LC-MS approaches for quantification of protein biomarkers

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Outlines

• Methodological overview
  • LBAs vs LC-MS
  • LC-MS: Targeted approaches vs untargeted approaches
• Analytical considerations for targeted quantification (protein biomarkers)
  • Sensitivity
  • Specificity
  • Accuracy
• Case: Quantification of mitochondrial protein frataxin by LC-HRAM/MS
# LBAs vs LC-MS assays for analysis of protein biomarkers

<table>
<thead>
<tr>
<th>Enzyme-linked immunosorbent assay (ELISA)</th>
<th>Stable isotope dilution liquid chromatography-mass spectrometry (SID-LC/MS) assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>High throughput</td>
<td>Low throughput</td>
</tr>
<tr>
<td>High sensitivity</td>
<td>High sensitivity with very expensive instrumentation</td>
</tr>
<tr>
<td>Inexpensive</td>
<td>Expensive</td>
</tr>
<tr>
<td>No proteolytic digestion</td>
<td>Proteolytic digestion</td>
</tr>
<tr>
<td>Easy sample pretreatment</td>
<td>Intensive sample pretreatment</td>
</tr>
<tr>
<td>Mono-specific (antigen-antibody recognition)</td>
<td>Multi-specific (affinity, retention time, correct mass)</td>
</tr>
<tr>
<td>Potential for cross-reactivity with other proteins</td>
<td>Cross-reactivity not relevant</td>
</tr>
<tr>
<td>Sensitive to antibody affinity</td>
<td>Affinity not relevant in the MS analysis</td>
</tr>
<tr>
<td>Analyst-dependent</td>
<td>Instrument-dependent</td>
</tr>
</tbody>
</table>
Targeted vs Untargeted Approaches

• Targeted approaches
  • Gold standard method (stable isotope dilution (SID)-LC-MS/MS)
  • Can be exquisitely sensitive and specific
  • Must know exactly the molecule of interest
  • Loss of information

• Untargeted approaches
  • Only need a general sense of target chemistry
  • Capture of a wider snapshot of metabolism
  • Less sensitive, less specific
  • High noise, data intensive
Analytical considerations for targeted quantification

Analytical consideration 1 -- Sensitivity

Analytical consideration 2 -- Specificity

Analytical consideration 3 -- Accuracy

Wang Q, Blair IA, Targeted biomarker quantitation by LC-MS (Wiley), 2016, Chapter 1.6
Analytical consideration 1 --Sensitivity

Analytical consideration 2 --Specificity

Analytical consideration 3-- Accuracy
Complexity of proteins present in plasma

Sample preparation strategies to improve sensitivity

• Removing high abundance proteins
  - Solvent precipitation: acetonitrile, methanol etc.
  - Immunoaffinity:
    - Seppro IgY 12/14 -- highly abundant proteins
    - SuperMix LC5 -- Moderately abundant proteins
    - Sepprro IgY + SumperMix (removing about 155 plasma proteins, Jones KA, Kim PD, Patel BB. J Proteome Res., 2013)

• Immunoaffinity enrichment
  - Protein level: immunoprecipitation
  - Peptide level: Stable Isotope standards and capture by antipeptide antibodies (SISCAPA)
Analytical consideration 1 -- Sensitivity

Analytical consideration 2 -- Specificity

Analytical consideration 3 -- Accuracy
Protein biomarker is not homogenous single molecule

- Protein biomarker is heterogeneity
  - Post-translational modification—glycosylation, phosphorylation, oxidation, deamidation, acetylation etc.
  - Chemically induced modifications – Cysteine 胱氨酸, Methionine 蛋氨酸, Tyrosine 酪氨酸 etc.
- Measurand – concentration + molecular identity

- Multi-specificity in bottom-up approach by LC-MRM/MS analysis
  - Immunoaffinity (if IP is employed)
  - Retention time
  - Precursor ions
  - Product ions
Specificity is an Issue ..... 

"One train can hide another one!"
Check twice!

Expected

Observed
Parallel Reaction Monitoring (PRM)

Q-Orbitrap instrument with PRM mode

Fingerprint to confirm the analyte identity

Chrom. traces for quantification

Parallel Reaction Monitoring (PRM)

Analytical consideration 1 -- Sensitivity

Analytical consideration 2 -- Specificity

Analytical consideration 3 -- Accuracy
Workflow of bottom-up protein analysis

- Biological samples (Urine, plasma/serum, CSF etc)
- Sample preparation for enrichment or any other purpose at protein level
- Enzymatic digestion
- Sample enrichment or preparation at peptide level
- LC-MRM/MS analysis
Introduction points of different INSTDs for quantification of protein biomarkers

- Biological samples (Urine, plasma/serum, CSF etc)
- Sample preparation for enrichment or any other purpose at protein level
- Enzymatic digestion
- Sample enrichment or preparation at peptide level
- LC-MRM/MS analysis

- Isotope-labeled recombinant standards (PSAQ, 2005 by Burn group)
- Quantification concatemer (QconCAT, 2005 by Beynon group)
- Absolute quantification peptide (AQUA, 2003 Gerber group)
AUQA peptides: synthesized peptides, are normally introduced to the protein sample after proteolytic digestion.

<table>
<thead>
<tr>
<th></th>
<th>AQUA Ultimate</th>
<th>AQUA QuantPro</th>
<th>AQUA Basic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formulation</td>
<td>5 pmol/µL in 5% (V/V) acetonitrile/H2O</td>
<td>5 pmol/µL in 5% (V/V) acetonitrile/H2O</td>
<td>Lyophilized</td>
</tr>
<tr>
<td>Concentration precision</td>
<td>± 5%</td>
<td>± 25%</td>
<td>NA</td>
</tr>
<tr>
<td>Isotopic enrichment</td>
<td>&gt; 97%</td>
<td>&gt; 97%</td>
<td>&gt; 95%</td>
</tr>
<tr>
<td>Peptide length</td>
<td>Up to 15 AAs</td>
<td>Up to 15 AAs</td>
<td>Up to 15 AAs</td>
</tr>
</tbody>
</table>
Assay variation when using AQUA peptides as internal standards.

*Pooled serum containing 10 human sera was processed with 5 replicates. Peak area ratios of peptides generated from serum ApoA1 (L) to corresponding AQUA peptides (H).

Quantification concatamer 多肽联合体: artificial proteins, are normally introduced to the protein sample before proteolytic digestion.

Spike-in SILAC: Stable isotope labeled protein is used as an INSTD for protein quantification. SILAC INSTDs are normally spiked into samples at beginning of sample preparation.

Development and optimization of a LC-MRM/MS assay

- Recombinant protein
- SILAC protein
- AUQA peptides

- Surrogate matrix
- Surrogate analyte
- Cohort screening

- Protein sequences

- Library: LC-MS spectral library
  Peptide Atlas
  iRT library

- Optimization and refinement (3-5 cycles)
  - Best peptides
  - LC gradient
  - Scheduling/multiplexing
  - Ion transitions
  - 3 peptides/pro
  - 3 ion trns/pep

- Initial LC-MRM assay
  Exhaustive list
  Proteins/peptides

- Validation with surrogate matrix/analyte

- Clinical samples
Quantification of mitochondrial protein, frataxin by LC-HRAM/MS

Wang Q, Blair IA et al. Candidate biomarkers to monitor the efficacy of new approaches for Friedreich’s ataxia treatment, Future Science OA, 2016
Friedreich’s ataxia (FRDA 共济失调)

- Genetically inherited
  - An autosomal recessive neurodegenerative disorder (染色体退行性神经性疾病)
  - Estimated prevalence is 1 in 50,000

- Most common inherited ataxia
  - Patients are often wheelchair bound by 10-15 years of age
  - Cardiomyopathy 心肌症 as the common cause of death

- Nucleotide triplicate expansion on FXN mRNA
  - Correlates with disease severity—Biomarker

- Deficiency in the protein frataxin—Biomarker
  - Involved in the assembly of iron-sulfur clusters
  - Mitochondrial iron accumulation, dysfunction of respiratory chain and electron transfer chain, oxidative stress (氧化应激)
Potential problems of analysis of frataxin

1. The average frataxin concentration has been found was 12 ng/mL in red blood cells, 3 pg/μg in PBMC cell lysate and 1 pg/μg in platelet cell lysate;

2. It requires sensitivity of <1 fmol on column by LC-MS system;

3. Need to distinguish four forms
Human platelets, PBMCs, or fibroblasts cell lysates

Magnetic beads-protein A/G-frataxin Ab

E. coli, SILAC medium: \([^{13}\text{C}_6,^{15}\text{N}_2]\)-lysine and \([^{13}\text{C}_6,^{15}\text{N}_1]\)-Leucine

LC-HRMS analysis

Frataxin

SILAC-labeled frataxin

Retention Time

Intensity (10^6)

18
16
14
12
10
8
6
4
2
0

Red

Heavy

15.9

Light

15.9

0

12
14
16
18

Human platelets, PBMCs, or fibroblasts cell lysates

Magnetic beads-protein A/G-frataxin Ab

DDT/Iodoacetamide

Trypsin

Heavy (labeled) peptides

Light (unlabeled) peptides

Dynabeads Protein A/G

Bind FXN antibody

Immunoprecipitation on frataxin (light/heavy, full length, intermediate, and mature forms)

On beads reducing

NuPAGE® Novex® Bis-Tris gels separation

Western blot

Immunoprecipitation

Further purification

On beads digestion

C18 spin column

Immunoprecipitation on frataxin (light/heavy, full length, intermediate, and mature forms)
LTQ Orbitrap-XL Hybrid

Resolution: 60,000 @ m/z 200
Mass range: 50 to 2,000 m/z
Mass accuracy: 0.002 Da at m/z 200
Scan mode: Positive or negative

Q-Exactive HF Hybrid Quadrupole-Orbitrap

Resolution: 240,000 @ m/z 200
Mass range: 50 to 6,000 m/z
Mass accuracy: 0.001 Da at m/z 200
Scan mode: Sequential positive and negative
Comparison: Orbitrap-XL and Q-Exactive HF

**Orbitrap-XL**

- **IS**: 32.7
- **MS**: 34.1
- **MS2**: 367.33
- **MS2**: 367.32
- **Relative Abundance**: 6.78E7

**Q-Exactive HF**

- **IS**: 34.3
- **MS**: 39.3
- **MS2**: 367.33
- **Relative Abundance**: 6.78E7

**m/z**

- **Orbitrap-XL**: 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100
- **Q-Exactive HF**: 50, 100, 150, 200, 250, 300, 350

**Relative Abundance**

- **Orbitrap-XL**: 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100
- **Q-Exactive HF**: 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100

**MS2**

- **Orbitrap-XL**: 367.33
- **Q-Exactive HF**: 367.3356

**MH+**

- **Orbitrap-XL**: 367.3363
- **Q-Exactive HF**: 367.3356
SIM and PRM on QE—Quan and Qual
Representative chromatograms
0.5 ug (23.8 pmol) STD frataxin, 10 µL (around 50 fmoL) INSTD in platelets lysate (total protein: 50 µg)

- m/z 607.287, 1.44E8, m/z 643.841, 7.18E7, m/z 625.343, 3.55E8
- m/z 611.965, 3.75E5, m/z 651.356, 2.42E5, m/z 651.356, 5.60E5

Peptides:
- S^81^GTLGHPGSLD ETTYER^3^+
- Q^{153}WLSSPSSGP^K^2^+
- L^{136}GDLGTYVINK^2^+
- S^81^GTLGHPGSLD ETTYER^3^+
- Q^{153}WLSSPSSGP^K^2^+
- L^{136}GDLGTYVINK^2^+
Summary

• Stable isotope dilution (SID) -LC-MRM/MS is a gold standard for quantification of protein biomarkers in biological matrices.

• High-resolution accurate mass (HRAM) quantification is an alternative to conventional LC-MRM/MS.

• Assay sensitivity, specificity and accuracy are the key factors in the method development and optimization.

• INSTD plays a critical role in the absolute quantification.

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THANK YOU FOR THE INSPIRATION, DRIVE, SUPPORT, PATIENCE, LOVE... EVERYTHING!
Backup slides
Targeted Approach: multiple reaction monitoring (MRM) mode on LC-MS/MS

Add Internal Standard → Sample preparation → Proteolytic digestion → Liquid Chromatography → Mass Spectrometry

Q1 → Q2 → Q3

Analyte (ng) → Ratio (Analyte / ISTD)

Time → m/z

Analyte

Internal Standard
Classical SILAC
