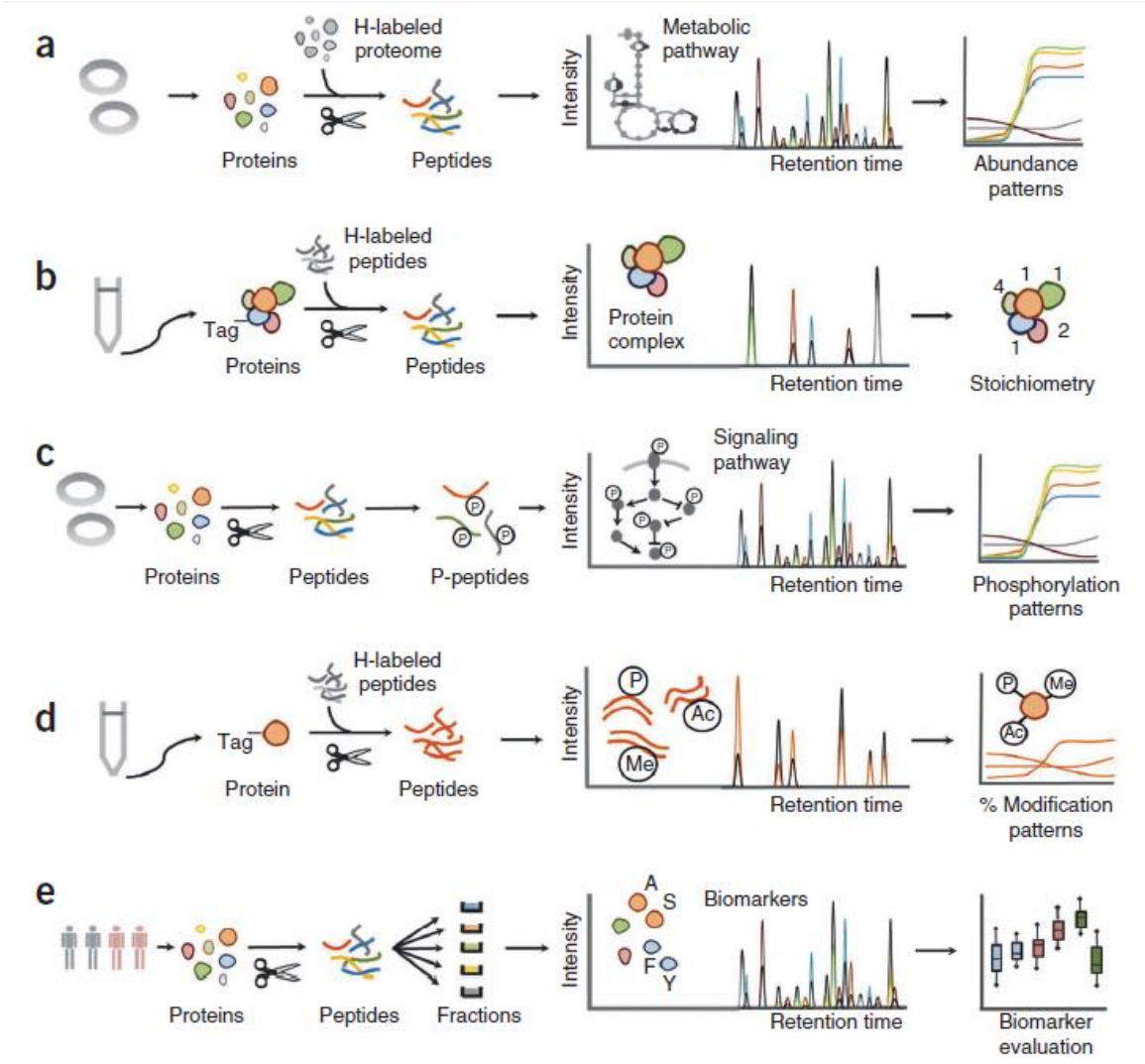
Metabolic pathway analysis

Protein complex subunit stoichiometry

Phosphorylation

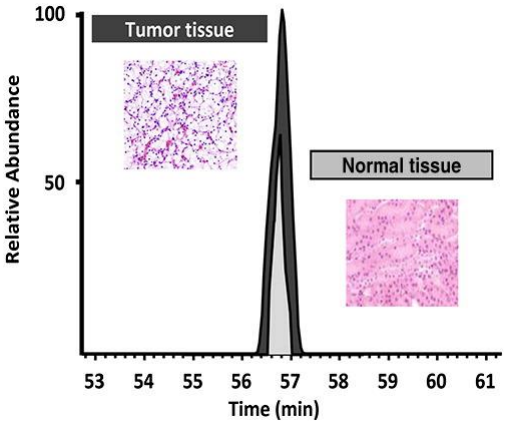
Modifications within protein

Biomarkers: protein indicator correlating to a disease state



MRM and Biomarker Verification

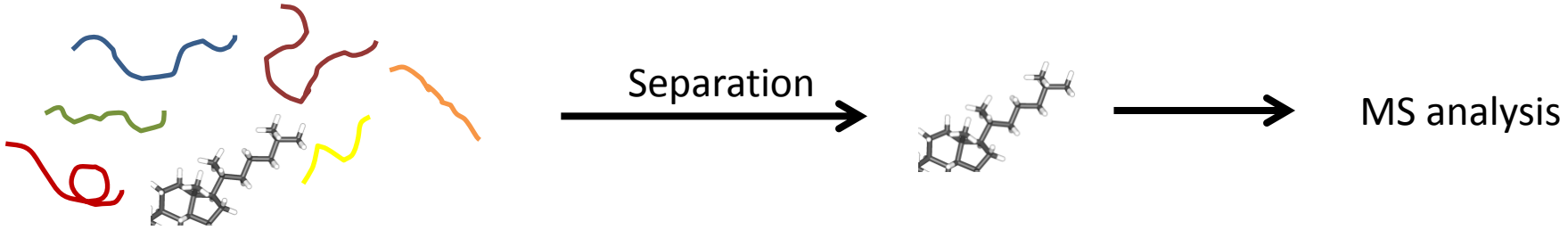
- Measurable indicator that provides the status of a biological state
  - Diagnosis
  - Prognosis
  - Treatment efficacy
- Shotgun proteomics → Biomarker Discovery (<100 patients)
- Targeted proteomics→ Biomarker Validation (~1000s patients)
  - Requires higher threshold of certainty
  - Remove high false positives from discovery phase
- Most often plasma/serum, but can be tissue-based biomarkers



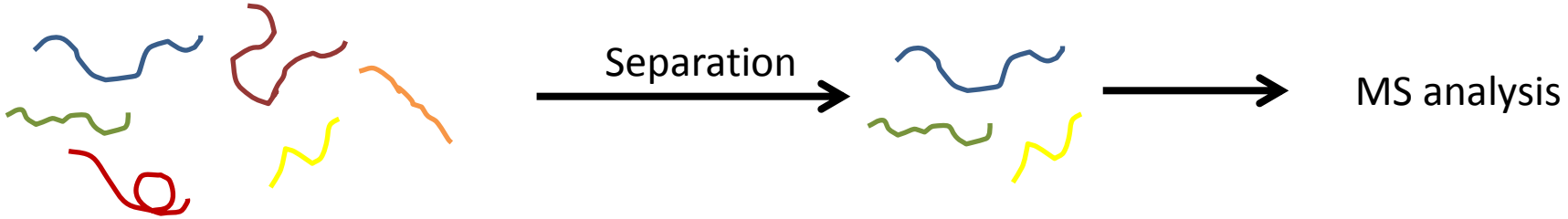
Meng Z and Veenstra TD, 2011

MRM and Biomarker Verification

- Originally used to analyze small molecules since the late 1970s
- More recently, used for proteins and peptide quantitation in complex biological matrices
- With small molecules, the matrix and analyte have different chemical natures so separation step is able to remove other components from analytes

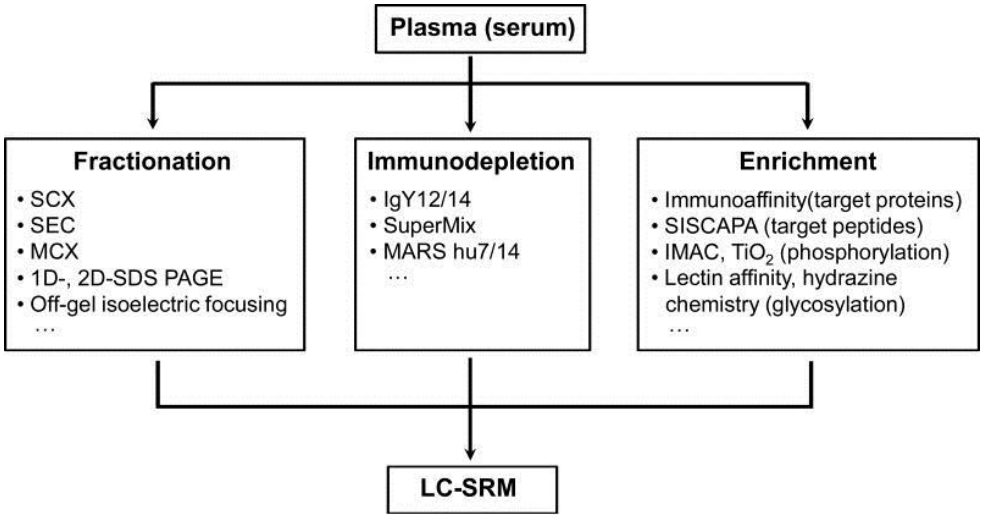


- With proteomics, both the analytes and the background matrix are made up of peptides, so this separation cannot occur. Leads to decreased sensitivity and increased interference.



Enhancing MRM Sensitivity for Biomarker Discovery

Sample Enrichment

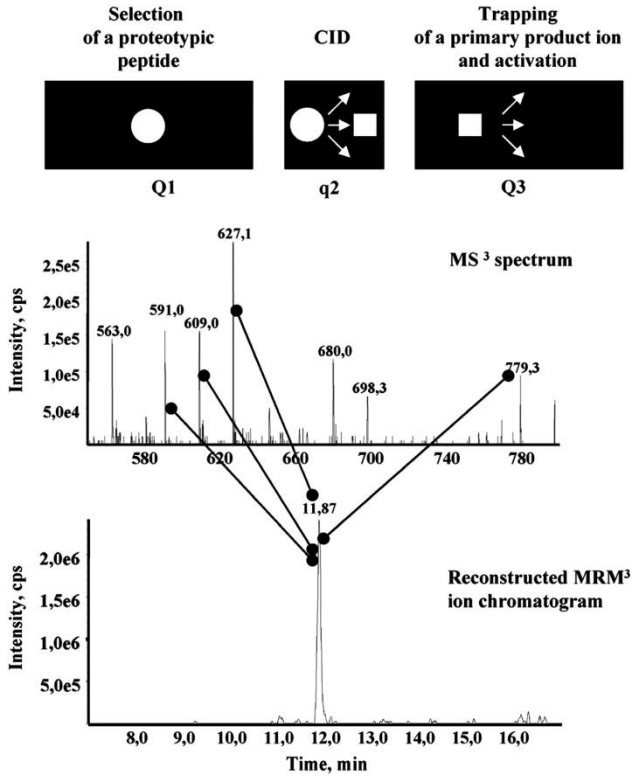


Shi T., et al. 2012

MRM3

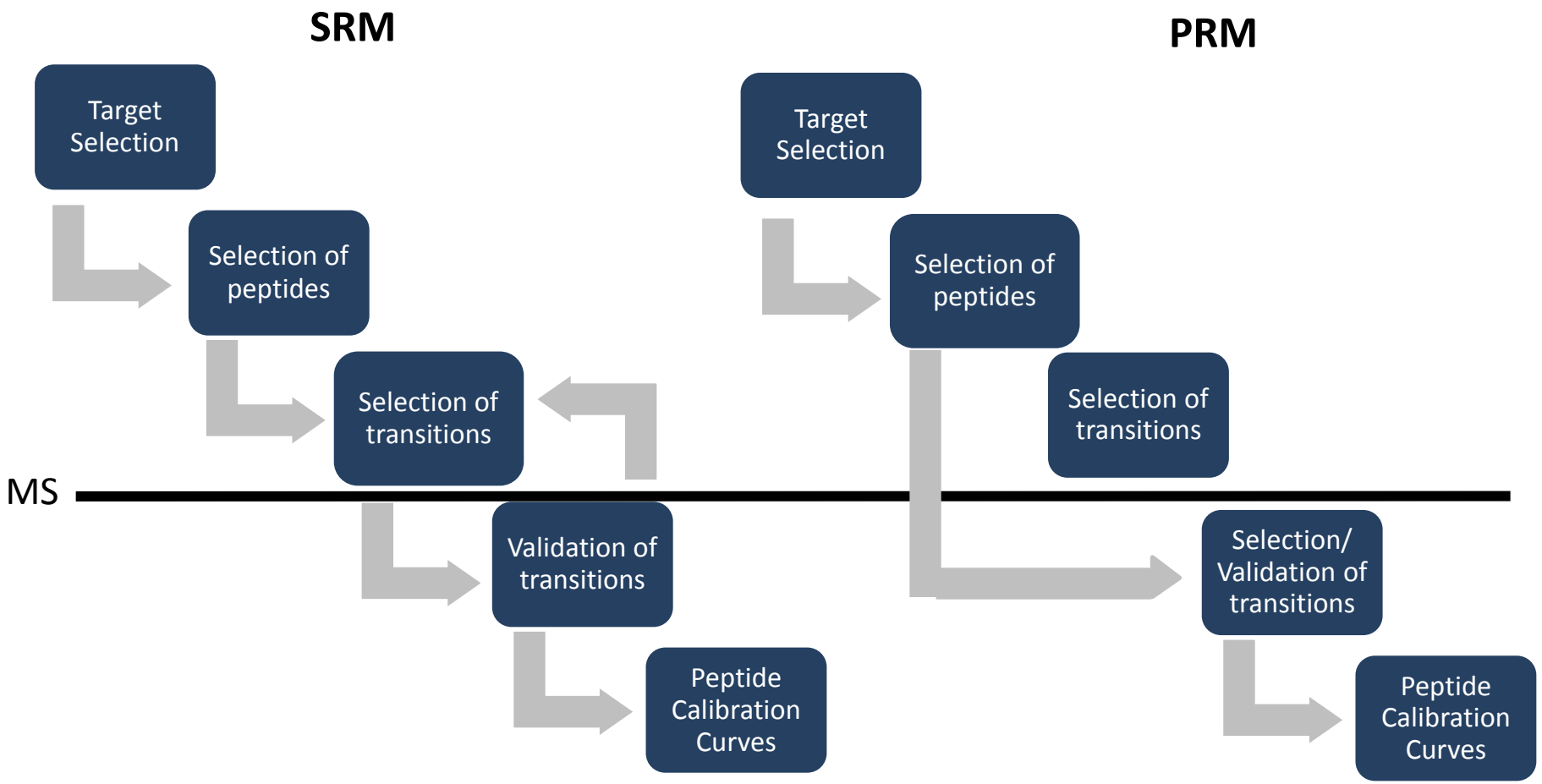
Further fragments product ions

Reduces background



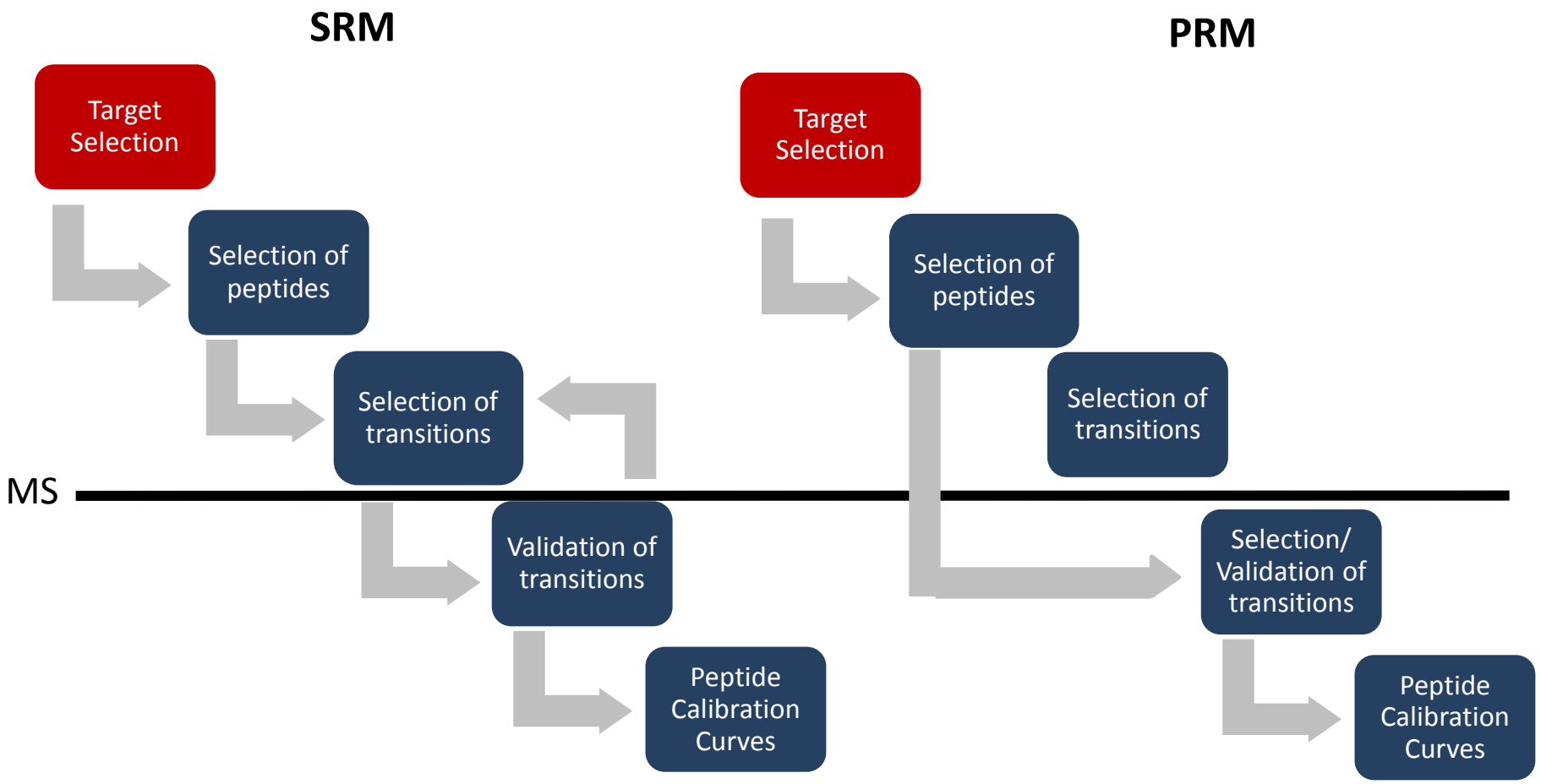
Meng Z and Veenstra TD, 2011

Workflow of MRM and PRM MS/MS



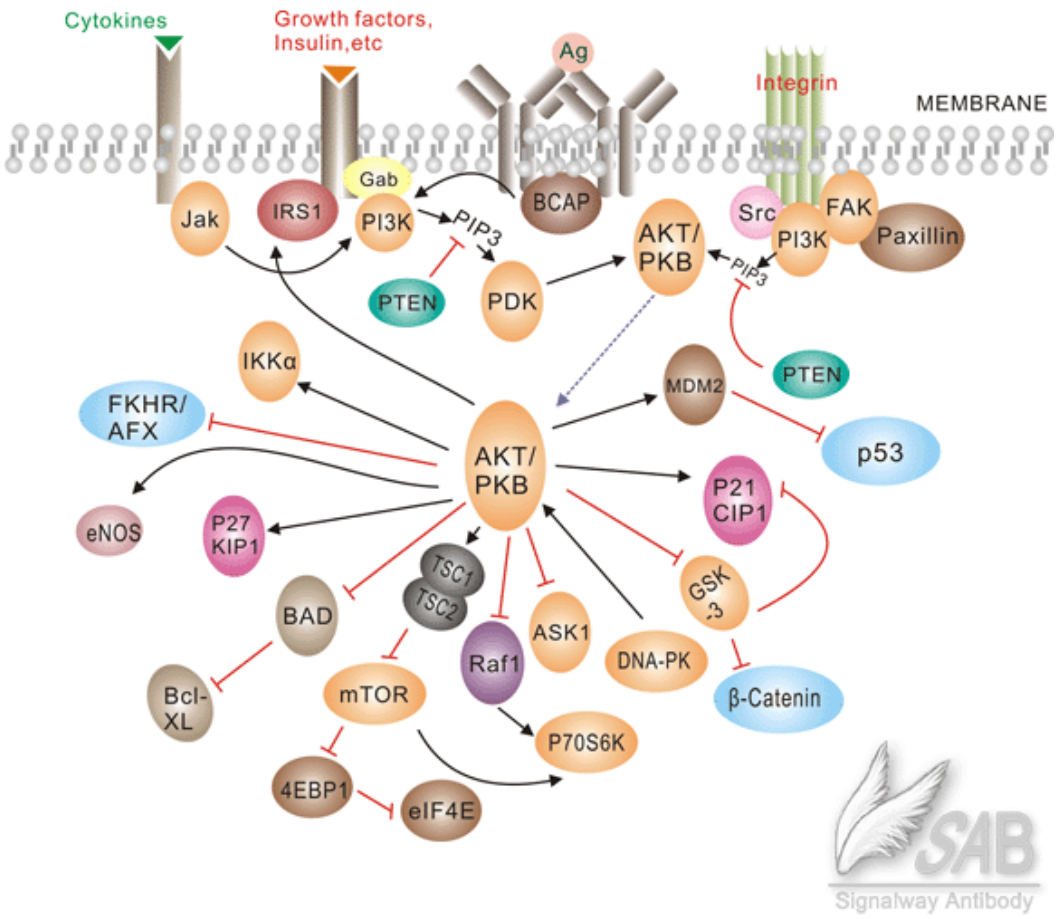
Slide from Dr. Reid Townsend, Washington University in St. Louis

Workflow of MRM and PRM MS/MS



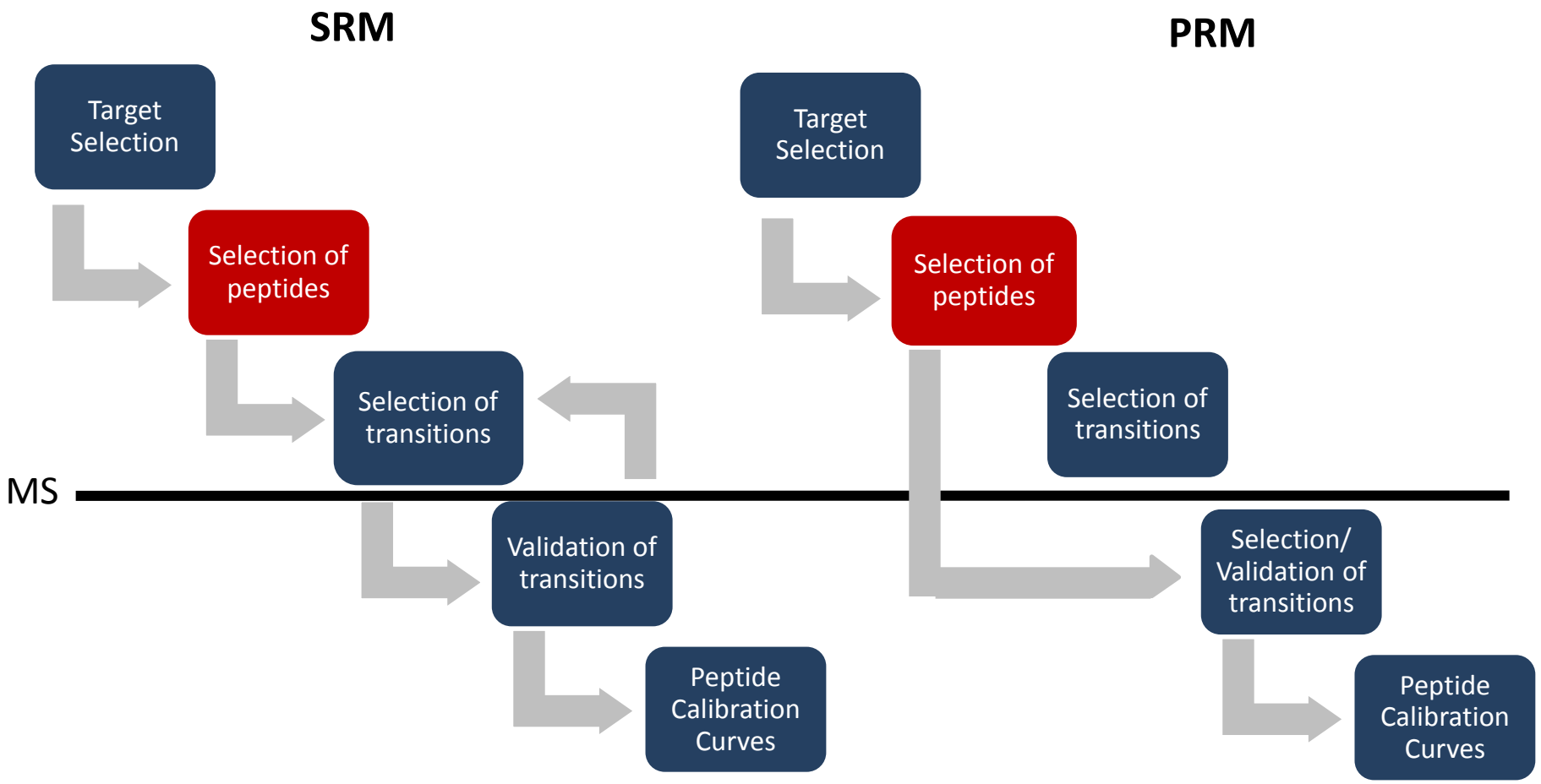
Define a set of proteins based on clinical/biological question

Motivating Example: AKT1 and Breast Cancer



- AKT
- PDK
- BAD
- MDM2
- GSK3
- mTOR
- RAF1

Workflow of MRM and PRM MS/MS



- Proteotypic
- Consistently observed by LC-MS methods

Selecting Peptides

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

Identifying Proteotypic Peptides

Set of Proteins

Trypsin

Peptides

RefSeq
Ensembl
Uniprot

Proteotypic Peptides

Step 1: Full protein sequence in FASTA format

```
MPKKKPTPIQLNPAPDGSAVNGTSSAETNLEALQKKLEELELDEQQRKRLEAFLTQKQKVGELKDDDFEK
ISELGAGNGGVVFKVSHKPSGLVMARKLIHLEIKPAIRNQIIRELQVLHECNSPYIVGFYGAFYSDGEIS
ICMEHMDGGSLDQVLKKAGRIPEQILGKVSIAVIKGLTYLREKHKIMHRDVKPSNILVNSRGEIKLCDFG
VSGQLIDSMANSFVGTRSYMSPERLQGTHYSVQSDIWSMGLSLVEMAVGRYPIPPPDAKELELMFGCQVE
GDAAETPPRPRTPGRPLSSYGMDSRPPMAIFELLDYIVNEPPPKLPSGVFSLEFQDFVNKCLIKNPAERA
DLKQLMVHAFIKRSDAEEVDFAGWLCSTIGLNQPSTPTHAAGV
```

Step 2: Tryptic Peptides

```
PTPIQLNPAPDGSAVNGTSSAETNLEALQK
LEAFLTQK
PSNIVLVNSR
LEELELDEQQR
DDDFEK.....
```

Step 3: Compare to human reference database
-Contain all peptide sequences
-Find all peptides that only map back to one gene

PTPIQLNPAPDGSAVNGTSSAETNLEALQK
LEAFLTQK
PSNIVLVNSR
LEELELDEQQR
DDDFEK.....

Match peptide to proteins
(Reference Protein DB)

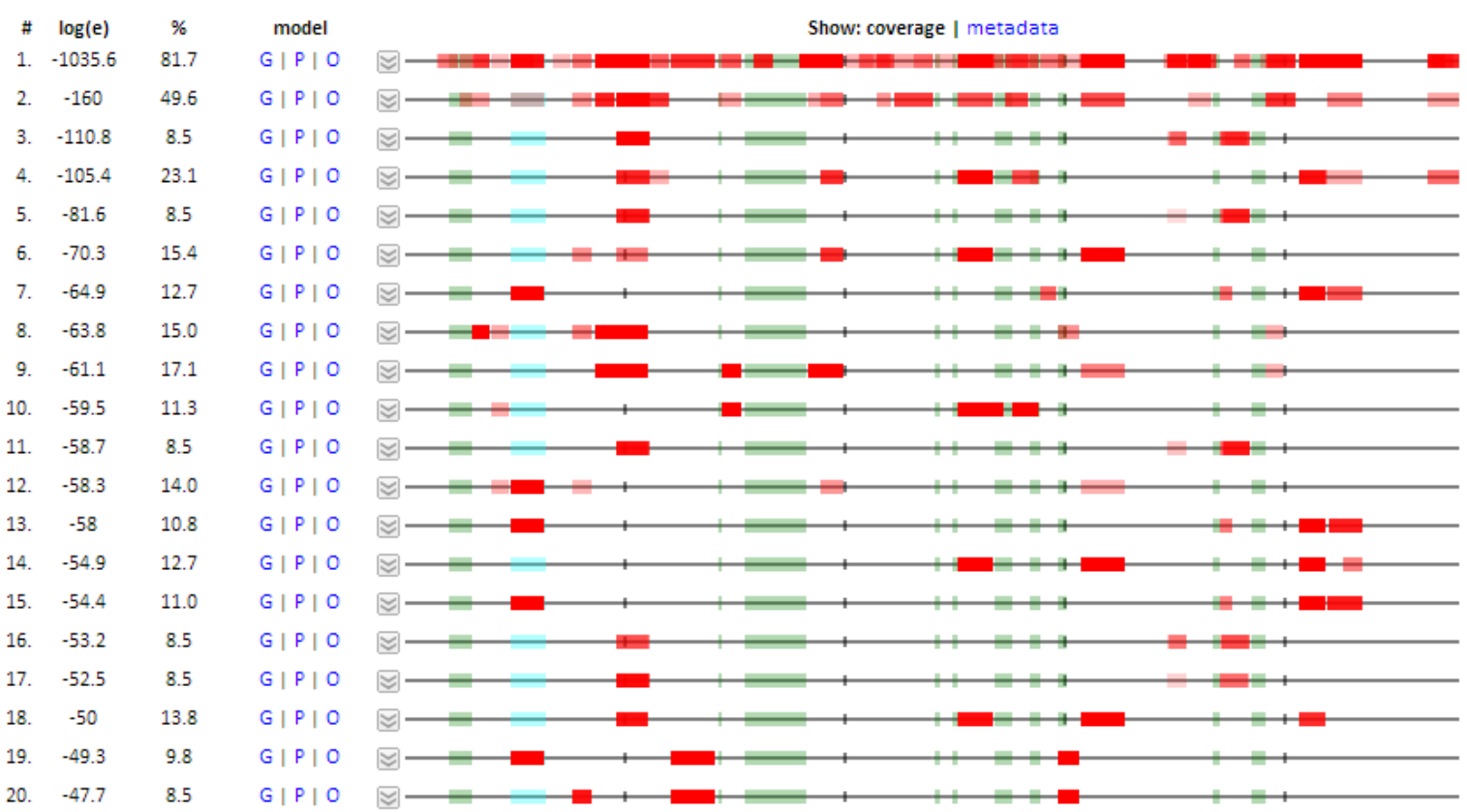
Match proteins to genes
(Using protein names and genomic DB)

LC/MS Properties: GPMDB

-Compares peptides to a collection of previously observed results

-Determines how many times the peptide has been observed by others

-<u>Most proteins show very reproducible peptide patterns</u>

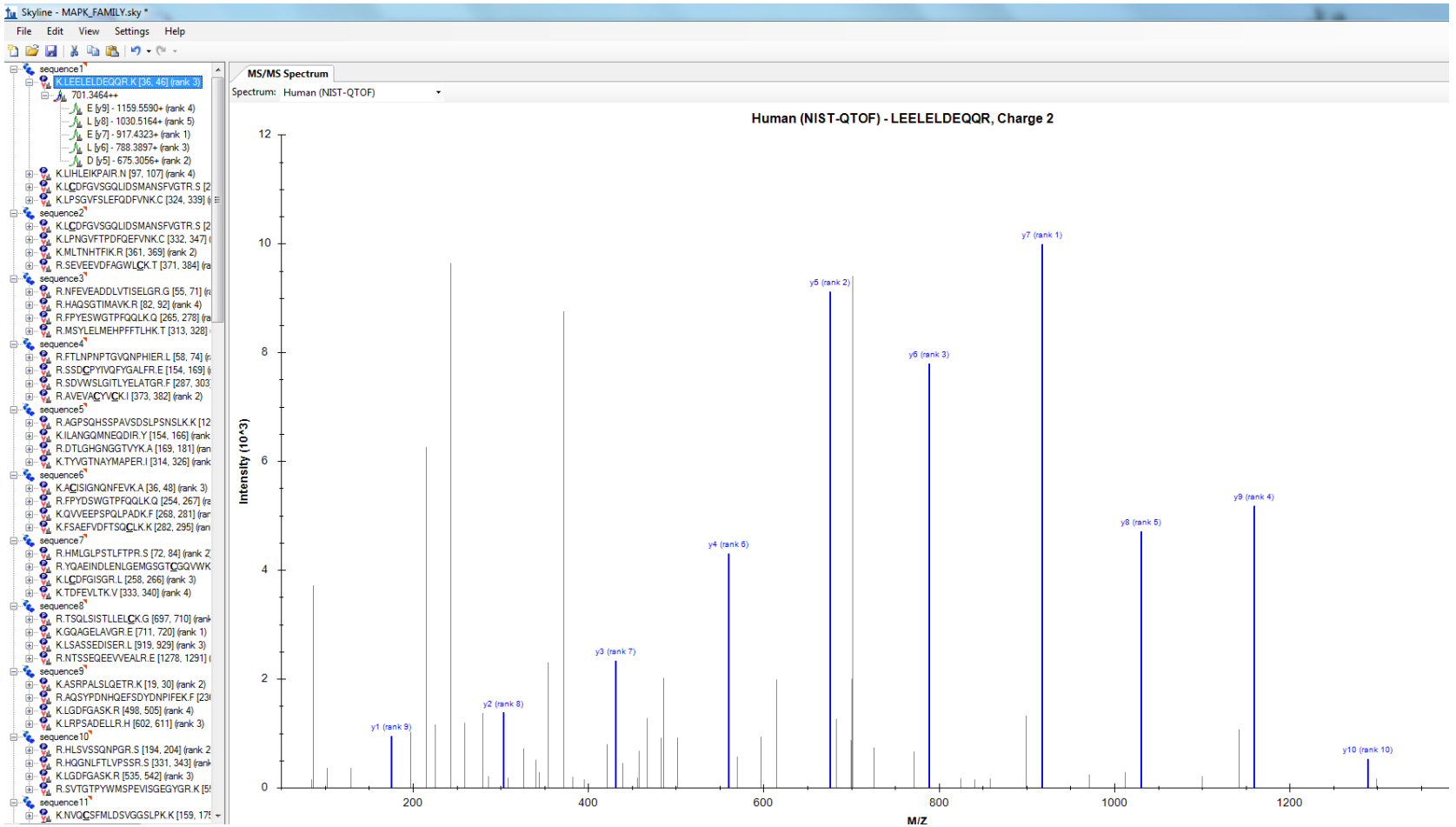


#	log(e)	%	model
1.	-1035.6	81.7	G | P | O
2.	-160	49.6	G | P | O
3.	-110.8	8.5	G | P | O
4.	-105.4	23.1	G | P | O
5.	-81.6	8.5	G | P | O
6.	-70.3	15.4	G | P | O
7.	-64.9	12.7	G | P | O
8.	-63.8	15.0	G | P | O
9.	-61.1	17.1	G | P | O
10.	-59.5	11.3	G | P | O
11.	-58.7	8.5	G | P | O
12.	-58.3	14.0	G | P | O
13.	-58	10.8	G | P | O
14.	-54.9	12.7	G | P | O
15.	-54.4	11.0	G | P | O
16.	-53.2	8.5	G | P | O
17.	-52.5	8.5	G | P | O
18.	-50	13.8	G | P | O
19.	-49.3	9.8	G | P | O
20.	-47.7	8.5	G | P | O

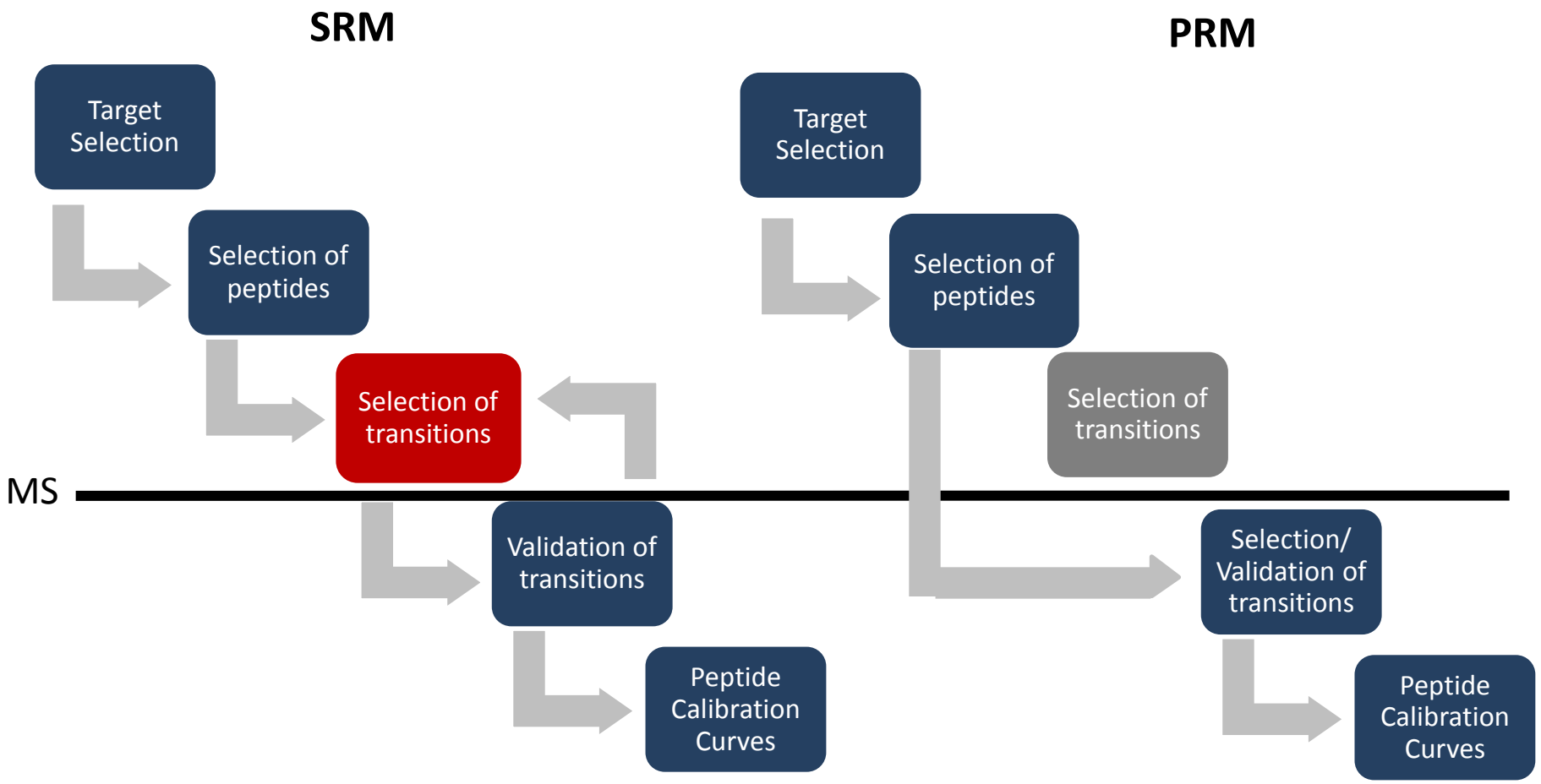
LC/MS Properties: Skyline

-Compares peptides to MS/MS spectral library

-Predicts most abundant transitions



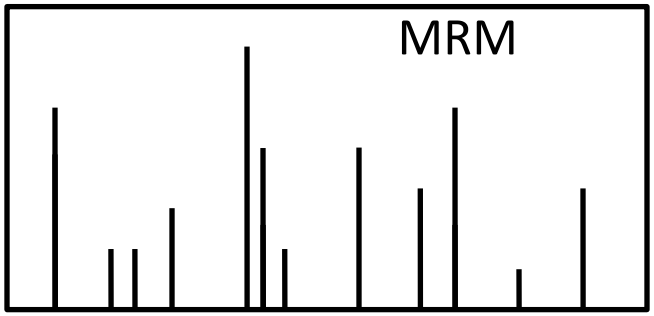
Workflow of MRM and PRM MS/MS



PRM allows for selection of transitions post-data acquisition

Selecting Transitions

- Limitation of MRM-MS: ~1-2 m/z unit window for precursor and fragment ion occasionally let in interfering peptides with similar characteristics
- If we want to use these transitions for quantitation, we need to be confident there are no interferences
- Largest always largest, smallest always smallest etc.
- b-fragments of high m/z are less represented on QqQ



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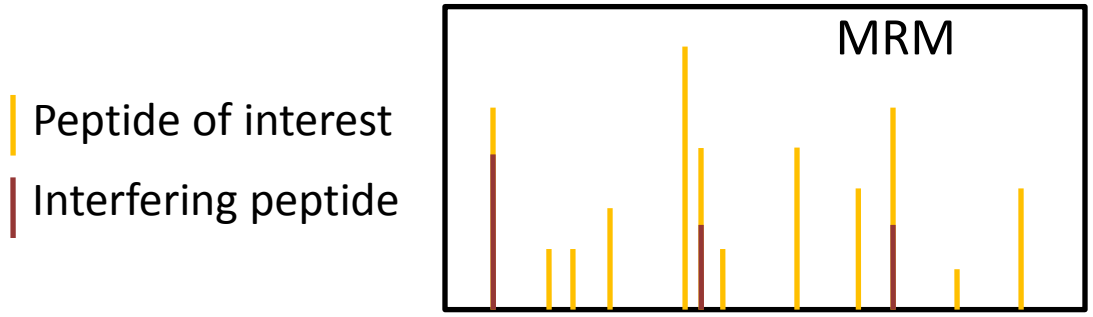
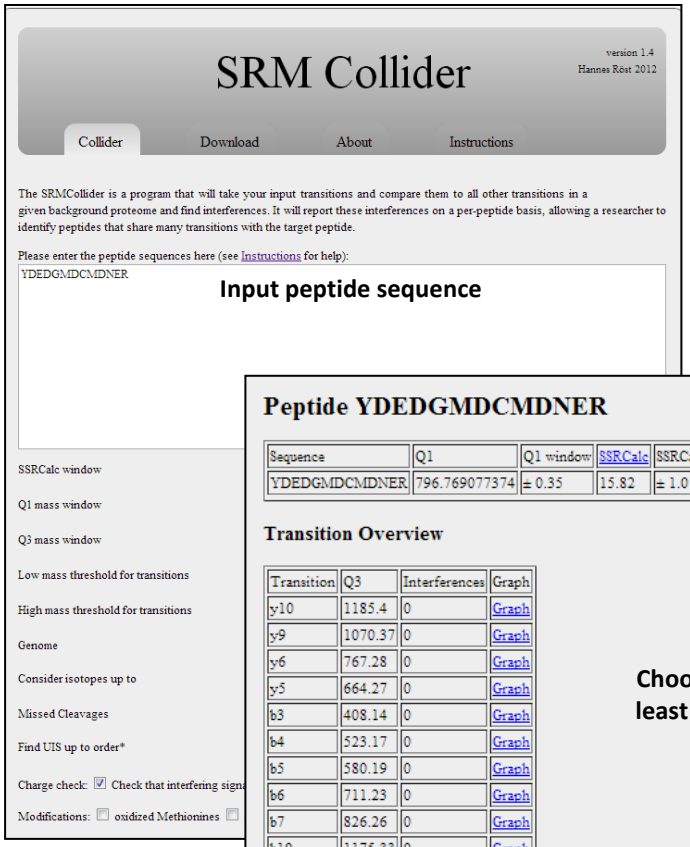


Selecting Transitions: SRMCollider

- 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



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

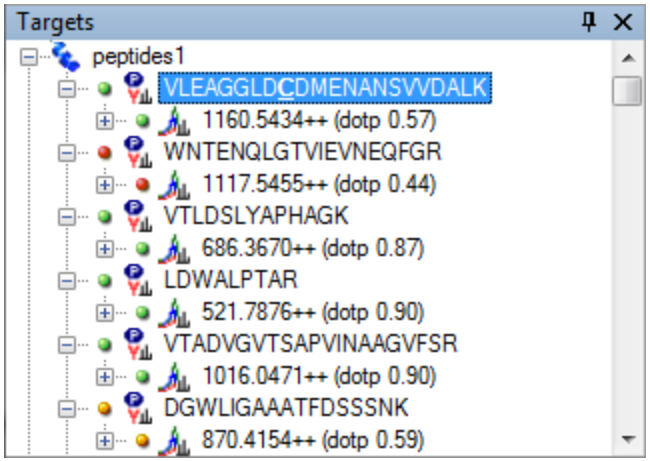
Transition Overview

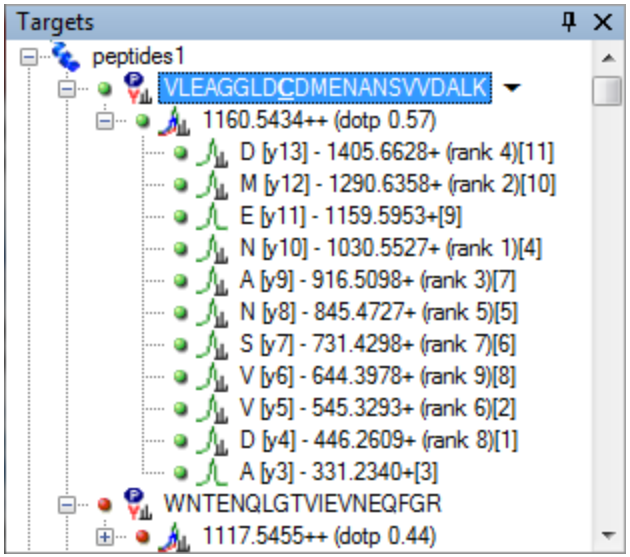
Transition	Q3	Interferences	Graph
y10	1185.4	0	Graph
y9	1070.37	0	Graph
y6	767.28	0	Graph
y5	664.27	0	Graph
b3	408.14	0	Graph
b4	523.17	0	Graph
b5	580.19	0	Graph
b6	711.23	0	Graph
b7	826.26	0	Graph
b10	1175.33	0	Graph
y11	1314.44	1	Graph
y8	1013.35	1	Graph
y7	882.31	1	Graph
y4	533.23	1	Graph
y3	418.21	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

Selecting Transitions: Skyline

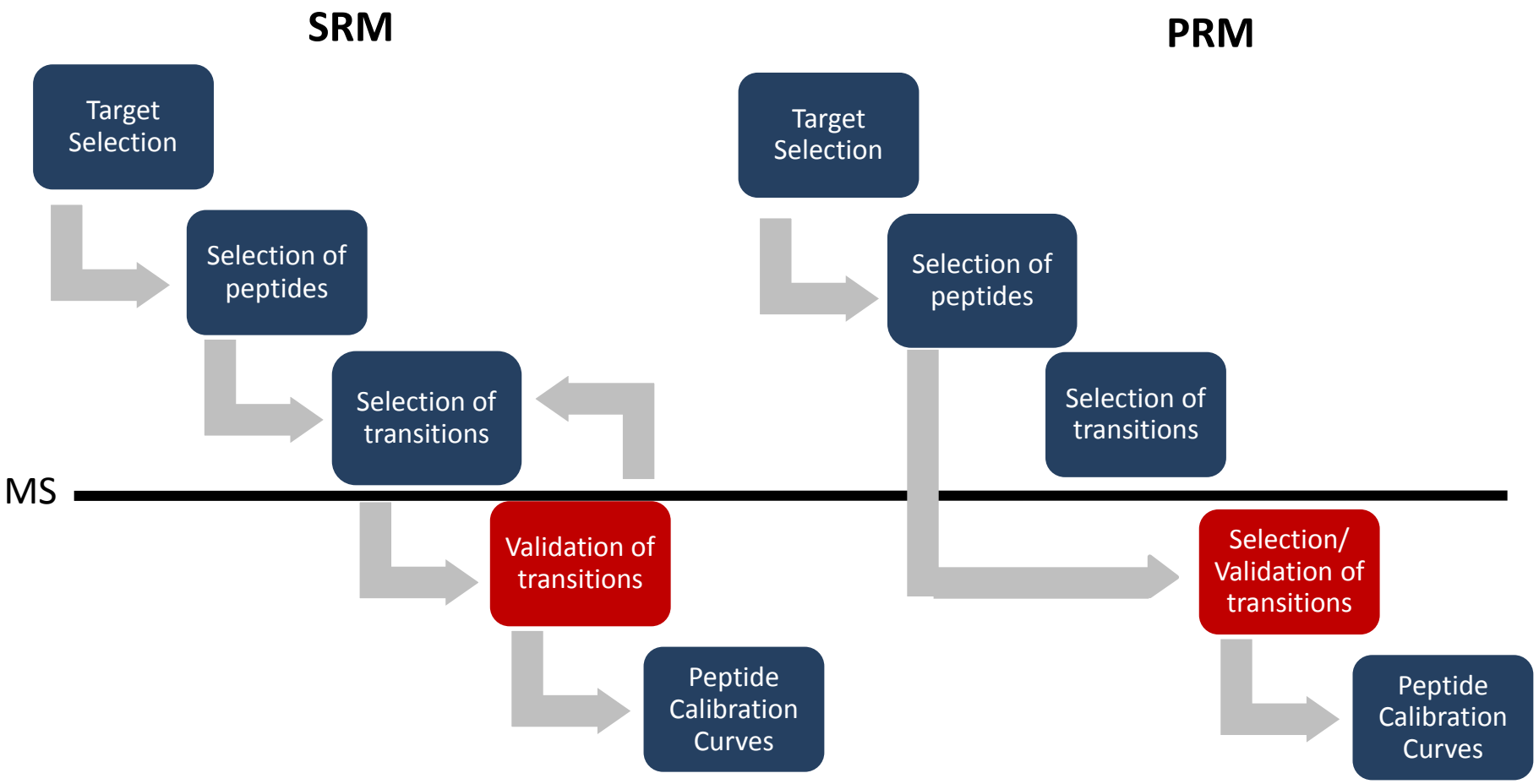
- Can use to find best transitions to pick
  - Intensity (rank)
  - Dot product (similarity to reference spectra)





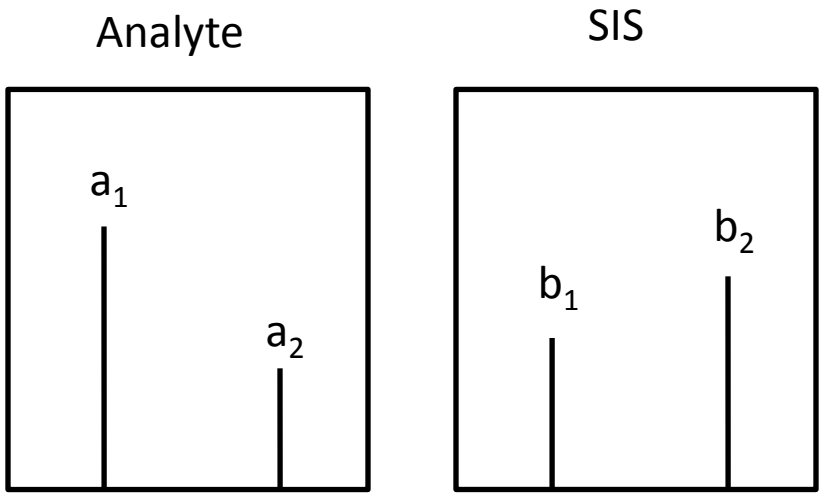
Want high rank and dotp close to 1

Workflow of MRM and PRM MS/MS

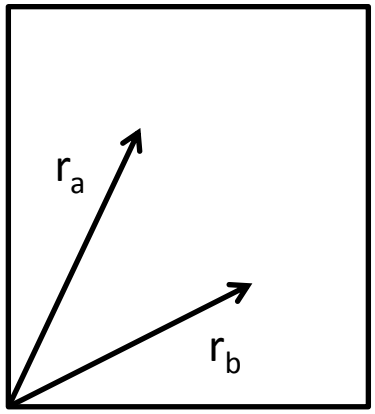


Validating Transitions: Contrast Angle

- Spectral Contrast Angle: each spectrum represented as a vector in N-dimensional space
- Spectra that resemble each other have vectors pointing in the same direction (θ ~ 0°)



$$cos\theta = \frac{\sum a_i b_i}{\sqrt{\sum {a_i}^2 \cdot \sum {b_i}^2}}$$



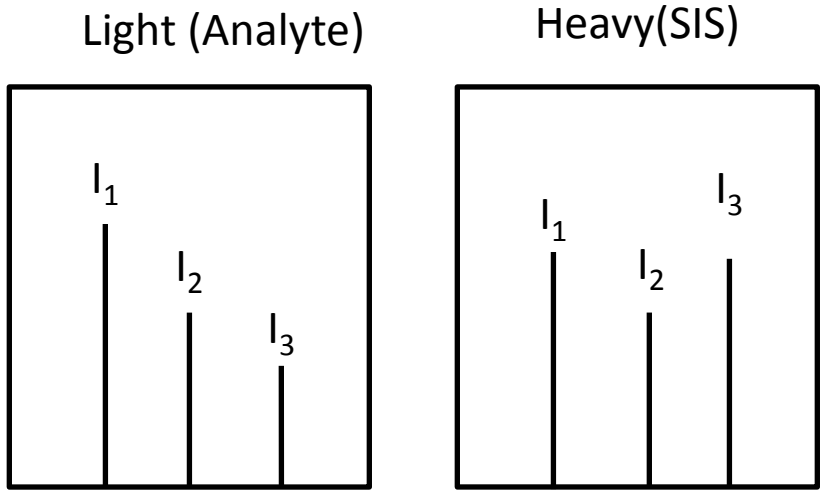
$$r_a = \sqrt{{a_i}^2}$$

$$r_b = \sqrt{{b_i}^2}$$

Validating Transitions: "Branching ratio"

Branching Ratio (BR): ratio of the peak intensities

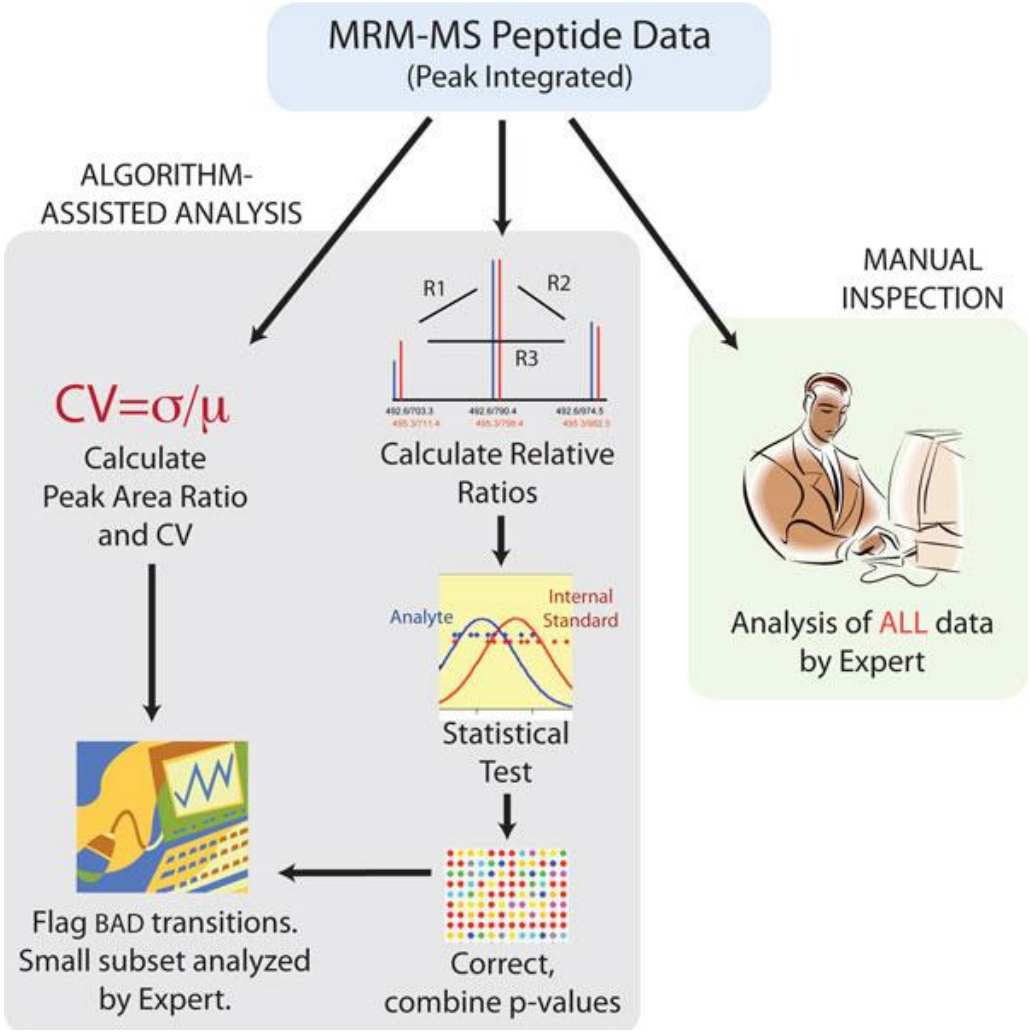
$$BR = ln\left\{\frac{\frac{I_{Ax}}{I_{Bx}}}{\frac{\sum\frac{I_{AxS}}{I_{BxS}}}{n}}\right\}$$



I_{Ax}, I_{Bx} : Peak areas of Analyte
I_{AxS}, I_{BxS} : Peak areas of SIS
n=number of SIS transitions

Kushnir, 2005

Validating Transitions in MRM: AuDIT

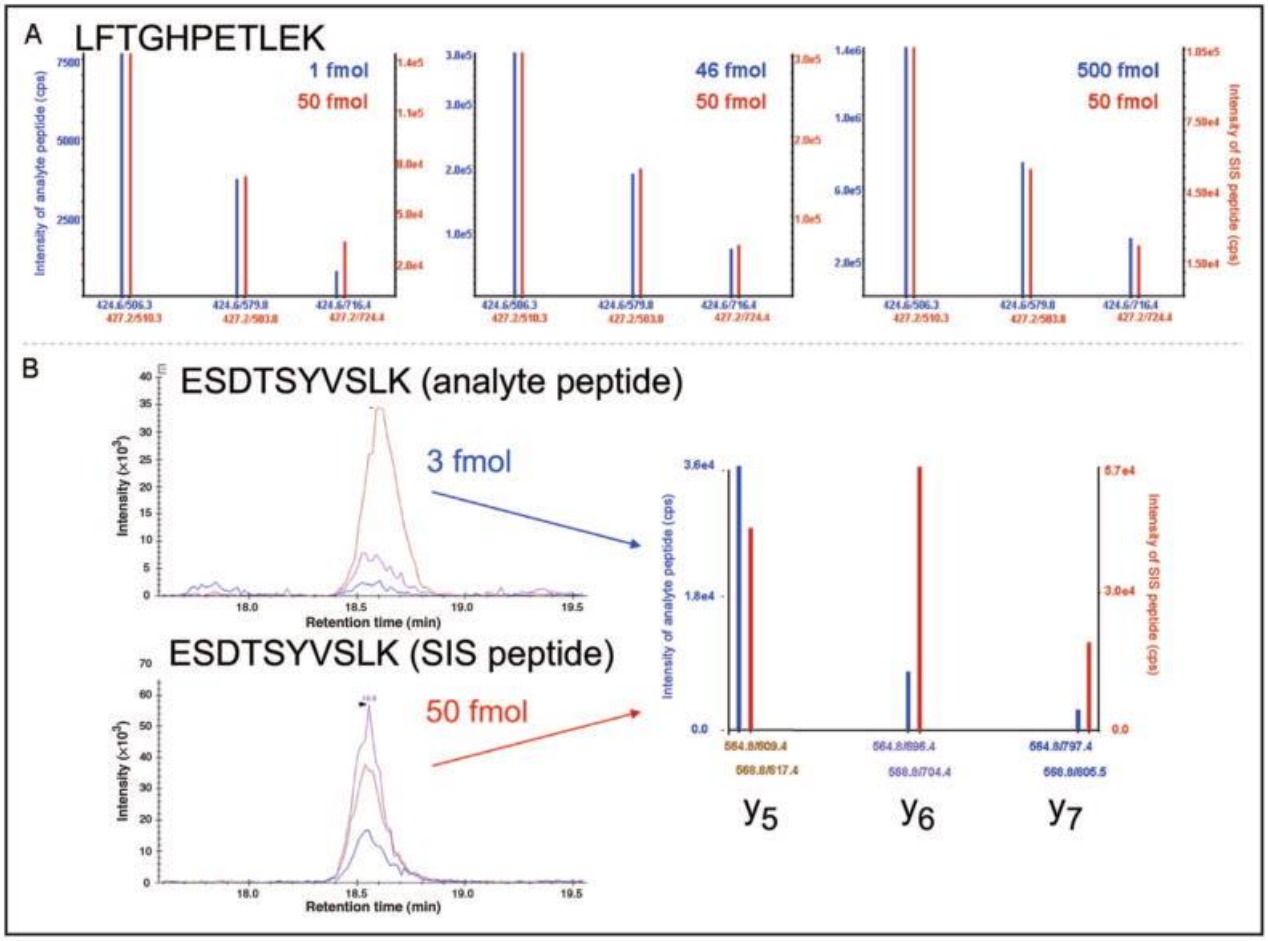


- AuDIT: Automated Detection of Inaccurate and imprecise Transitions
- Uses "branching ratio"

1. Calculate relative ratios of each transition from the same precursor
2. Apply t-test to determine if relative ratios of analyte are different from relative ratios of SIS

http://www.broadinstitute.org/cancer/software/genepattern/modules/AuDIT.html.

Validating Transitions in MRM: AuDIT



Blue: Light
Red: Heavy

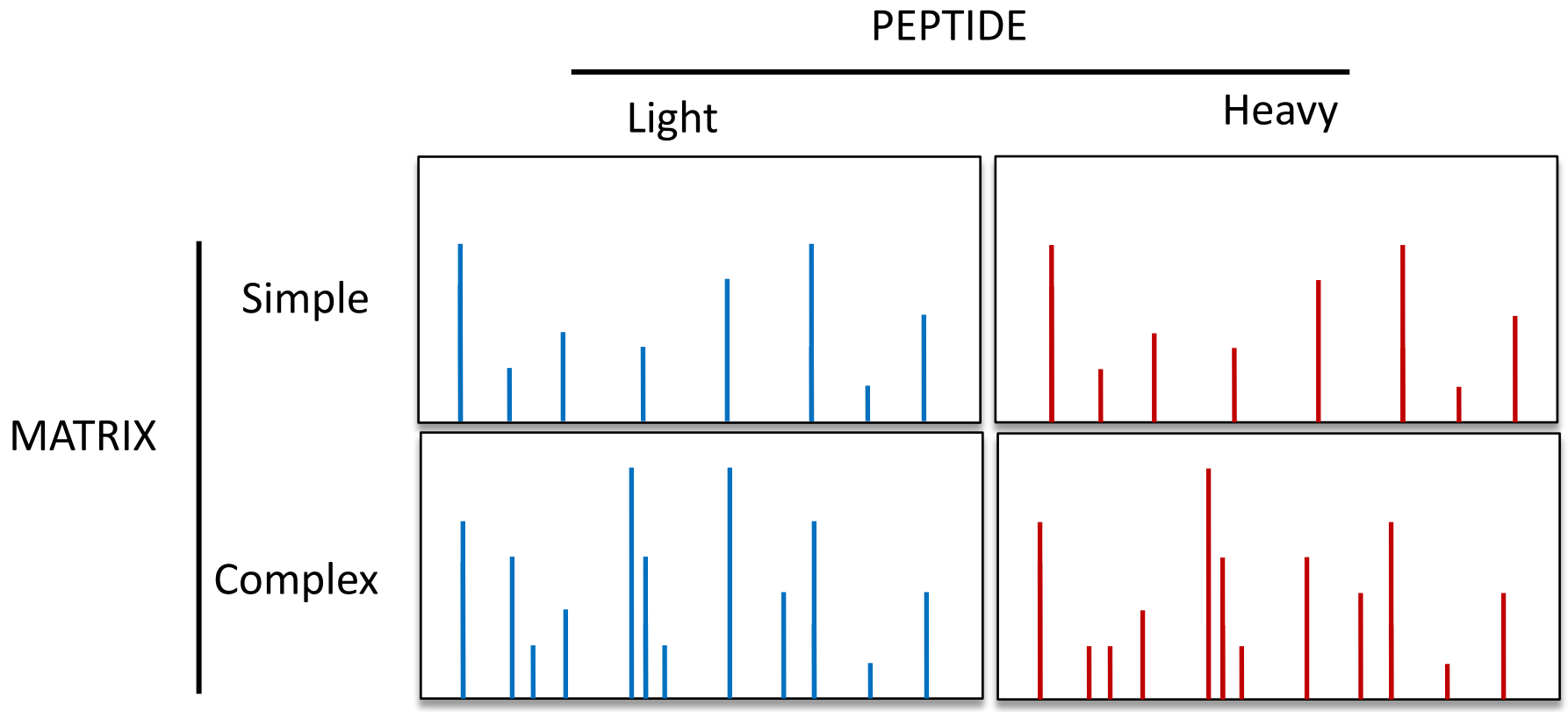
Relative product ions should have a constant relationship

Abbatiello, 2009

Validating Transitions in PRM: CRAFTS

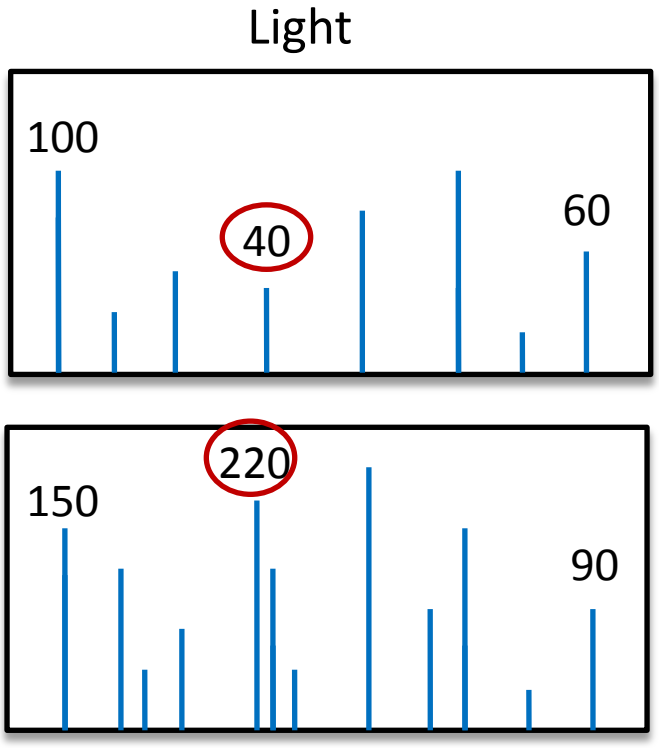
- PRM and MRM are most useful when quantifying protein in a complex matrix
  - Tumor lysate
  - Plasma
- Simple Matrix (buffer) should have no interferences
- Compare the transitions in complex to those in simple
- Ratio close to 1 indicates low interference

Validating Transitions in PRM: CRAFTS



- Simple matrix: peptide carrier solution
- Complex matrix: unfractionated tumor digest
- Simple matrix should have minimal interference- use this as reference
- Transitions in complex buffer should have the same relative intensities of transitions within the spectra
- Transitions in complex with relative intensities different from simple → interference

Validating Transitions in PRM: CRAFTS



Transition	Simple	Complex
y2	100	150
y5	40	220
y10	60	90

Ratio of Transitions

	y2	y5	y10
y2	1	y5/y2	y10/y2
y5	y2/y5	1	y10/y5
y10	y2/y10	y5/y10	1

Simple Matrix

	y2	y5	y10
y2	1	0.4	0.6
y5	2.5	1	1.5
y10	1.67	0.67	1

Complex Matrix

	y2	y5	y10
y2	1	**1.47**	0.6
y5	**0.68**	1	**0.41**
y10	1.67	**2.44**	1

Validating Transitions in PRM: CRAFTS

Ratio of Transitions

	y2	y5	y10
y2	1	y5/y2	y10/y2
y5	y2/y5	1	y10/y5
y10	y2/y10	y5/y10	1

Simple Matrix

	y2	y5	y10
y2	1	**0.4**	0.6
y5	**2.5**	1	**1.5**
y10	1.67	**0.67**	1

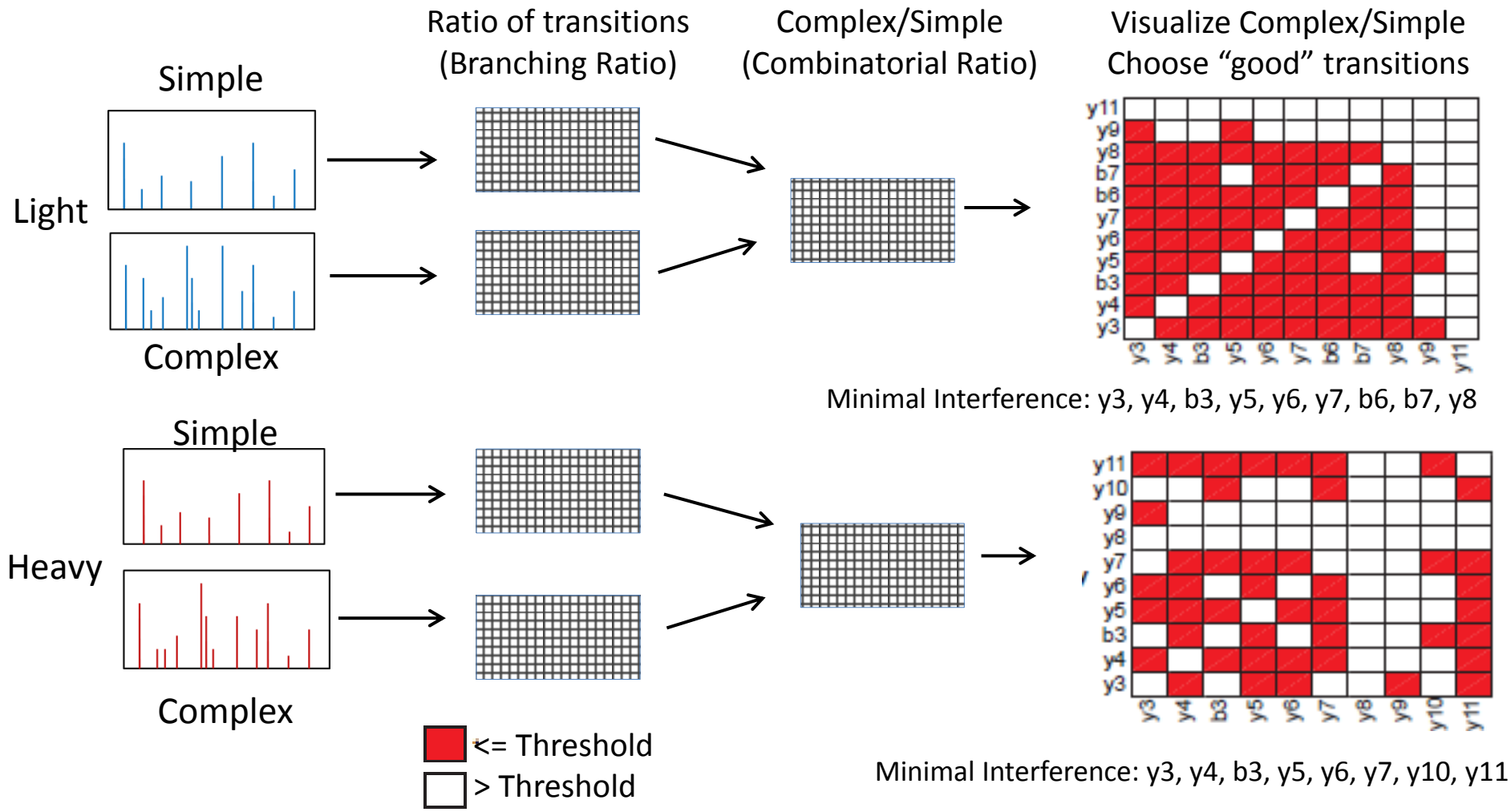
Complex Matrix

	y2	y5	y10
y2	1	**1.47**	0.6
y5	**0.68**	1	**0.41**
y10	1.67	**2.44**	1

Complex/Simple

	y2	y5	y10
y2	1	**3.675**	1
y5	**0.272**	1	**0.273**
y10	1	**3.641**	1

Validating Transitions in PRM: CRAFTS

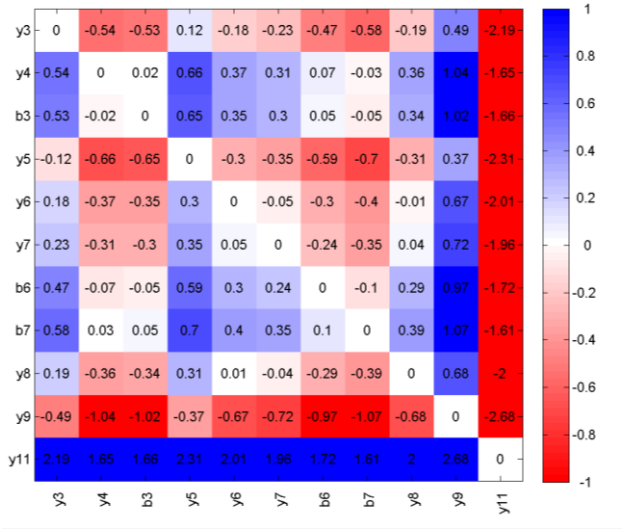


Minimal Interference: y3, y4, b3, y5, y6, y7, b6, b7, y8

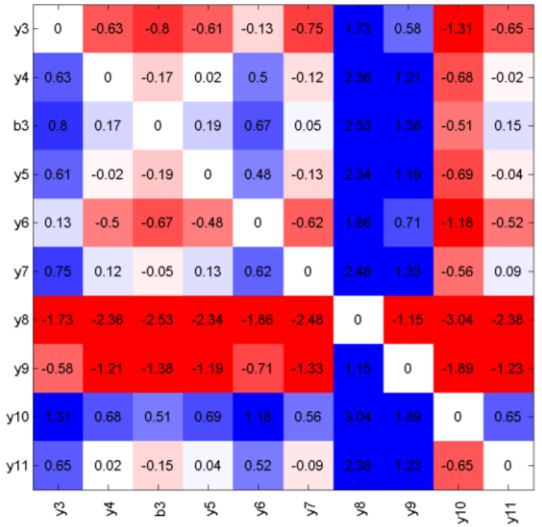
Minimal Interference: y3, y4, b3, y5, y6, y7, y10, y11

Validating Transitions in PRM: CRAFTS

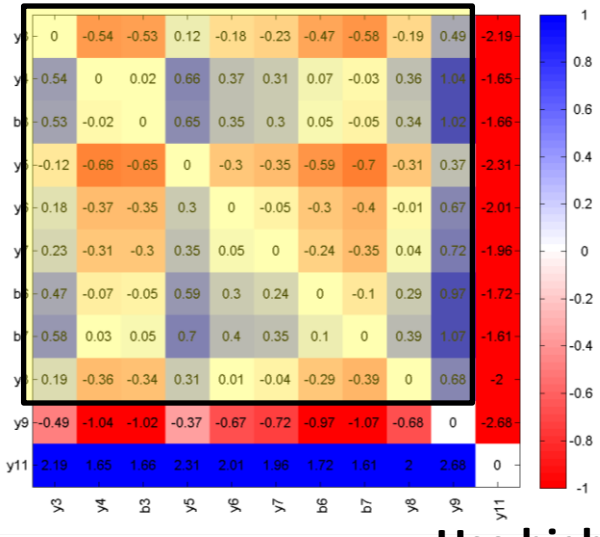
Light



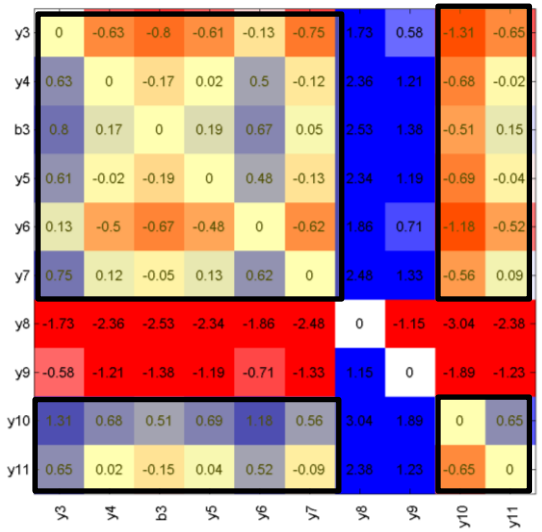
Heavy



Minimal Interference: y3, y4, b3, y5, y6, y7, b6, b7, y8
With Interference: y9, y11

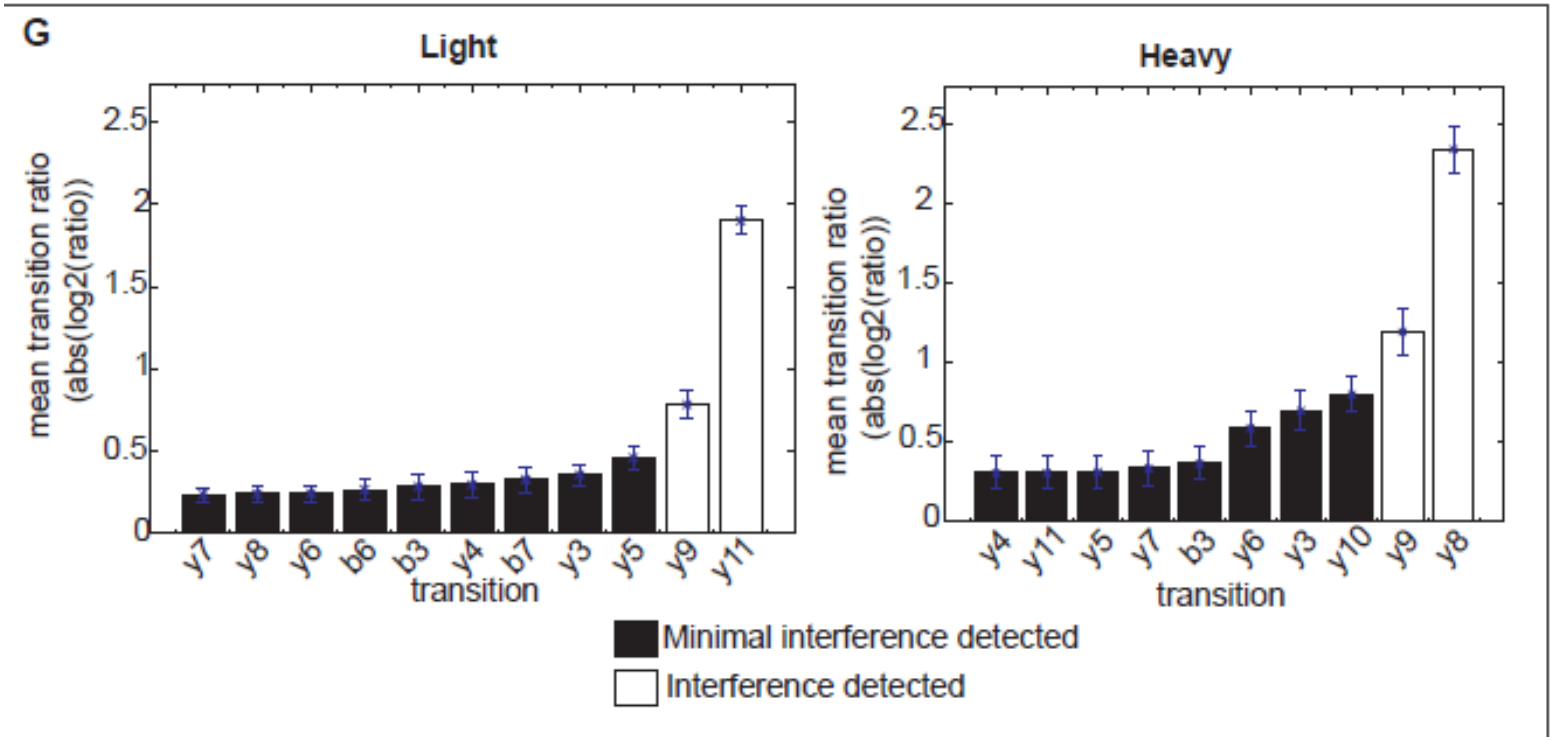


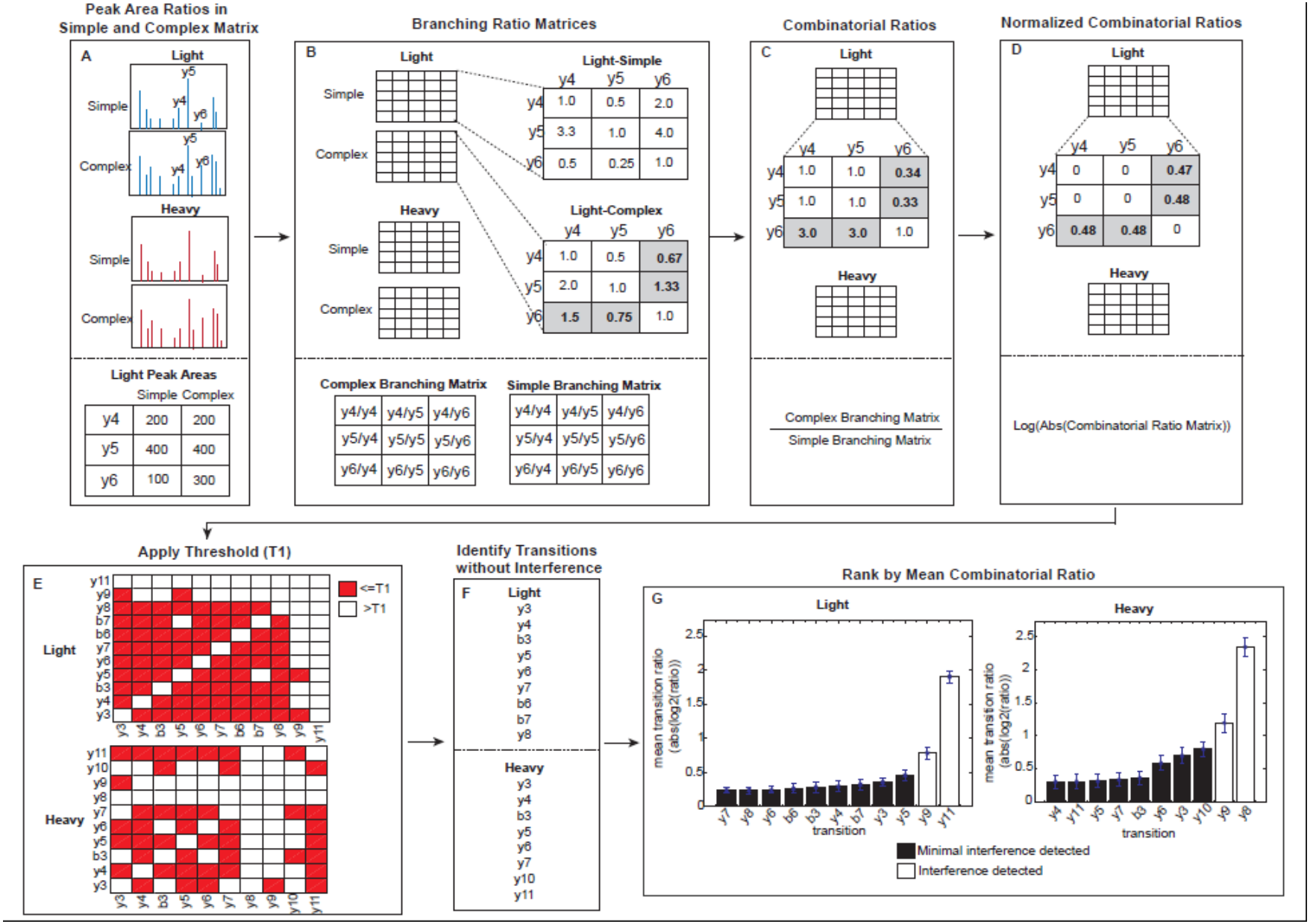
Minimal Interference: y3, y4, b3, y5, y6, y7, y10, y11
With Interference: y8, y9



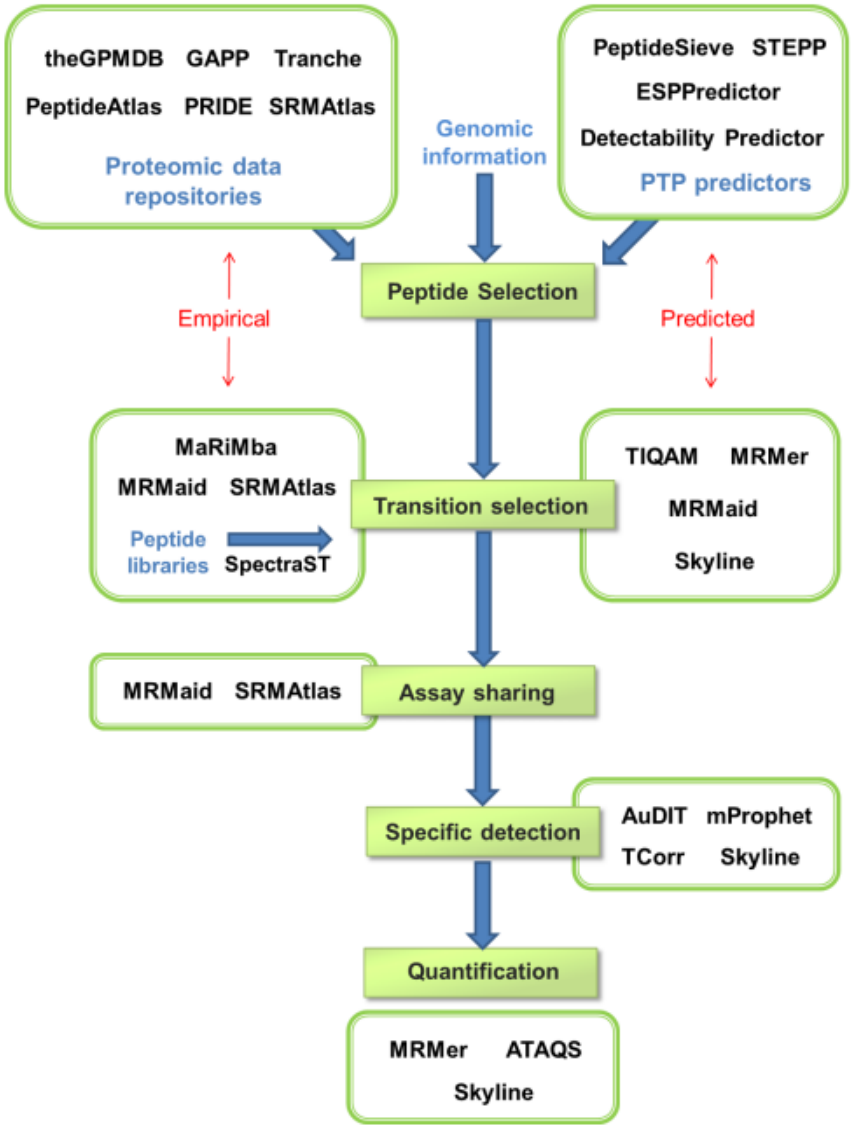
Use highlighted values to get mean ratio

CRAFTS: Ranking Transitions by Mean Combinatorial Ratio



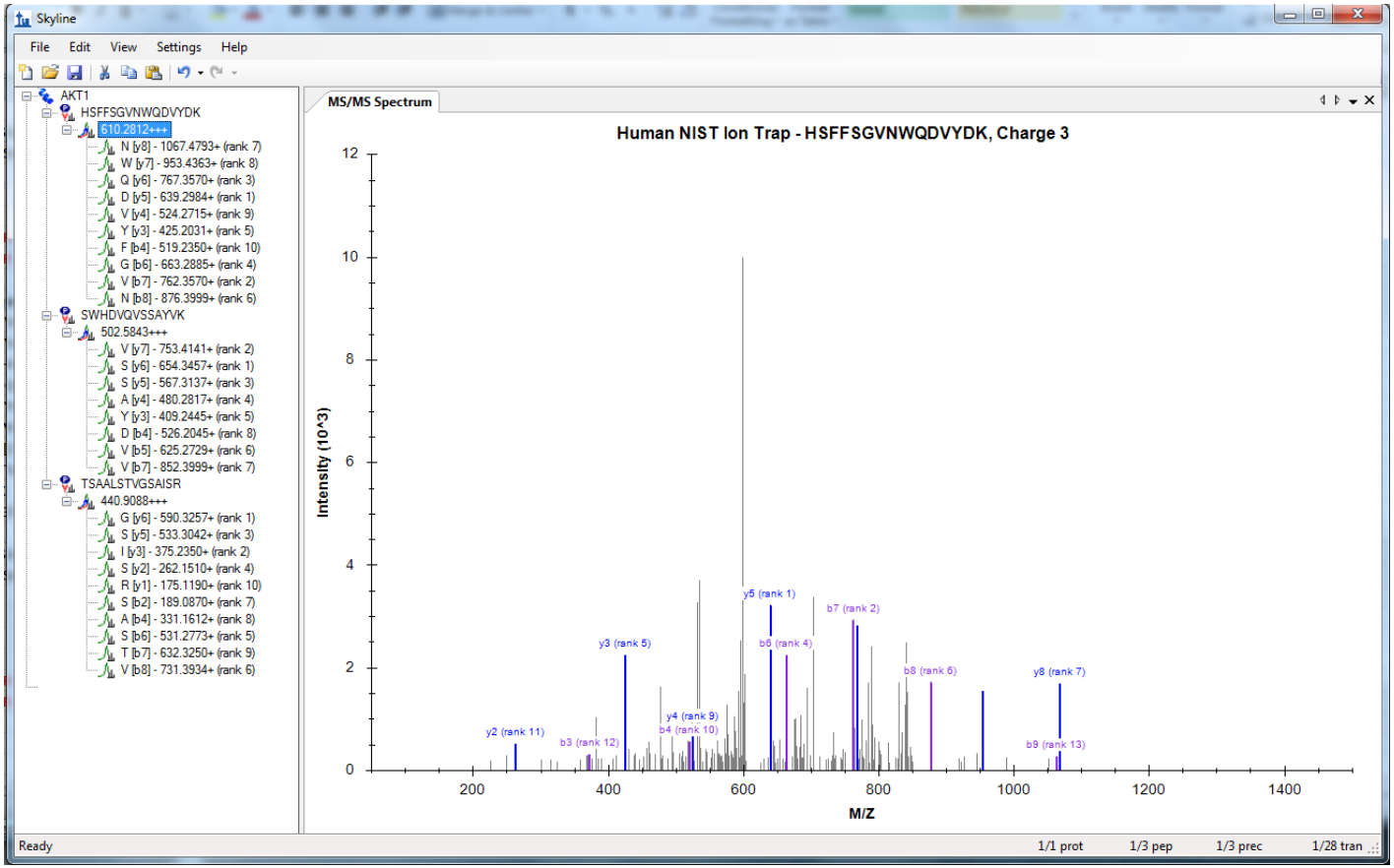

Peak Area Ratios in Simple and Complex Matrix
A
Light
Simple
Complex
Heavy
Simple
Complex
y5
y4
y6
Light Peak Areas
Simple Complex
y4 200 200
y5 400 400
y6 100 300
Branching Ratio Matrices
B
Light
Simple
Complex
Heavy
Simple
Complex
Light-Simple
y4 y5 y6
y4 1.0 0.5 2.0
y5 3.3 1.0 4.0
y6 0.5 0.25 1.0
Light-Complex
y4 y5 y6
y4 1.0 0.5 0.67
y5 2.0 1.0 1.33
y6 1.5 0.75 1.0
Complex Branching Matrix
y4/y4 y4/y5 y4/y6
y5/y4 y5/y5 y5/y6
y6/y4 y6/y5 y6/y6
Simple Branching Matrix
y4/y4 y4/y5 y4/y6
y5/y4 y5/y5 y5/y6
y6/y4 y6/y5 y6/y6
Combinatorial Ratios
C
Light
y4 y5 y6
y4 1.0 1.0 0.34
y5 1.0 1.0 0.33
y6 3.0 3.0 1.0
Heavy
Complex Branching Matrix
Simple Branching Matrix
Normalized Combinatorial Ratios
D
Light
y4 y5 y6
y4 0 0 0.47
y5 0 0 0.48
y6 0.48 0.48 0
Heavy
Log(Abs(Combinatorial Ratio Matrix))
Apply Threshold (T1)
E
Light
Heavy
<=T1
>T1
Identify Transitions without Interference
F
Light
y3
y4
b3
y5
y6
y7
b6
b7
y8
Heavy
y3
y4
b3
y5
y6
y7
y10
y11
Rank by Mean Combinatorial Ratio
G
Light
Heavy
mean transition ratio (abs(log2(ratio)))
transition
Minimal interference detected
Interference detected

Open Source MRM analysis tools

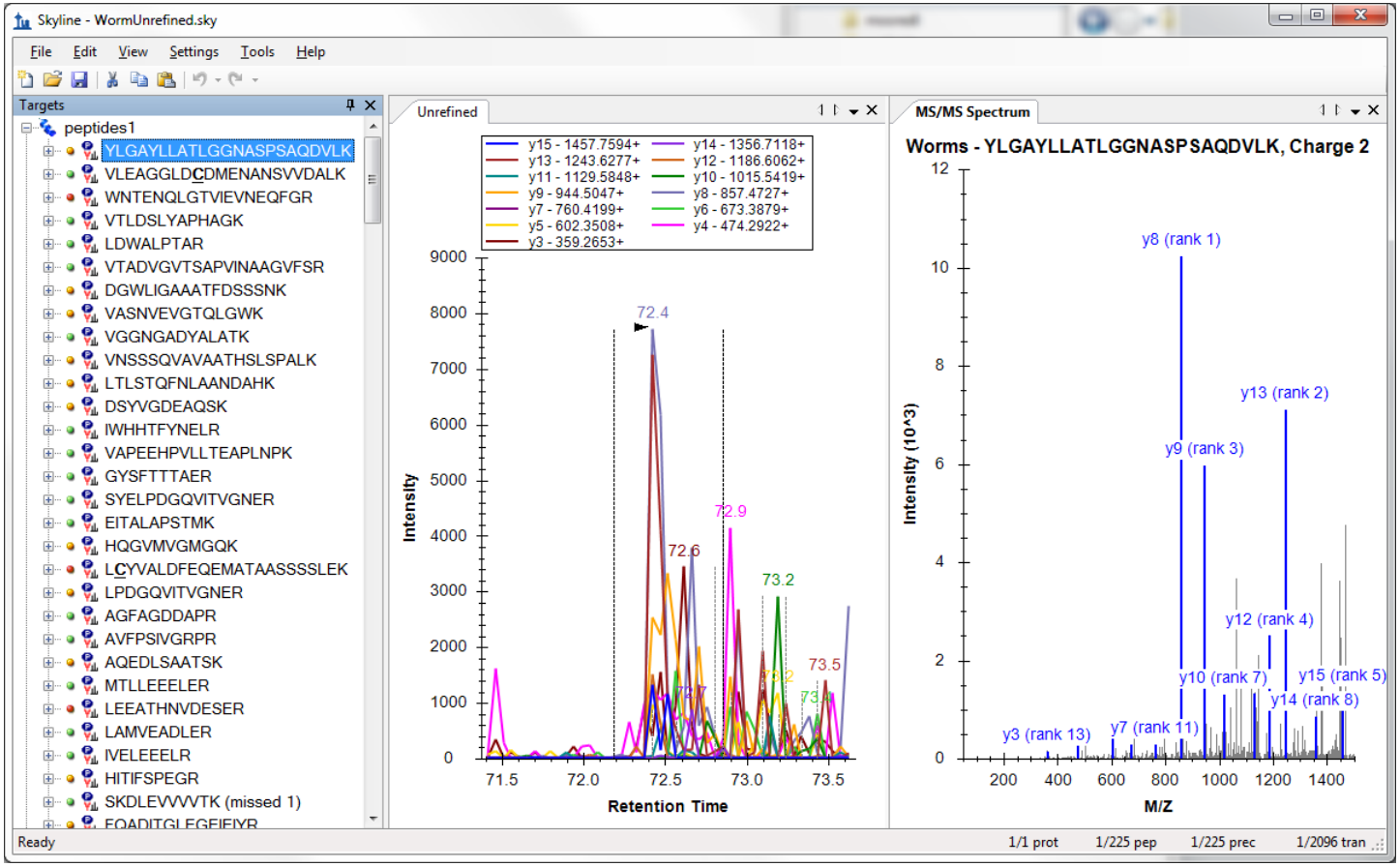


SKYLINE for creating targeted MS/MS methods

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



Skyline for MRM: Method Building



Input all peptides of interest

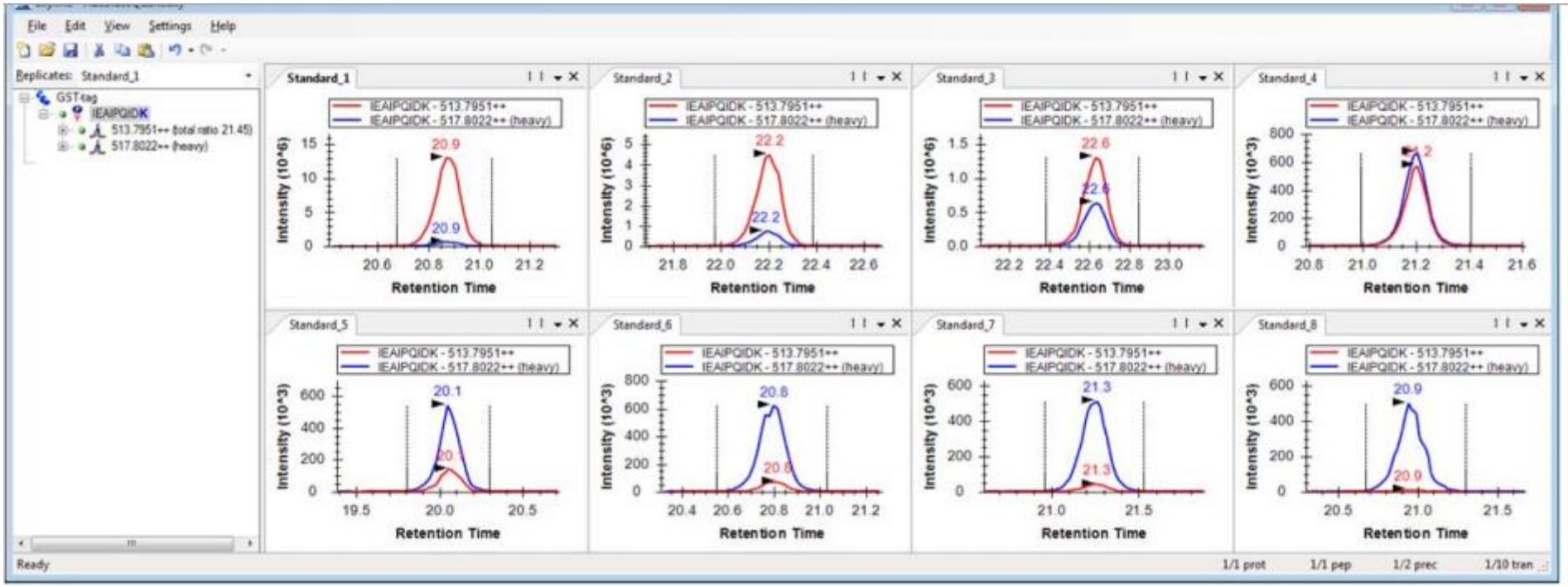
Shows graphs of MS/MS spectra from spectral library

Skyline for MRM: Method Building

- Helps generate protetypic 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 experiements

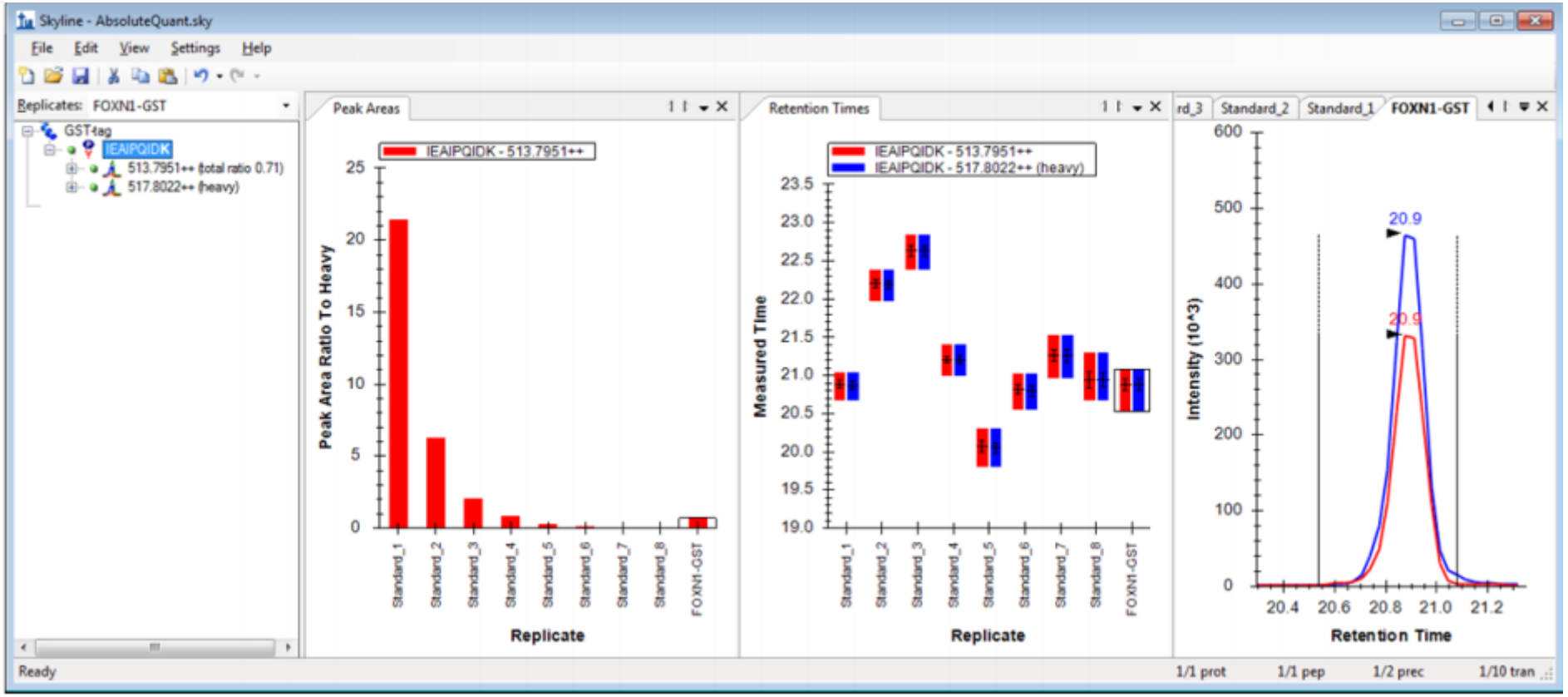
Skyline for MRM: Quantification

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



Skyline for MRM: Quantification

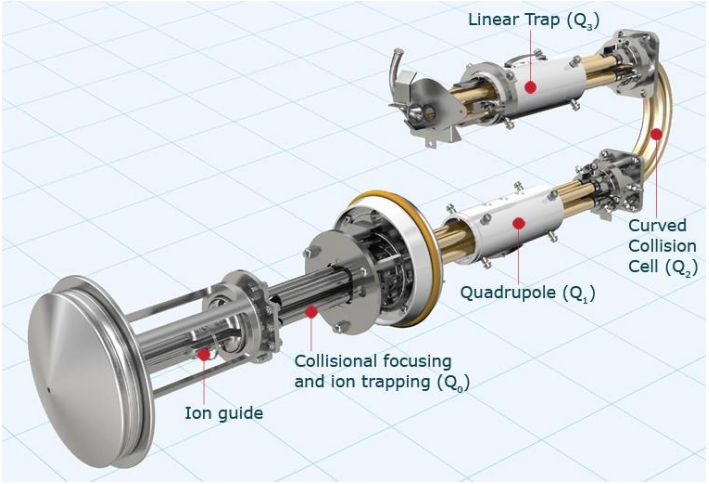
- Use the heavy standard PAR to make calibration curve
- Determine sample quantity based on curve



Questions?

MRM Instrumentation

Triple Quadrupole



Quadrupole Time-of-Flight (Qqtof)