GETTING STARTED WITH PROTEIN QUANTIFICATION: OF PROTEOFORMS AND MEASURANDS

CHRISTOPHER M. SHUFORD
THE ALTERNATIVE TO MASS SPECTROMETRY

"Friends don’t let friends do immunoassays"

- Dan Holmes

Specificity
Poor standardization
Hook effect
Anti-reagent antibodies
Autoantibodies
Microclots
Single (low)-plex

...Why Not?

MASS SPECTROMETRY = MOLECULAR SPECIFICITY

Insulin Assays

C-peptide Assays

BINDING PARTNERS ≠ INTERFERENCES

Immunoassay vs MS

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<td>AIP</td>
<td>63</td>
<td>0.81</td>
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C.E. Bystrom & co-workers, *PLOS 2012*, 7 (9), e43457.
WHAT IF MY PROTEIN IS TOO BIG FOR (ROUTINE) MS?

Buy a bigger MS?
WHAT IF MY PROTEIN IS TOO BIG FOR MS?

The detected molecular species are **proteolytic peptides** (not intact proteins)

Digestion-based MS workflows are less susceptible to binding interferences
The “Signature Peptide” defines the “Measurand”

APOLIPPOPROTEINS

APO-E2

KVEQAVETEPEPELRQQTEWQSGQRWELALGRFDYLRLVQVTSEQVQEEQLSSQVTQEL
RALMDETMKELKAYKSELEEQLTPVAEEETRALSKELQAAQARLGADMEDVCGRGLVQYRG
EVQAMLQGSTEELRVRLASHLRKLRKRLLRAADDLQKCLAQVQAGAREGAGERGLSAIRER
LGPLVEQGRVRAATVGSLAGQPLQERQAQLGERLRARMEEMGSTRTRDRLDEVKEQVAEVR
AKLEEQAAQIQRLQAEAFQARLKSWFEPPLVEDMQRQWAGLVEKVQAAVGTSAAPVPSDNH

APO-E3

KVEQAVETEPEPELRQQTEWQSGQRWELALGRFDYLRLVQVTSEQVQEEQLSSQVTQEL
RALMDETMKELKAYKSELEEQLTPVAEEETRALSKELQAAQARLGADMEDVCGRGLVQYRG
EVQAMLQGSTEELRVRLASHLRKLRKRLLRAADDLQKCLAQVQAGAREGAGERGLSAIRER
LGPLVEQGRVRAATVGSLAGQPLQERQAQLGERLRARMEEMGSTRTRDRLDEVKEQVAEVR
AKLEEQAAQIQRLQAEAFQARLKSWFEPPLVEDMQRQWAGLVEKVQAAVGTSAAPVPSDNH

APO-E4

KVEQAVETEPEPELRQQTEWQSGQRWELALGRFDYLRLVQVTSEQVQEEQLSSQVTQEL
RALMDETMKELKAYKSELEEQLTPVAEEETRALSKELQAAQARLGADMEDVGRGRLVQYRG
EVQAMLQGSTEELRVRLASHLRKLRKRLLRAADDLQKCLAQVQAGAREGAGERGLSAIRER
LGPLVEQGRVRAATVGSLAGQPLQERQAQLGERLRARMEEMGSTRTRDRLDEVKEQVAEVR
AKLEEQAAQIQRLQAEAFQARLKSWFEPPLVEDMQRQWAGLVEKVQAAVGTSAAPVPSDNH

Signature peptides can be selected to target/quantify specific proteoforms
Use to quantify E3 in E3/E3 or E3/E4 genotypes
POLYMORPHISMS

APO-E2

Use to quantify E3 in E3/E3 or E3/E2 genotypes

APO-E3

APO-E4
## BEYOND TRYPsin

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<th>LysC</th>
<th>LysN</th>
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<td>R</td>
<td>D</td>
<td>F/Y/L/W/M</td>
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**mature PSA**

```
IVGGWE CEKHSQPWQV LVASRGRAVC GGVLVHPQWV
LTAAHCIRNK SVILLGRHSL FHPEDTGQVF QVSHSFPHPL YDMSLLKNRF LRPGDDSSHD
LMLRLSEPA ELTDAVKVMD LPTQEPALGT TCYASGWGSI EPEEFLPKK LQCVDLHVIS
NDVCAQVHPQ KVTKFMLCAG RWTGGKSTCS GDSGGPLVCN GVLOGITSWG SEPCALPERP
SLYTKVVHYR KWIKDTIVAN P
```

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**mature PSA**

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**mature PSA**

![mature PSA diagram]

SELECTING SIGNATURE PEPTIDES FOR “TOTAL” MEASURES

MALVLEIFILLASICQVSAN IFEFQYQVDAPLRPCQELQRET AFLKQADYVPCPCLCDGSPQTV QQCPQNDGRSCWCVSGANGSEV LGSRQFGPVPACLSCQCLQKQ
QQLILSGYINSDTQLPCC DSDGYAQPCQCVQQCWWC VDAEGMENVYGRQLRGPKRC FRSLREIRNLHLVGGDKSP QCSCAEQGFMVQCFVNTT
DMMITLHLVHPPFPQFVT FFSSFRCOFPPYSYCHCHADS QGRELSTGLQELLEDQTYD IPAGDLIPSTFTETETYLIR QRPFLAQSVQGCRPFCRT
CEVERATIFSPHYSEVS CQKRDQGVCQQGQGQGQGA EQLCSCAQASSQSLRSLYFGTS GYFQSHQDFSSPRQRFKASPR
VARFATCPPTKELVFDSG LRRPMQKQGQSFVSSEML HKEATRAFPRGSLRALRLQT TTNPKRLQQLNFGKFLNVN QQNLGALGTRGFMPNFQF
FQQLQGSLFMRGQREDAK LPSVGLDNSSTGGTPAEAK DTGMNTPKTVQGFGEINPQNE QNIALKFLASLLELPELFF LQAISQVPEDVARDLDQVME
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DLIQGQSPWQLHDSKFTPAE TPHLQDGDFHTGQTRWFCQ SGEFQYVYLAQSEAOSQQLGC KPCPESYSQDEECICPGVGF YEQQAGSACVCPGCVPGRTI
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VVVDPVSDHRDFVADVPAF NFSSARDVLRLCSOSHRAC LIILTLOQPGVRMCMYTD QSCBHLQOGNCRLLLIEAA THYRKYRGLISLYQAVSYS
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VPTSHHGRGQARQAOQVT SWQPDQoriGVPYAAAPLA RESQAPFPQYMPWQDSASK RASQCPGIGRTSTSGPVSED CLYNIFTPQONAPNASVLF
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KVEVDIVLIGPSQRFSSTTSKTA FYALQCSQIGFLGSDVAGAQATQMMYHVF YTHYKGRPFLLSYAVSYPV
SELECTING SIGNATURE PEPTIDES FOR "TOTAL" MEASURES

MALVLE1FTLASS1EQVSAK 1FQYVQADVQRPRPCEREQSM LFQCLGADVFYCPQGASQDTPG Q0CLGAG0LTQDERCTQPLQG 2Q0DVTQ0QCDQ0Q0Q0Q0Q0

Gene Variants
- Missense Mutations
- Nonsense Mutation
- Splice Variant

Frequency > 0.5%

17
SELECTING SIGNATURE PEPTIDES FOR “TOTAL” MEASURES

MALVLE1FTLLASICQVSA N IFDQVDAQRQLRPCQELQRET APLQDAPPVQPGAEDGSPQT VQCQNDGRSCWCVGSGSEV LGSSRQPGRVPACCLFQOLQ
QOLLGSYINSTDTLYLPC QDGDYAPVQCDVQVQCWC VDAEMGEXGQRQLGPRKRC FRSCREIRNLRRHVGQDKSP QCQSAEGEPVQQCFVWTT
DMITTDLVHSFIVFQATT ANFGRQGGFVPEGSCHCAK QGRLAELGTELLEDLYTDT IFAPGDLGSLFTETTLRYL QRQLFALVSQISFCRPTC
CEVERATASGHPYFVSPC RQGDYAVQQCQTEGCPCWVC AQQKEMHGTRQQGEPSSCAE QCNQASERQQALRSQFYGTQS YFQSQHDLFSSFSEKRNASPR
VARFATCSPPTKKFLVDSG LRRPMVEQGKSQSVSNMLL KEARAFIFPSRGLALRQLAF TTNPKRLQONLFGQKLNQV QROQALGAGTRFPNQFQ
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TVLSQSCQFTCFLLEPVSF QTYEFQGCSQFQGSCQWNV SWKCELPSRSSRQGQPVLPDC ECEQKARMQSIMQDQAGS TILVFPCCTSEGHQFLCVNQ
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KLLVRKMSYREAEGGSLFL STLYLAQDQQVDFPQVSYP SQLQVFPALEKRGQPREN IPFRRQILWQMLNGQDQV KYSSPDSTFPLAHFDFLRCWC
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DLIQSQQGHCQLDHSVQFPAE TIFLQGDHOTIPSTWFGQ SEFGYQVLTSEAMQGQCVK CEQEPGSQDEEICPGQPGF QYEQAGASLCVCPGQVGRRTI
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MQLCTIIDTEDACSSFVTST TEPEISCDFYAWTSDNVAC TSDQKDALLGNKATSGSL RCQQVVRSHQOQFPSVYLVK QQGSTTRLLQRFEPFGQNM
LSGLYNIPVSAQ SDLRCLACDLCDCFQVLF QVQQHAIICGSSQAVOLLC KQNWDMDPEAEAWATCPGV TQYQSQHGLVRQDGFQIKPQK
SLTPEGLGTQDTTPQFQVLW WKSDDMNGRSEPMSQCDKTV PRPASPTETAGLTTESPDVL LNQVIVNQGSLQSKQHDLF KHLFSAQANLWCLSCVQE
HSGFCLVATIESLQYFT LYPEAECVCDSMQNASQRCR LILQPQRAFLKVRWLEDK RFVMYRFLPTFLQGRAMTQ KPVSSMKSQNGFFCCERRC
DADPOCTCGFGFLVQVQKGG EVVLCTLINSIGQCMSRENG GWAILDCQGDPEVIDPFWF QGWYQKRIPANNNASFRCPLVL WPILSETVSLDSQRSLLASS
VVDVSPPSHIFDFVWAVSNFSFALTVDLCRQSSQHAR LQCTLTQQAQRKMYQADT QCSTSLQCGNCRLLREA THYKIRGFSLYSSAVPS
VP化QFQDQFGLPFPMAALPE RQFQAEPELHRGQGDSKPS RASRWQGTPRTSTSPQVSED CLYNVFTQPQVRAAASVLYL
FFHNTMRDSESGGWADPSL FLAQLGTVLYTASYVRGQVF GPQSSGGRQCVGQWNLALG AALTVWQTHIRGGSDFPRKV SLAADDGADVASIHLLTIAC
ATNSQLEQAVLMSGSSLPQ AIVISHAREQIIAQIAKKEV SCPHSSQQQVQSCLRQFQAN DLNQATKLQSAQFPHNYKQ PVQIDHGQREPQALARKSL
KVEVDJQEGREFQSRGTSKTAQ QALQSNLSQGDSLQKQAHQRQWYSLQHSTDQCAFSIRAL FAFRFAICIPDAQKPK
AKRAGSNWMPHAYQNHG SLELLADAVFAQALGPLFPPAY EQQFSEQLESRLLKQIMYQPH QHSQSNFNPQYPF SVPATPWFDFPPRAQGENYKEF
SELLSNRQGLKPCDFSWK YSLLRSTRASGAKGQSQAES EEREELTACGRELDDLSIQE FGSQSTFK 2768

PTMs
- N-linked Glycosylation
- O-linked Glycosylation
- Proteolytic Cleavage
- Iodination
SELECTING SIGNATURE PEPTIDES FOR “TOTAL” MEASURES

Methionine (M) Residue
- Cysteine (C) Residue
SELECTING SIGNATURE PEPTIDES FOR "TOTAL" MEASURES

Fully Tryptic
6 – 25 aa Residues
BLAST (unique)

Others Considerations
SELECTING SIGNATURE PEPTIDES FOR "TOTAL" MEASURES

Not omitted
Whiteaker et al., MCP, 10(4):M100.005645
EMPERICAL PEPTIDE SELECTION

Assay Workflow

In-Silico Candidates

Poor “flyers”

Poor retention

Poor Digesters

Slow Digesters & Instability

Sensitivity & Specificity

Development Workflow

Mass Spectrometry
- Precursor Ion
- Product Ion
- Source Tune
- Optics Tune

Chromatography
- Solvent
- Ion Pair
- Stationary Phase
- Dimension

Protein Enrichment
- not peptide dependent?

Digestion
- Additive
- pH
- Incubator
- Trypsin Type
- Trypsin
- Time

Peptide Enrichment
- Sorbent
- pH (L/W/E)
- Buffer (L/W/E)
- Recon Solvent

Protein Enrichment

Digestion

Peptide Enrichment

Chromatography

Mass Spectrometry

Courtesy Dr. Jim Bollinger
SIGNATURE PEPTIDE COMPARISON

Correlation (Agreement?) Infers Equivalent Molecular Specificity

Mass Spectrometry improves selectivity

Mass Spectrometry reduces interferences

**Top-down**
- Absolute Proteoform Specificity Plausible
- Limited to small (<30 kDa) and/or abundant (~mg/mL) proteins

**Bottom-up**
- $\Sigma$ Measured Proteoform(s) = “Measurand”
- Signature Peptides define the Measurand
- Confirming the Measurand requires validation
- Don’t Assume databases are right! (we don’t know everything – yet).
GETTING STARTED WITH PROTEIN QUANTIFICATION: CHEAPER, FASTER, BETTER SAMPLE PREPARATION

CHRISTOPHER M. SHUFORD
The Human Plasma Proteome (What a Pain!)

Total Protein Mass Distribution in Plasma

- ~ 6% (~10^6 Proteins)
- ~ 54% (HSA)
- ~ 40% (Top 13)

Graph showing the dynamic concentration range and distribution of plasma proteins.

- Albumin
- Transferrin
- Alpha-2-macroglobulin
- Alpha-1-acid glycoprotein 1
- Apolipoprotein C-III
- Clusterin
- Apolipoprotein C-I
- Complement component 7
- Tetraneutrin
- EGF receptor
- Calciitonin

ENRICHMENT OPTIONS

Protein-Level Enrichment
- Size Exclusion
- Precipitation
- Solid-Phase Extraction
- Depletion
- Affinity
- Immunoaffinity

Peptide-Level Enrichment
- Size Exclusion
- Precipitation
- Solid-Phase Extraction
- Depletion
- Affinity
- Immunoaffinity

LC-MS Detection

A:IS

time
Dynamic Range of LC-MS

Plasma Protein Concentrations (pg/mL)

- Serum Albumin
- Transferrin
- C-Reactive Protein
- Thyroxin Binding Globulin
- Lysozyme
- Ferritin
- Myoglobin
- Prostate Specific Antigen
- NT-proBNP
- Insulin
- BNP-32
- Interleukins

Abundant Protein Depletion OR SPE
Plasma Protein Concentrations (pg/mL)

- Serum Albumin
- Transferrin
- C-Reactive Protein
- Thyroxin Binding Globulin
- Lysozyme
- Ferritin
- Myoglobin
- Prostate Specific Antigen
- NT-proBNP
- Insulin
- BNP-32
- Interleukins

Abundant Protein Depletion AND SPE
Plasma Protein Concentrations (pg/mL)

- Serum Albumin
- Transferrin
- C-Reactive Protein
- Thyroxin Binding Globulin
- Lysozyme
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- BNP-32
- Interleukins

Dynamic Range of LC-MS

Imunoaffinity Enrichment

Log scale of plasma protein concentrations ranging from $10^{-1}$ to $10^{11}$ pg/mL.
HEROIC

Plasma Protein Concentrations (pg/mL)

- Serum Albumin
- Transferrin
- C-Reactive Protein
- Thyroxin Binding Globulin
- Lysozyme
- Ferritin
- Myoglobin
- Prostate Specific Antigen
- NT-proBNP
- Insulin
- BNP-32
- Interleukins

Dynamic Range of LC-MS

Protein Immunoaffinity AND Peptide Immunoaffinity
SPIKE ORDER EXPERIMENTS FOR OPTIMIZATION/DEVELOPMENT

Analyte Peptide
FSPDDSAGASALLR

1

Enrich

Digested Serum

LC-SRM

Measured response reflects incurred loss

SPIKE ORDER EXPERIMENTS FOR OPTIMIZATION/DEVELOPMENT

Analyte Peptide
FSPDDSAGASALLR

1
Enrich
Digested Serum

LC-SRM

Measured response reflects incurred loss

A1

Digested Serum

Analyte Peptide
FSPDDSAGASALLR

2
Enrich

LC-SRM

Measured response reflects no loss (100% recovery)

A2

SPIKE ORDER EXPERIMENTS FOR OPTIMIZATION/DEVELOPMENT

Study may be confounded by endogenous signature/analyte peptide (solution: over-spike)

Measured response reflects incurred loss

$A_1 \times 100 = \%\ recovery$

Measured response reflects no loss (100% recovery)

SPIKE ORDER EXPERIMENTS FOR OPTIMIZATION/DEVELOPMENT

**SIL peptide IS**
FSPDDSAGASALLR

1. **Enrich**
   - Digested Serum

2. **Enrich**
   - Digested Serum

**need more replication due to large experimental variance (& drift) of absolute response**

**IS**

- IS1: IS Response reflects incurred loss
- IS2: IS Response reflects no loss (100% recovery)

\[
\text{IS1} \times 100 = \% \text{ recovery}
\]

SIL peptide IS enrichment LC-SRM

SIL peptide IS enrichment LC-SRM

Response ratio reflects 100% recovery given both analyte and IS peptide experience the same loss.

Response ratio reflects analyte peptide recovery given IS peptide experiences no loss.

Highly robust assessment of recovery (resistant to matrix effects & MS drift)

SIMPLE OPTIMIZATION WITH SPIKE ORDER

Recovery (Relative A:IS Ratio)

Baseline (pre-IP) 1ug/10 ug 2/20 ug 3/30 ug 4/40 ug 6 ug

A1:IS1

A1:IS2

Ab1 (monoclonal) Ab2 (monoclonal) Ab3 (polyclonal)
MASS BALANCING: WHERE DID MY ANALYTE GO?

Ab1 (monoclonal)

Ab2 (monoclonal)

Re-extracted after IS2 addition
MASS BALANCING: WHERE DID MY ANALYTE GO?

**A1:IS1**

**A1:IS2**

Recovery (Relative A:IS Ratio)

- Not re-extracted after IS2 addition (non-specific contaminants)

- Ab1 (monoclonal)
- Ab2 (monoclonal)
Does the absence of protein infer the presence of signature peptides?

An Ideal World

The path taken depends on many variables!

The Real World

DIGESTION EFFICIENCY VARIES WITH TIME

Always consider digestion time during optimization/development.
Optimal for “discovery” proteomics ≠ optimal for targeted proteomics
DIGESTION EFFICIENCY VARIES WITH ENZYME CONCENTRATION

Faster is better....
DIGESTION EFFICIENCY VARIES WITH ENZYME CONCENTRATION

Faster is better….sometimes
DIGESTION EFFICIENCY VARIES WITH DENATURANT

Experiment! (titrate denaturant concentrations)

*optimal conditions will be protein- and peptide-specific: see C.H. Borchers and co-workers, J. Proteome Res. 2010, 9, 5422-5437.
Different signature peptides digest differently (does a plateau = “completion”?)
DIGESTION EFFICIENCY VARIES WITH SAMPLE MATRIX

Peptide Recovery

Digestion Time (min)

Chicken Serum

60 mg/mL HSA in PBS

Human Serum

Implications regarding accuracy of external calibration with surrogate matrices

*digestion can even vary between samples of the same matrix - I. van den Broek, et al., J. Proteome Res. 2013, 12, 5760-5774.
DIGESTION EFFICIENCY VARIES WITH MIXING?

Which one is “right”?  

Courtesy Dr. Clark Henderson & Dr. Andy Hoofnagle
High sensitivity measures requires enrichment

Leverage “spike-order” experiments for optimization

Digestion is complicated...don’t assume “completion”

Enrichment
- Top-down susceptible to interferences from binding-interactions
- Leverage orthogonality of protein and peptide-level enrichment to improve sensitivity/selectivity

Digestion
- There is no universal optimum for all proteins/peptides – experiment!
- Always consider time as a co-variable
- Be cognizant of peptide degradation
GETTING STARTED WITH PROTEIN QUANTIFICATION: CALIBRATORS ARE NOT INTERNAL STANDARDS

CHRISTOPHER M. SHUFORD
Calibration defines relationship between concentration and analytical response (i.e., response factor).

Response Factor of the analytical measurement ≠ Response Factor of the mass spectrometer.
**CALIBRANT VS INTERNAL STANDARD (IS)**

**External Calibration**
- without Internal Standardization
- with Internal Standardization

**Internal Calibration**
- defacto Internal Standardization

\[ R_A = m[A] + b \]
\[ \frac{R_A}{R_{IS}} = m[A] + b \]
\[ \frac{[A]}{[IS]} = m \frac{R_A}{R_{IS}} + b \]
**CALIBRANT VS INTERNAL STANDARD (IS)**

**IMMUNOASSAY**

- **External Calibration with Internal Standardization**
- **Internal Calibration**
  - *defacto* Internal Standardization

\[
R_A = m[A] + b
\]
\[
\frac{R_A}{R_{IS}} = m[A] + b
\]
\[
\frac{[A]}{[IS]} = m \frac{R_A}{R_{IS}} + b
\]
IMMUNOASSAY

Analyte Response vs. Analyte Concentration

$$R_A = m[A] + b$$

ISOTOPE DILUTION

A:IS Response Ratio vs. Analyte Concentration

$$\frac{R_A}{R_{IS}} = m[A] + b$$

Internal Calibration

defacto Internal Standardization

A:IS Concentration Ratio

$$\frac{[A]}{[IS]} = m \frac{R_A}{R_{IS}} + b$$

Internal Calibration

(IS Internal Standard ≠ Calibrant)

(IS Internal Standard = Calibrant)
$$R_A = m[A] + b$$

$$\frac{R_A}{R_{IS}} = m[A] + b$$

$$\frac{[A]}{[IS]} = m \frac{R_A}{R_{IS}} + b$$

$$m = 1 \& b = 0 \text{ (assumed)}$$
Protein - Level Enrichment

Cleavable Surrogate

Peptide Surrogate

Digestion

Peptide-Level Enrichment

LC-MS Detection

A-IS

time

m/z
RESPONSE FACTOR & RECOVERY LOSS

“Calibrators” should experience the same recovery/loss as samples
COMPARISON OF “INTERNAL CALIBRATORS”

COMPARISON OF “INTERNAL STANDARDS”

*external full-length protein calibrator

(GOOD & BAD) CALIBRANT OPTION

Intact (top-down)
- Protein Calibrant

Digest (bottom-up)
- Cleavable Surrogate Calibrant
- Peptide Surrogate Calibrant
(GOOD & BAD) INTERNAL STANDARD OPTIONS

Intact (top-down)
- Analog IS
- Protein IS

Digest (bottom-up)
- Cleavable IS
- Peptide IS
ALL OF THE (GOOD & BAD) OPTIONS

Intact (top-down)
- Analog IS
- Protein Calibrant
- Protein IS

Digest (bottom-up)
- Cleavable IS
- Peptide IS
- Peptide Surrogate Calibrant
- Cleavable Surrogate Calibrant
THE ONLY (GOOD) OPTIONS

Intact (top-down)

Protein Calibrant

Protein IS

Analog IS

Digest (bottom-up)

Cleavable IS

Peptide IS

Protein IS
THE ONLY (GOOD) OPTIONS

Intact (top-down)

Protein Calibrant
Analog IS
Protein IS

Digest (bottom-up)

Cleavable IS
Peptide IS
Comparison of 4 Different LC-MS/MS Methods Between 4 Labs

<table>
<thead>
<tr>
<th>Lab</th>
<th>Signature Peptide</th>
<th>Calibrant</th>
<th>Calibrator Matrix</th>
<th>Internal Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mayo</td>
<td>FSP</td>
<td>BCR®457</td>
<td>Human Serum</td>
<td>peptide</td>
</tr>
<tr>
<td>U. Wash.</td>
<td>FSP</td>
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</tr>
<tr>
<td>ARUP</td>
<td>VIF</td>
<td>Beckman Tg</td>
<td>Synthetic</td>
<td>cleavable</td>
</tr>
<tr>
<td>LabCorp</td>
<td>FSP</td>
<td>Beckman Tg</td>
<td>Synthetic</td>
<td>cleavable</td>
</tr>
</tbody>
</table>

All full-length Proteins (traceable to BCR®457)
ABSOLUTE QUANTIFICATION = HARMONIZATION

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</table>

Example of Bad Calibration

"Lab X" | FSP | cleavable peptide | Human Serum | cleavable peptide
Purified Proteins (…. and peptides)

- Gravimetrically (>10 mg …. often impractical)
  - How much of the solid is protein?
  - Nitrogen Analysis
  - Water Content (Karl-Fisher)
- AAA (Gold Standard)
- Nitrogen Analysis
- Spectrophotometry (UV/Vis or HPLC-UV/Vis)
- Bradford/BCA/Lowery Method (Bad)

Impure Proteins (e.g., Human Sample Calibrators)

- Reference Methods
  - Reference Method Procedure (RMP … few for proteins)
  - Immuno(cough)assays

Ensure quality of reference method!
ESTIMATING PROTEIN (IM) PURITY BY SDS-PAGE

From Certificate of Analysis

Vendor States > 98% Pure

Do you believe the Vendor?

- High MW Contaminants
  - Contaminants >30 kDa?
- Low MW Contaminants?
  - Contaminants <10 kDa?
- Dynamic Range of Stain
  - Can you observe 2% impurity?

MW Ladder

Calibrant Protein

MW (Da)

26.6
17.0
14.2
ESTIMATING PROTEIN (IM) PURITY BY SDS-PAGE

Your SDS-PAGE ....

How pure now? >98%?
ESTIMATING PROTEIN (IM) PURITY BY SDS-PAGE

How pure now? ~85%?
INTACT MASS (DIS)QUALIFICATION

Full-Scan (MS1)

Confirm AA Composition
(\textit{MS}^2 \text{ for Sequence})

Larger proteins may need bottom-up sequence analysis

Courtesy or Dr. Cory Bystrom
PROTEIN FOLDING: DOES IT MATTER?

Are recombinant proteins good calibrants for endogenous protein analytes?

Courtesy or Dr. Cory Bystrom
**MATRIX EFFECTS & SURROGATE MATRIX**

**Response Factor of Analyte in the Calibrator Matrix ≠ Response Factor of Analyte in the Sample Matrix**

**Sources of Matrix Effects:**
- Ion Suppression/Enhancement
- Enrichment Efficiency
- Digestion Efficiency
- Adsorptive Loss

**Other Considerations:**
- Interferences (signature peptide?)
- Background Noise

**Surrogate Serum/Plasma Matrices:**
- BSA/HSA/rHSA in PBS
- Animal Human/Serum
- Analyte-depleted Human Serum
- Mixtures

**Solutions:**
- Good Internal Standards
- Good Internal Calibrants
- **Human Sample/Pool Calibrator**

*Validate with Parallelism, Spike & Recovery, and/or Mixing Studies*
Calibrate a protein assay with a protein

Internal Standards less important
(in the absence of matrix effects)

Protein Calibration

- *Purified* Native Protein
- *Purified* Recombinant Protein
- Native Sample
- Surrogate Matrix is Important Too!

Internal Standards

- Tryptic Peptides: control for peptide degradation & peptide enrichment
- Cleavable Surrogates: not much better than tryptic peptides
- Full-length proteins: control for everything (*protein binding?*)