Quantitation of Proteins and Monoclonal Antibodies In Serum by LC-MS/MS Using Full-Length Stable Isotope Labeled Internal Standards

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MilliporeSigma
Outline

• Why Quantitative MS vs ELISA

• Quantitative MS workflow

• Why SIL protein as an internal standards

• Expression and characterization of SIL proteins and antibodies

• Quantitative MS assays using SIL proteins and antibodies
Serum Protein Measurement Methods

**ELISA / LBA**

**Pros:**
- High sensitivity
- High throughput
- Traditional methodology
- Minimal sample prep

**Cons:**
- Assay specific reagents
  - Long lead times
  - Poor standardization
- Specificity concerns
- Difficult to multiplex

**LC-MS/MS**

**Pros:**
- Highly selective
- Faster assay development
- Ability to multiplex
- Can be combined with immunoaffinity enrichment

**Cons:**
- Expensive instrumentation
- Extensive sample prep
- Requires an internal standard
General Design of an Internal Standard

- Not be present in any of the samples
- Similar in physiochemical properties to the target analyte
- Added as early on in the procedure as possible
  - recovery during transfer and clean-up
  - variability in extraction efficiency
  - injection volume variability
  - matrix suppression
- for LC-MS, preferably an isotopically labeled version of the analyte (SIL)
Typical Quantitative MS Workflow

Sample Preparation
- Dissolution/Denaturation

Protein Extract

Digestion
- Enzymatic
- Chemical

Peptide Digest

Protein Fractionation
- 1D or 2D Gels
- Abundant Protein Depletion
- Antibody Enrichment

Peptide Fractionation
- Anti-peptide antibodies (SISCAPA)
- Cation-exchange LC

Add SIL peptide

LC-MS

QQQ Mass Spectrometer

SIL peptides are typically added late in the workflow
Protein Internal Standard Workflow

Sample Preparation
Dissolution/Denaturation

Protein Fractionation
• 1D or 2D Gels
• Abundant Protein Depletion
• Antibody Enrichment

Protein Extract

Digestion
Enzymatic Chemical

Peptide Digest

LC-MS

Peptide Fractionation
• Anti-peptide antibodies (SISCAPA)
• Cation-exchange LC

Add SILuMab or SIL Protein

SIL protein is added early in the workflow
Accuracy and Precision with Three SIL-IS’s

Desired SIL-Protein Properties

- High protein purity
- Matches native protein sequence
- High incorporation of stable isotopes
- Similar PTM to native protein (i.e., glycosylation)
- Digestion kinetics same as native protein
- Similar enrichment or fractionation as native protein
SIL Protein and SILuMab Development

Available Cell Lines

CHO
HEK
E. coli

Cell Lines Diagram:
- Electroporate
- DNA
- Scale Up
- Identify top 10 producers
- Heavy media adaptation
- Protein production
- Isotope incorporation analysis by LC-MS/MS
- Tryptic digestion
- Protein purification
SIL-Protein Purity by SDS-PAGE

Purity greater than 95% achieved
Characterization of SILUMab
Sequence confirmation by peptide mapping

SILuMab Heavy Chain

Apolipoprotein A1 (APOA1)

High sequence coverage obtained
RP-LC-MS Analysis of Intact SIL-IGF1

Sequence of IGF-1 consisting of 70 amino acids in a single chain and three intramolecular disulfide bonds

Sequence and structure verified by RP-LC-UV-MS
Isotope Incorporation

$^{13}\text{C}_6^{15}\text{N}_2$ Lys in SILuMab and $^{13}\text{C}_6^{15}\text{N}_4$ Arg in SIL-Thyroglobulin

**VVSVLTVLHQDQLNGK**
- Heavy: $[\text{M+3H}]^+^3$ 606.0117
- Light: $[\text{M+3H}]^+^3$ 636.3403
- % Incorporation > 99%

**VIFDANAPVAVR**
- Heavy: $[\text{M+2H}]^+^2$ 641.3631
- Light: $[\text{M+2H}]^+^2$ 636.3590
- % Incorporation = 98.2%

Incorporation > 98%
Desired SIL-Protein Properties

- High protein purity
- Matches native protein sequence
- High incorporation of stable isotopes
- Similar PTM to native protein (ie, glycosylation)
- Digestion kinetics same as native protein
- Similar enrichment or fractionation as native protein
Glycosylation similar to native human protein
SIL Protein Digestion Kinetics
APOA1 in Human Serum, TFE Denaturation

Kinetics are similar after 4 hours of digestion
SIL Protein Digestion Kinetics
APOA1 in Human Serum, Urea Denaturation (FASP)

Kinetics are similar after denaturation
Desired SIL-Protein Properties

- High protein purity
- Matches native protein sequence
- High incorporation of stable isotopes
- Similar PTM to native protein (i.e., glycosylation)
- Digestion kinetics same as native protein
- Similar enrichment or fractionation as native protein
Protein Internal Standard Workflow

Sample Preparation → Protein Extract

Protein Fractionation
- 1D or 2D Gels
- Abundant Protein Depletion
- Antibody Enrichment

Dissolution/Denaturation → Digestion → Enzymatic Chemical → Peptide Digest

Peptide Fractionation
- Anti-peptide antibodies (SISCAPA)
- Cation-exchange LC

Add SILuMab or SIL Protein

LC-MS

SIL protein should normalize enrichment variability
SIL Protein Characterization: Ligand Binding Affinity
SIL-Infliximab vs Remicade on Biacore

TNF-α sensorgrams

SIL-Infliximab
KD = 0.15 nM

Remicade
KD = 0.17 nM

Ligand binding equivalent to therapeutic antibody
Desired SIL-Protein Properties

- High protein purity
- Matches native protein sequence
- High incorporation of stable isotopes
- Similar PTM to native protein (ie, glycosylation)
- Digestion kinetics same as native protein
- Similar enrichment or fractionation as native protein
Immuno-affinity enrichment LC-MS assay
Erythropoietin (EPO) in dog serum

Beagle serum with h-EPO at 1 pg/ml to 10 ug/ml, 50 ng/ml SIL-EPO
Variable capture efficiency, saturation above 1 ug/ml
Immuno-affinity enrichment LC-MS assay
Erythropoietin (EPO) in dog serum

Response normalized by SIL protein ISTD
Comparison of Peptide and Protein Internal Standards
Erythropoietin (EPO) in dog serum

Accuracy Table

<table>
<thead>
<tr>
<th>[EPO] ng/mL</th>
<th>Protein IS</th>
<th>Peptide IS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VNFYAWK</td>
<td>SLTTLLR</td>
</tr>
<tr>
<td>5</td>
<td>88.8</td>
<td>88.6</td>
</tr>
<tr>
<td>10</td>
<td>107.6</td>
<td>99.6</td>
</tr>
<tr>
<td>50</td>
<td>109.8</td>
<td>113.6</td>
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<tr>
<td>100</td>
<td>93.1</td>
<td>101.2</td>
</tr>
<tr>
<td>250</td>
<td>100.7</td>
<td>97.1</td>
</tr>
</tbody>
</table>

Red highlight: >20% deviation from expected

Greater accuracy achieved with SIL protein ISTD
Universal Peptide Strategy
Quantification of Human MAb in Pre-Clinical Model Plasma

- Surrogate tryptic peptide from Fc region of human MAb
- Second tryptic peptide from light chain of human MAb

Generalized preclinical PK assay employing surrogate peptides from constant regions
Immuno-affinity enrichment LC-MS PK assay
Humira (adalimumab) in monkey serum

100 µL Serum → Wash PBS → In plate Digestion Trypsin → LC-MS

Anti hu-Fc SILuMab Capture Plate

Monkey serum with ADA at 10 ng/ml to 50 ug/ml, 2 ug/ml SILuMab
Human Mab in Monkey Serum
Peptide VVSV at LOQ

Injection #1

Injection #2

Injection #3

6% CV @ 100 ng/mL
Human Mab in Monkey Serum

Assay Statistics

Accuracy: 85-115%, Precision: <10%

Range: 100 ng/mL to 50 ug/mL
## SILuMab Standards

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SILu™MAB Stable-Isotope Labeled Universal Monoclonal Antibody Standard</td>
<td>Human IgG1 – lambda</td>
</tr>
<tr>
<td>SILu™MAB K1 Stable-Isotope Labeled Universal Monoclonal Antibody Standard</td>
<td>Human IgG1 – kappa</td>
</tr>
<tr>
<td>SILu™MAB K4 Stable-Isotope Labeled Universal Monoclonal Antibody Standard</td>
<td>Human IgG4 – kappa</td>
</tr>
<tr>
<td>SILu™MAB Infliximab Stable-Isotope Labeled Universal Monoclonal Antibody Standard</td>
<td>Human IgG1 – kappa</td>
</tr>
<tr>
<td>SILu™MAB Mouse Stable-Isotope Labeled Universal Monoclonal Antibody Standard</td>
<td>Mouse IgG1 – kappa</td>
</tr>
</tbody>
</table>

![Graph](image)
## SILuProt Standards

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SILu™Prot <strong>APOA1</strong> Apolipoprotein A-I</td>
<td>MSST0001</td>
</tr>
<tr>
<td>SILu™Prot <strong>PTX3</strong> Pentraxin-related protein</td>
<td>MSST0003</td>
</tr>
<tr>
<td>SILu™Prot <strong>VEGFA</strong> Vascular endothelial growth factor A</td>
<td>MSST0005</td>
</tr>
<tr>
<td>SILu™Prot <strong>CLU</strong> Clusterin</td>
<td>MSST0007</td>
</tr>
<tr>
<td>SILu™Prot <strong>MAPK1</strong> Mitogen activated protein kinase 1</td>
<td>MSST0009</td>
</tr>
<tr>
<td>SILu™Prot <strong>ALB</strong> Albumin</td>
<td>MSST0011</td>
</tr>
<tr>
<td>SILu™Prot <strong>AMBP</strong> Alpha-1 microglycoprotein</td>
<td>MSST0013</td>
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<tr>
<td>SILu™Prot <strong>B2M</strong> Beta-2-microglobulin</td>
<td>MSST0015</td>
</tr>
<tr>
<td>SILu™Prot <strong>IL6</strong> Interleukin 6</td>
<td>MSST0017</td>
</tr>
<tr>
<td>SILu™Prot <strong>MAPK3</strong> Mitogen activated protein kinase 3</td>
<td>MSST0019</td>
</tr>
<tr>
<td>SILu™Prot <strong>CRP</strong> C-reactive protein</td>
<td>MSST0021</td>
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<tr>
<td>SILu™Prot <strong>APOA2</strong> Apolipoprotein A-II</td>
<td>MSST0029</td>
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<tr>
<td>SILu™Prot <strong>MAPT</strong> Microtubule-associated protein tau-441</td>
<td>MSST0031</td>
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<tr>
<td>SILu™Prot <strong>IFNG</strong> Interferon Gamma</td>
<td>MSST0039</td>
</tr>
<tr>
<td>SIL-Thyroglobulin Certified Reference Material</td>
<td>T-109</td>
</tr>
</tbody>
</table>

[sigma-aldrich.com/silutions](http://sigma-aldrich.com/silutions)
Summary

- LC-MS can address the shortcomings of LBA’s associated with long assay development time and specificity

- Immunoaffinity enrichment can be combined with LC-MS to improve sensitivity

- Stable isotope labeled SIL proteins have been produced in human and *e. coli* cells and characterized for quantitative MS applications

- Use of SIL proteins and SILuMab standards reduces error and variability associated with enrichment and enzymatic digestion
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