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**
1. Records M/Z
2. Selects peptides based on abundance and fragments
3. Protein database search for peptide identification

**LC-MS**
- **Digestion**
- **Fractionation**
- **Lysis**

**Targeted MS**
1. Select precursor ion
2. Precursor fragmentation
3. Use Precursor-Fragment pairs for identification

Uses predefined set of peptides

**Data Dependent Acquisition (DDA)**
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

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

Analyte concentration = PAR \times \text{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 $\rightarrow$ Biomarker Discovery (<100 patients)
• Targeted proteomics $\rightarrow$ 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

Separation → MS analysis

• 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.

Separation → MS analysis
Enhancing MRM Sensitivity for Biomarker Discovery

Sample Enrichment

- Plasma (serum)
  - Fractionation
    - SCX
    - SEC
    - MCX
    - 1D-, 2D-SDS PAGE
    - Off-gel isoelectric focusing
  - Immunodepletion
    - IgY12/14
    - SuperMix
    - MARS hu7/14
  - Enrichment
    - Immunoaffinity (target proteins)
    - SISCAPA (target peptides)
    - IMAC, TiO₂ (phosphorylation)
    - Lectin affinity, hydrazine chemistry (glycosylation)

- LC-SRM


MRM3
Further fragments product ions
Reduces background

Meng Z and Veenstra TD, 2011
Define a set of proteins based on clinical/biological question
Motivating Example: AKT1 and Breast Cancer

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

**Step 1:** Full protein sequence in FASTA format

```
HPKHPPTPIQLNPAPDGSAVNGTSSAETNLEALQKEELLEELDEQRHRELAEFTQKQKV5ELKDDFEK
ISELSAGNGSVVFNYSVKHP5LGVMARKELLEIKRIRNQITRELQVHECN3PTFYGAFLYSEGEIS
ICHEKMDGSSLQVRKAEIR1QET1LQGSKV1TAVKGLTYLREKHKMRVDKPSNLVNSRGR1KLCDG
VSQLIDSMANSFYGRYSNSFERLQQGTHSYQSDWSMGLSLVENAVGRTPIPEPDAKEELLMFGCQVE
GDAEETFPRPRTFRSLSSYGNDSRPMAIHELDDYTVNEPPKLGSEVSLEFQDFVNRKCLIRNPAPAER
DLQILMVHAFIKRSDAEVEFAGKLGQTLIGNDTSTPTFAAISV
```

**Step 2:** Tryptic Peptides

```
PTPIQLNPAPDGSAVNGTSSAETNLEALQK
LEAFLTQK
PSNIVLNVNR
LEELELDEQQQR
DDDFEK.....
```

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

**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
- Most proteins show very reproducible peptide patterns
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
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.
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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
Selecting Transitions: Skyline

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

[Image of peptide sequences with intensity and dot product values]

Want high rank and dotp close to 1
Workflow of MRM and PRM MS/MS

SRM
- Target Selection
- Selection of peptides
- Selection of transitions
- Validation of transitions
- Peptide Calibration Curves

PRM
- Target Selection
- Selection of peptides
- Selection of transitions
- Selection/Validation of transitions
- Peptide Calibration Curves
Validating Transitions: Contrast Angle

- Spectral Contrast Angle: each spectrum represented as a vector in \( \mathbb{N} \)-dimensional space
- Spectra that resemble each other have vectors pointing in the same direction (\( \theta \sim 0^\circ \))

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

\[
r_a = \sqrt{a_i^2}
\]

\[
r_b = \sqrt{b_i^2}
\]
Validating Transitions: “Branching ratio”

Branching Ratio (BR): ratio of the peak intensities

\[
BR = \ln \left( \frac{\frac{I_{Ax}}{I_{Bx}}}{\frac{\sum I_{AxS}}{I_{BxS}n}} \right)
\]

- \(I_{Ax}, I_{Bx}\): 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

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

MATRIX

PEPTIDE

Light

Heavy

Simple

Complex

- 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

Light

Simple

Complex

Ratio of Transitions

<table>
<thead>
<tr>
<th></th>
<th>y2</th>
<th>y5</th>
<th>y10</th>
</tr>
</thead>
<tbody>
<tr>
<td>y2</td>
<td>1</td>
<td>y5/y2</td>
<td>y10/y2</td>
</tr>
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<tr>
<td>y10</td>
<td>y2/y10</td>
<td>y5/y10</td>
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Simple Matrix

<table>
<thead>
<tr>
<th></th>
<th>y2</th>
<th>y5</th>
<th>y10</th>
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</thead>
<tbody>
<tr>
<td>y2</td>
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<td>0.6</td>
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<tr>
<td>y5</td>
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<tr>
<td>y10</td>
<td>1.67</td>
<td>0.67</td>
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</table>

Complex Matrix

<table>
<thead>
<tr>
<th></th>
<th>y2</th>
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</thead>
<tbody>
<tr>
<td>y2</td>
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<td>0.6</td>
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<tr>
<td>y5</td>
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<tr>
<td>y10</td>
<td>1.67</td>
<td>2.44</td>
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Transition | Simple | Complex
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>y2</td>
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<td>150</td>
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<tr>
<td>y5</td>
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<td>220</td>
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<tr>
<td>y10</td>
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### Validating Transitions in PRM: CRAFTS

#### Ratio of Transitions

<table>
<thead>
<tr>
<th></th>
<th>(y_2)</th>
<th>(y_5)</th>
<th>(y_{10})</th>
</tr>
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<tbody>
<tr>
<td>(y_2)</td>
<td>1</td>
<td>(y_5/y_2)</td>
<td>(y_{10}/y_2)</td>
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<tr>
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<td>(y_{10}/y_5)</td>
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<tr>
<td>(y_{10})</td>
<td>(y_2/y_{10})</td>
<td>(y_5/y_{10})</td>
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#### Simple Matrix

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<td>1</td>
<td>1.5</td>
</tr>
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<td>0.67</td>
<td>1</td>
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#### Complex Matrix

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<td>1</td>
<td>0.41</td>
</tr>
<tr>
<td>(y_{10})</td>
<td>1.67</td>
<td>2.44</td>
<td>1</td>
</tr>
</tbody>
</table>

#### Complex/Simple

<table>
<thead>
<tr>
<th></th>
<th>(y_2)</th>
<th>(y_5)</th>
<th>(y_{10})</th>
</tr>
</thead>
<tbody>
<tr>
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<td>3.675</td>
<td>1</td>
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<tr>
<td>(y_5)</td>
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<td>1</td>
<td>0.273</td>
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<tr>
<td>(y_{10})</td>
<td>1</td>
<td>3.641</td>
<td>1</td>
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</tbody>
</table>
Validating Transitions in PRM: CRAFTS

Light

- Simple
- Complex

Heavy

- Simple
- Complex

Ratio of transitions (Branching Ratio)

Complex/Simple (Combinatorial Ratio)

Visualize Complex/Simple
Choose “good” transitions

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

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

\[ \leq \text{Threshold} \]

\[ > \text{Threshold} \]
Validating Transitions in PRM: CRAFTS

Light

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

Heavy

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
Open Source MRM analysis tools

theGPMDB  GAPP  Tranche
PeptideAtlas  PRIDE  SRMAAtlas
Proteomic data repositories

Genomic information

Peptide Selection

PeptideSieve  STEPP
ESPPredictor
Detectability Predictor
PTP predictors

MaRiMba
MRMaid  SRMAAtlas
Peptide libraries
SpectraST

Transition selection

TiQAM  MRMer
MRMaid
Skyline

MRMaid  SRMAAtlas

Assay sharing

Specific detection

AuDiT  mProphet
TCorr  Skyline

Quantification

MRMmer  ATAQS
Skyline
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 prototypic 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
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)