Quantitative Analysis of monoclonal antibodies at the low ng/mL range from serum samples via affinity enrichment coupled LC-MS/MS

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How to quantitate a monoclonal Antibody ?

Technologies used up to now → LIGAND BINDING ASSAYS

- ELISA (Biotin - POD)
- ECL

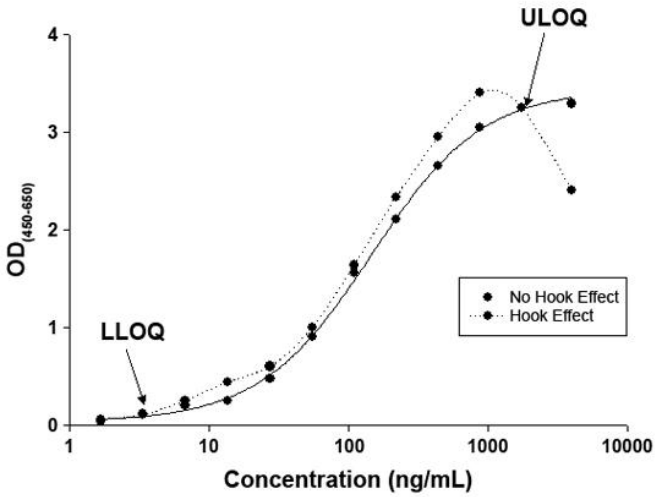
- HTRF (under evaluation)
- Luminex (under evaluation)

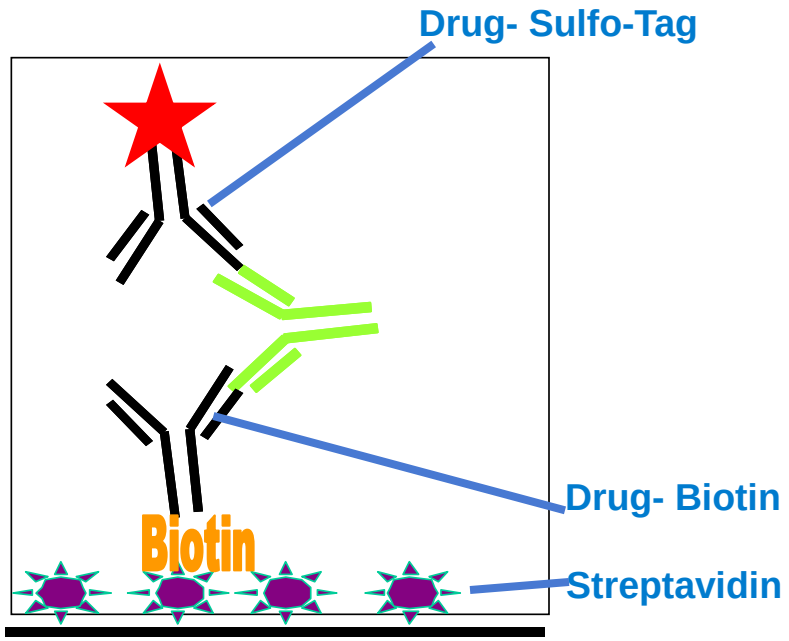
Ligand binding assays (LBA)

Based on high affinity protein-protein or similar inter-molecule interactions

Standard assay for Pharmacokinetic (PK) and Anti-Drug-Antibody (ADA) analysis

- Non-linearity of response/ Dilution linearity
- Matrix effects
- (Assay range)





ECL-based bridging immunoassay

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How to quantitate a monoclonal Antibody ?

Technologies used up to now

LIGAND BINDING ASSAYS

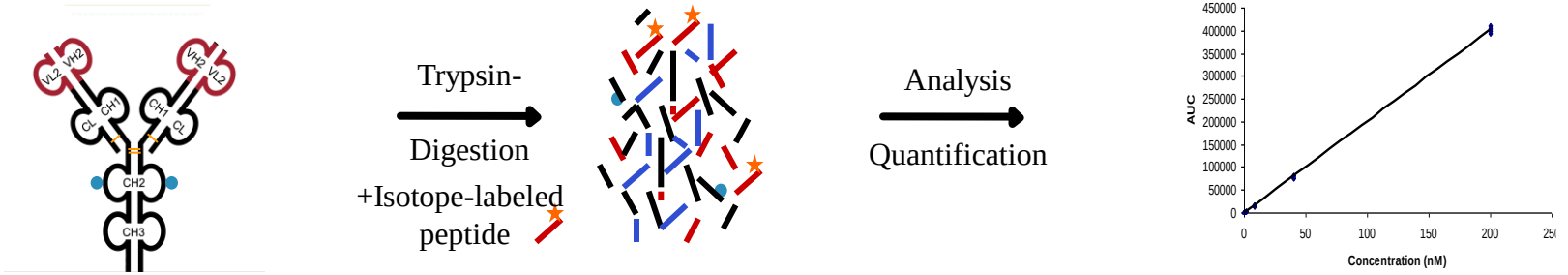
- ELISA (Biotin - POD)
- ECL

- HTRF (under evaluation)
- Luminex (under evaluation)

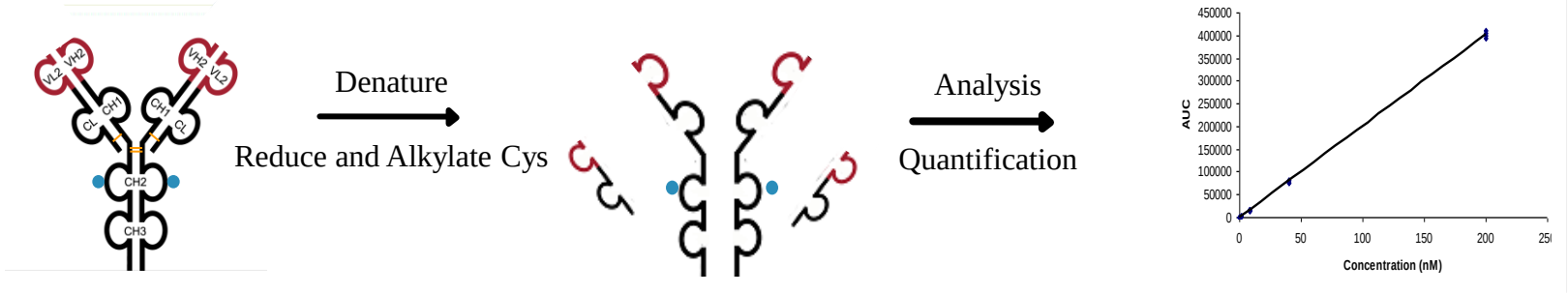
- Mass-spectrometry?

Theoretical approaches

- Bottom-up (Measurement of tryptic peptides of the Antibody)



- Top-down (Measurement of intact Antibody)



Peptides of antibodies

Heavy Chain

AGISKRQRST AGISKRQRST AGISKRQRST AGISKRQRST AGISKRQRST
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TSRQ

Light Chain

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ISKQGASTRA TVE

aa-sequence is only a fake

Variable Domain	**CDR**
Linkers	
Constant Region	

Strategy for Selecting Peptides for Quantitation

Theoretical # of peptides: 25-30 from LC, 45-50 from HC

ID “unique” peptides from CDR and linker regions

Filter unique peptides based on composition, ideally

- MW ~1500 Da
- No oxidizeable residues (C, M, W)
- No unstable sequences (DG, N-term Q, KK, RR)
- Proline is desirable

Search databases to ID duplicate sequences

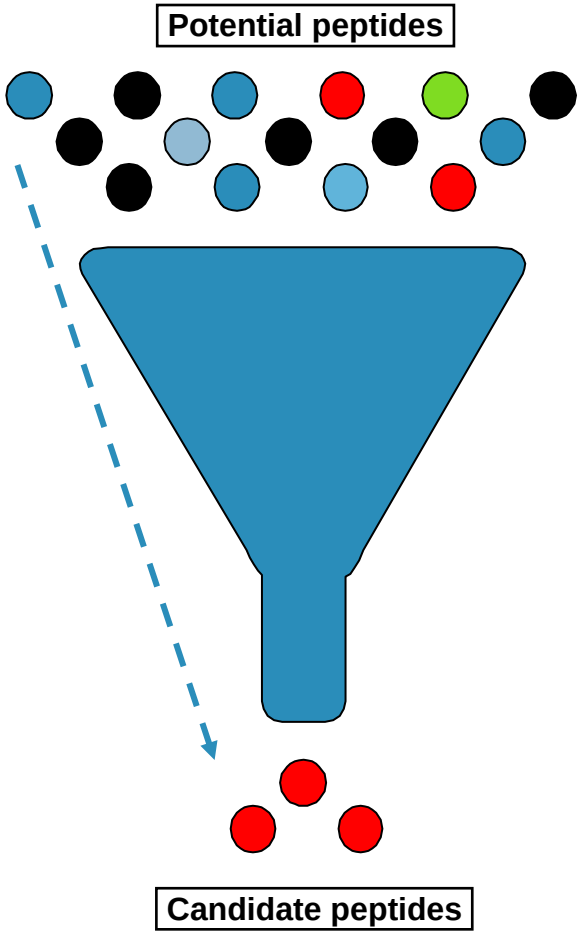
- Are the peptides species-specific?
- Will sample prep remove duplicate peptides?

Protein Digestion

- Confirm that expected peptides are produced
- Incomplete cleavage minimized

MS evaluation

- Select peptides that ionize and fragment appropriately



Database Searching – are the Peptides Unique?

GenomeQuest

Used to ID prototypic peptides that do not match other known amino acid sequences

- 18 databases composed of 37,982,376 protein sequences from 7 species
- 12 databases composed of 191,433,748 nucleotide sequences translated into protein (6 frames)

Peptide-Protein Alignment Summary

	Peptide Sequence	# aa	Chain	# hits	Align % identity	Considerations
1	AGISWRQRST_AGISKRQRST_AGISKRWRSR	24	H	393	100-80	Oxidation: 2 W
2		14	H	1968	92.86-81.25	
3		24	H	423	88.0-76.92	Oxidation: M; Pyro-glu formation; undercuts
4		22	H	1888	90.91-69.23	Contains DG
5		16	L	2032	100-93.75	100% identical to IgG Kappas
6		26	L	2040	96.15-84.62	Oxidation: C

aa-sequence is only a fake

Provided by Advanced Tech group

Candidate Peptides (from CDR/ Linker region)

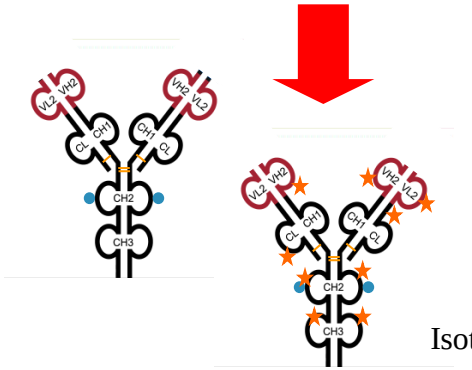
What is left after candidate selection?

Sequence	Residues	CDR or Linker	Unique Seq.	Heavy (H)/ Light (L) Chain	MW (Da)
AGISKRQRST AGISKRQRST AGISKRQRST	71-80	CDR	Yes	HC	2662.85
AGISKRQRST AGI	135-149	Linker	Yes	HC	1440.62
KRQRST AGISKRQRST AG	113-165	CDR	Yes	HC	2933.16
ISKRQRST AGISKRQRST AGISKRQRST A	238-257	CDR	Yes	HC	2219.35
AGISKRQRST AGISKRQRST AGISKR	61-96	CDR	Yes	LC	1675.95
KRQRST AGISKRQRST AGISKRQRST AGIS	209-225	CDR	Yes	LC	2853.16

aa-sequence is only a fake

MS Approach for Absolute Antibody Quantitation

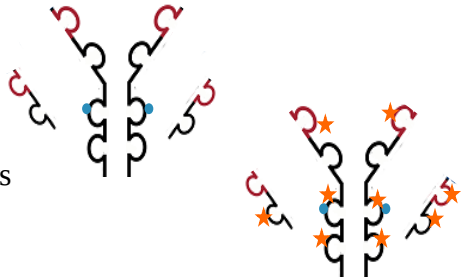
Antibody digestion



Isotope-labeled Ab

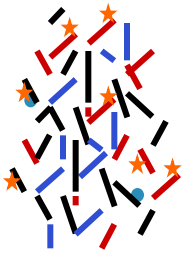
Denature

Reduce and Alkylate Cys



Trypsin-

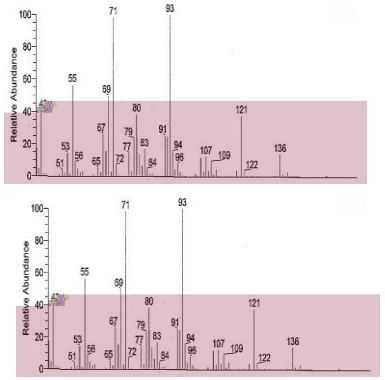
Digestion



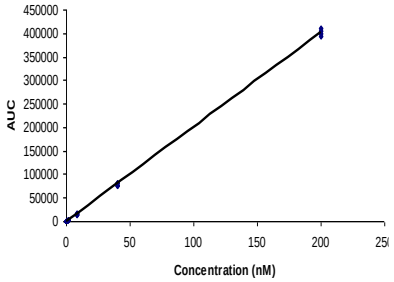
Analysis of peptides by LC-MS and quantitation

Quenching

Analysis/Quantification



MS/MS spectrum



MS quantitation

Production of Labeled Antibody

A stable CHO cell line expressing monoclonal antibody is adapted to and cultured in the following medium:

- DMEM-F/12 Flex medium (Invitrogen) supplemented with glucose, L-glutamine, dialyzed low IgG FBS, penicillin G, streptomycin sulfate, and methothrexate

Cells grown in 2L roller bottles

Cells were transferred in

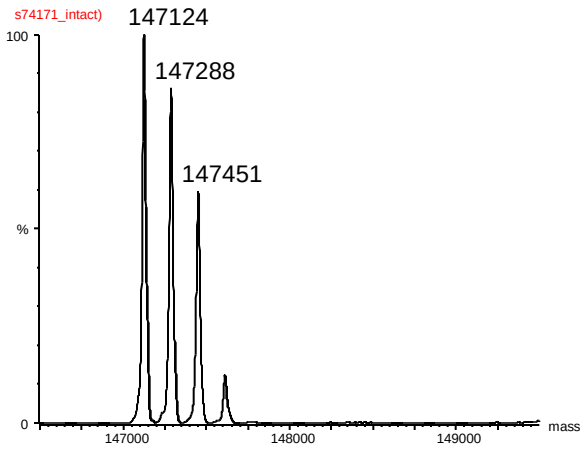
- SILAC Advanced DMEM-F/12 Flex medium (Invitrogen) supplemented as above. This medium lacks the amino acids lysine and arginine, and was supplemented with [U-$^{13}C_6$]-L-lysine and [U-$^{13}C_6$, $^{15}N_4$]-L-arginine

Cells cultured for 15 days in the labeled medium; supernatant harvested

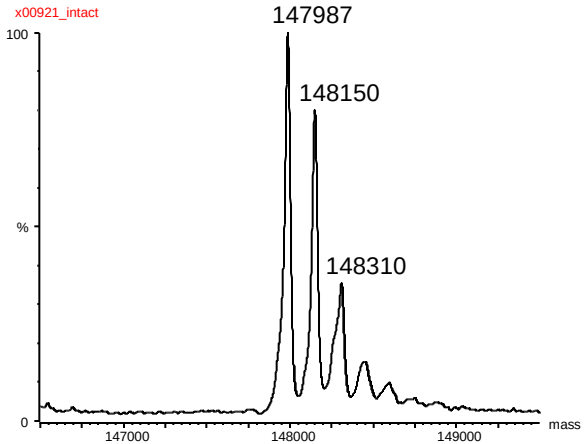
MS showed uniform incorporation of labeled amino acids in greater than 98% of the sample

★ **- Isotope labeled Lys or Arg**

Unlabeled Ab



Labeled Ab



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Method development: Ion chromatogram of Tryptic Digest

Digestion protocol:

2,5 µL monoclonal antibody

+ 2 µL DTT

+ 10 µL Iodoacetic acid

+ 0.1 M Tris , pH 8

+ Trypsin (Promega)

=> overnight, 37 ° C

+ 5 N HCl

LC/MS protocol:

-Thermo Accela U-HPLC

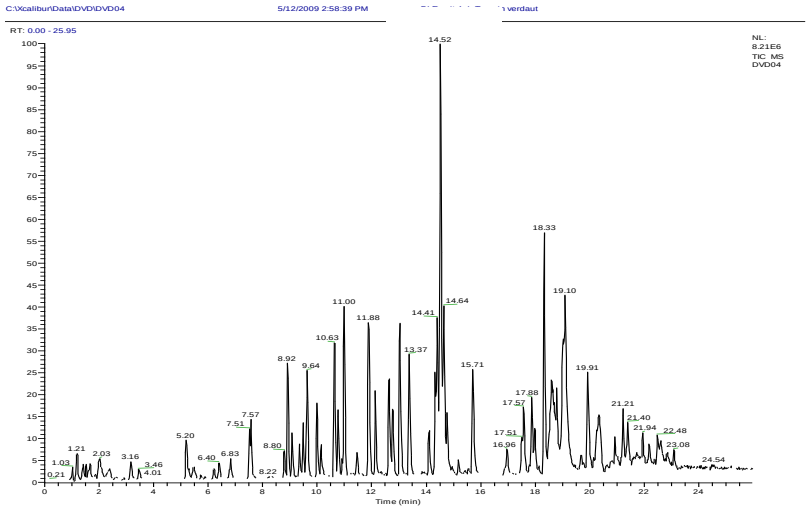
- BEH300 C18 150mm*2.1mm*1.7µm

- Thermo Orbitrap LTQ XL

Flow: 250µL/min

Linear gradient profile

Full Scan 500-3000 (LTQ)



Calculated sequence coverage:

LC: 95%

HC: 92%

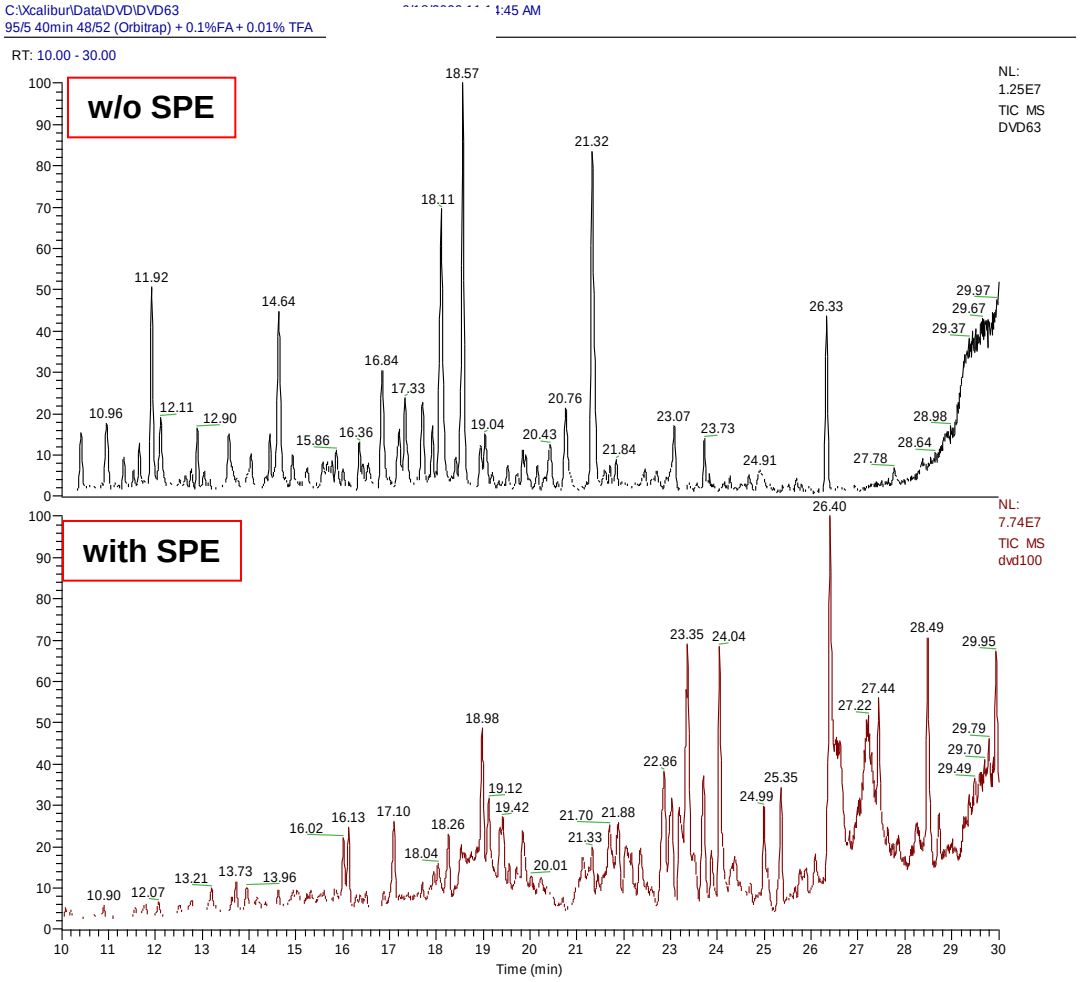
Missing regions result from:

- Peptides too small to detect or too large to fragment
- Glycosylated peptides

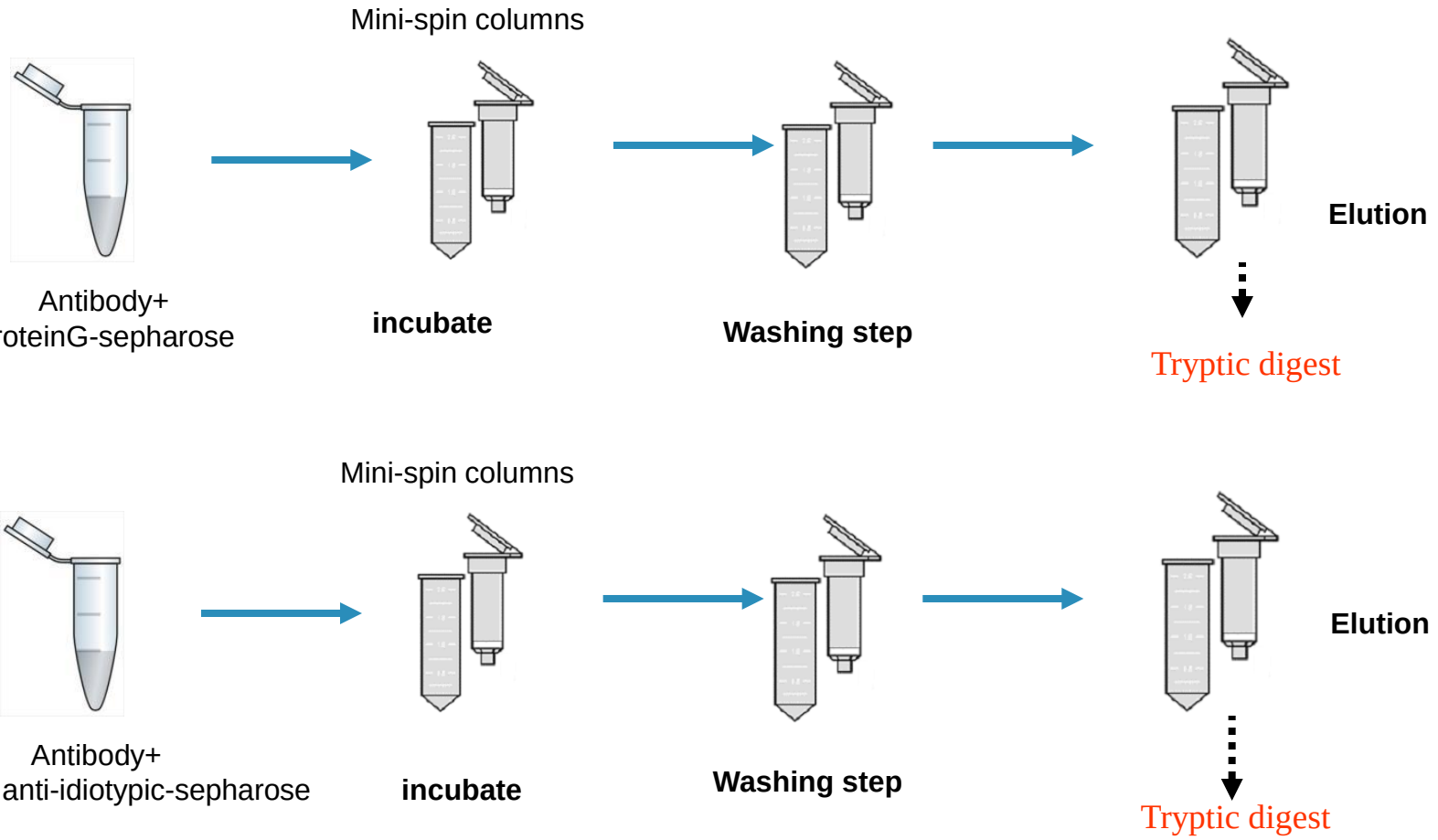
Method Improvement: Additional SPE Purification Step

Additional solid phase extraction

led to improved background signal

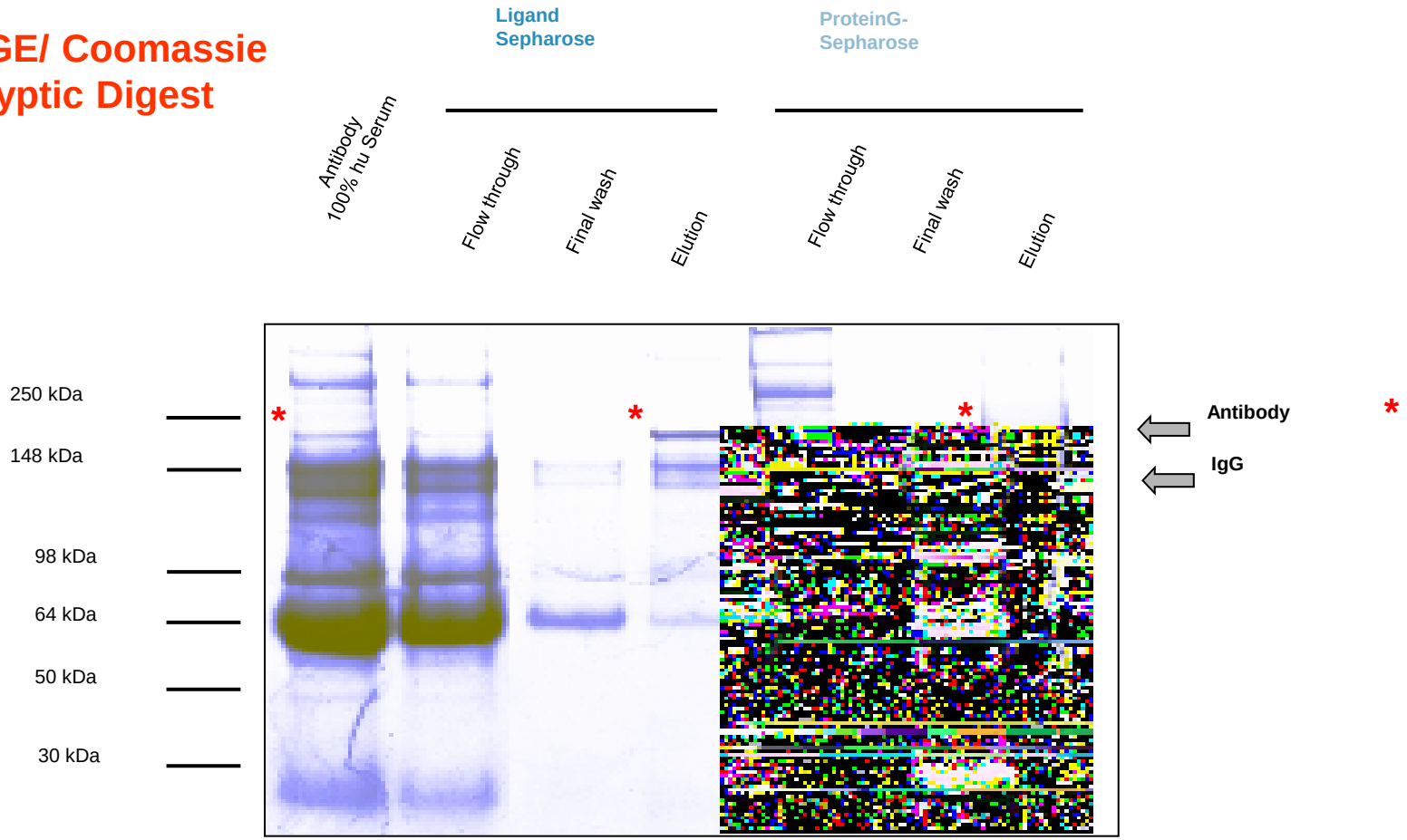


Evaluate affinity enrichment



Peptide Recovery after affinity enrichment

SDS-PAGE/ Coomassie and Typtic Digest



Antibody in 100% human serum

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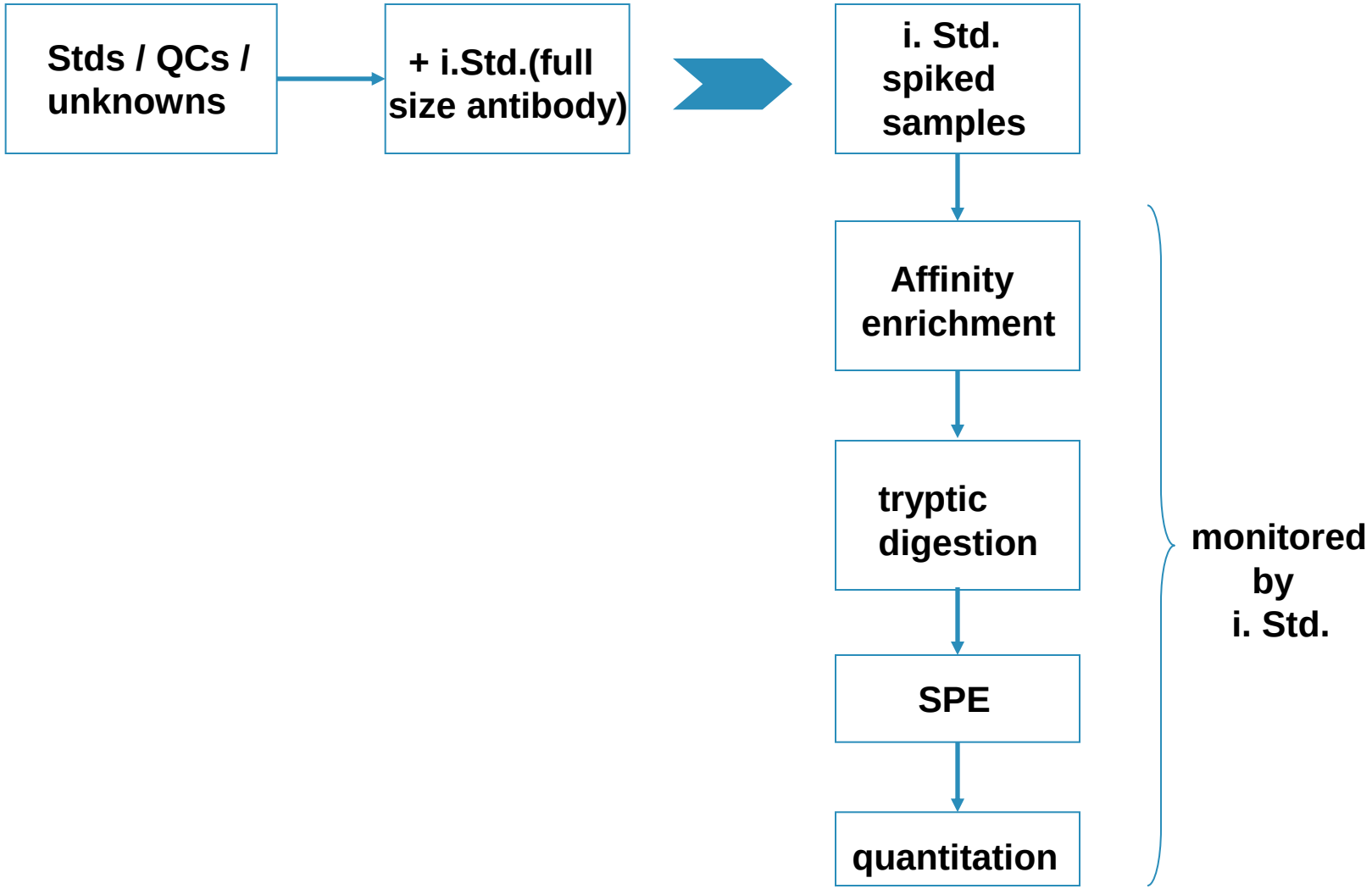
Improvements for high throughput

- Affinity chromatography is done in 96-well plate format
- Tryptic digest is done in 96-well plate format
- Tryptic digest in 60 min
- Separation via SPE (automated, ready to inject)
- Chromatographic run 5 min

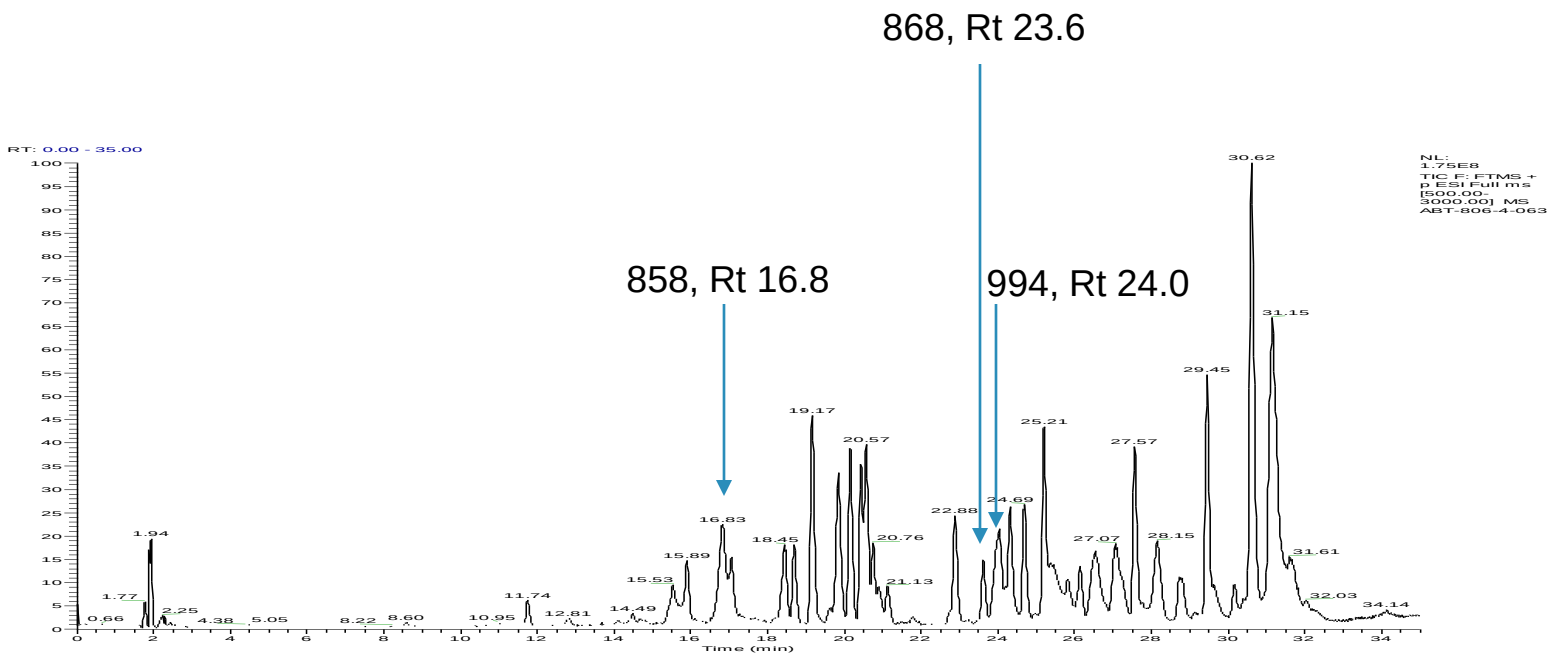
Method scheme



Peptides on focus for quantitation

Transitions for the three peptides on focus

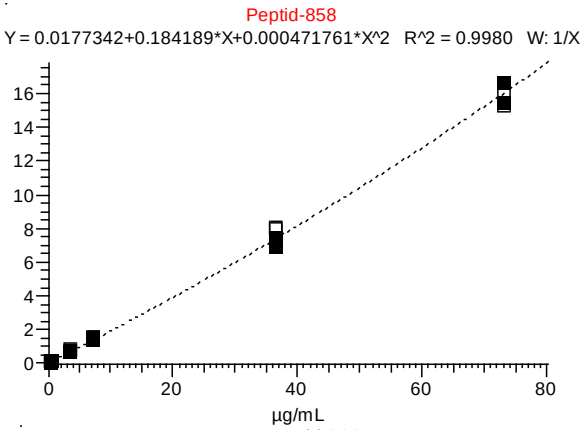
- 858: 857.94 → 1130.91
- 868: 867.96 → 951.36
- 994: 994.11 → 893.00

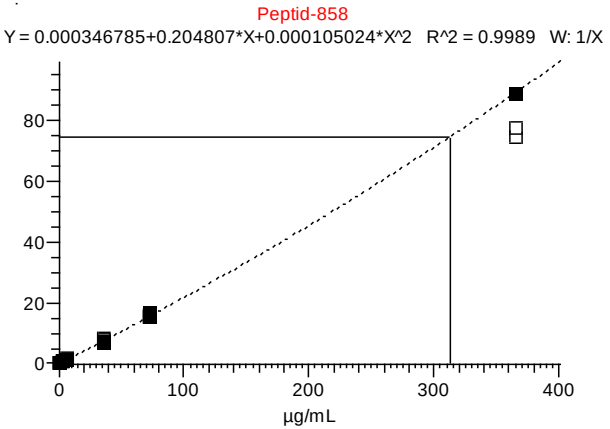


Quantitation via Tryptic Peptides – Quantitation is achievable at low ng/mL Levels

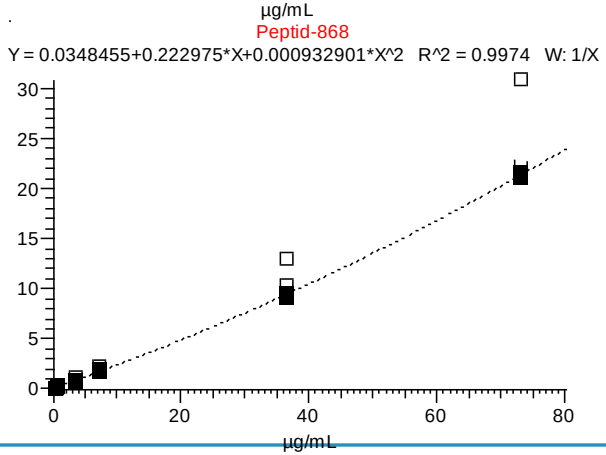
- **Antibody was purified from serum using Anti-idiotypic Ab coupled to sepharose**
- **After tryptic digestion, samples were analyzed using MS/MS (Orbitrap LTQ XL)**
- **The main fragment ion of each peptide was used for quantitation**

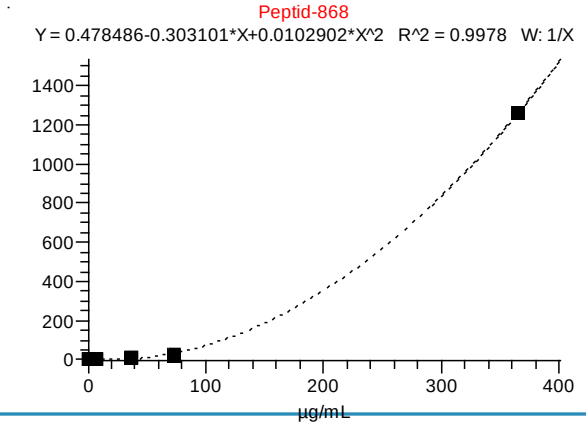
Peptide 858





Peptide 868





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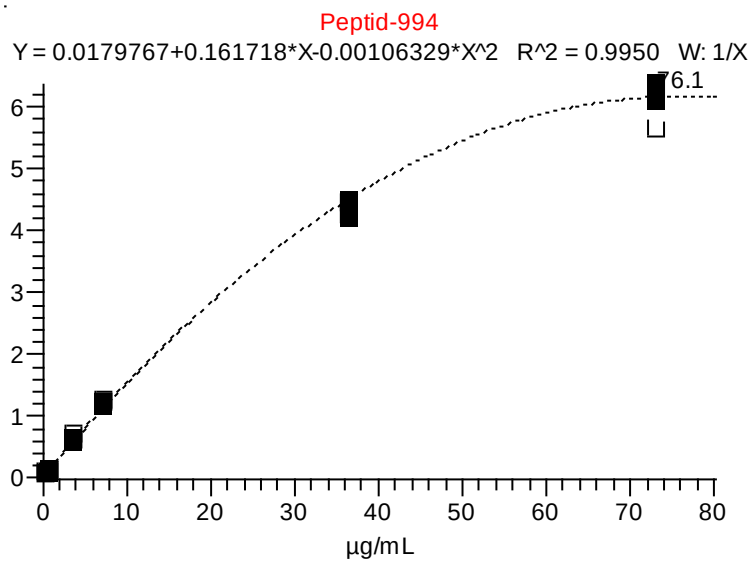
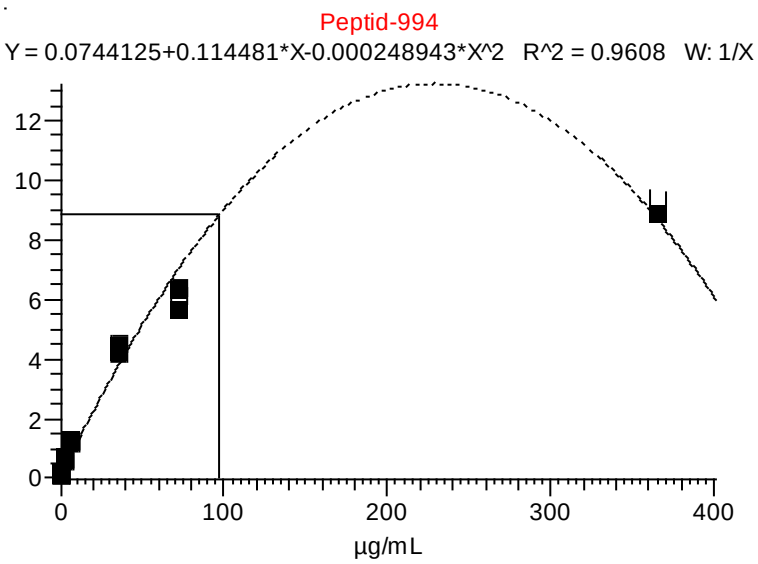
Quantitation via Tryptic Peptides – Quantitation is achievable down to lower ng/mL Levels

For peptide 994



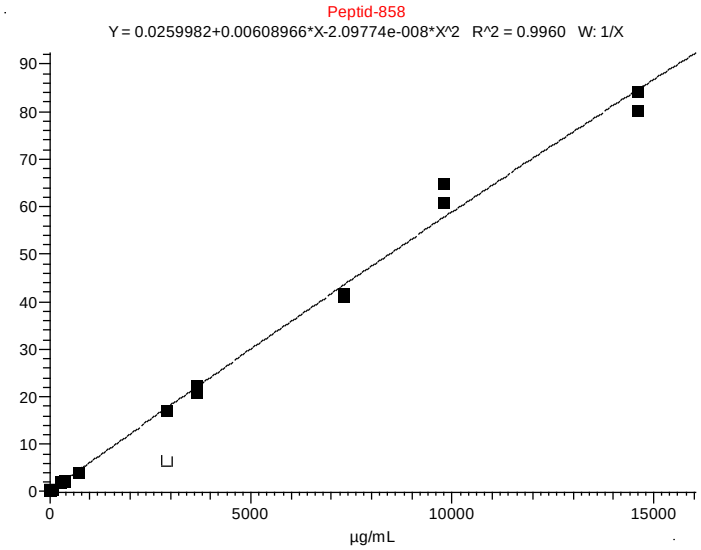


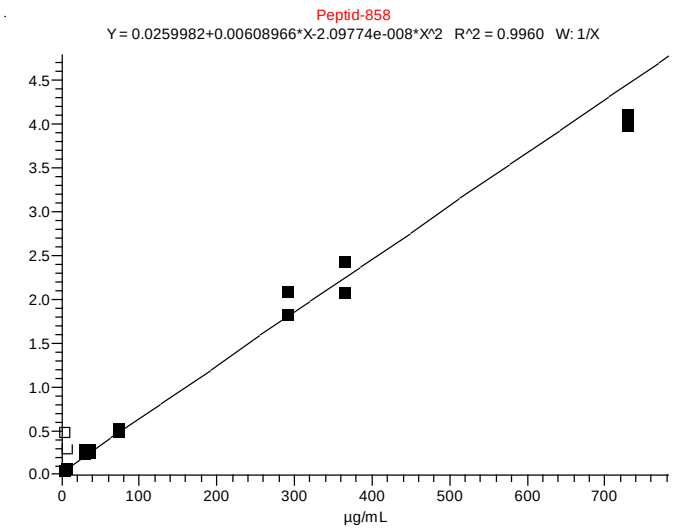
Lowest level: 0.365 µg/mL

Highest level: 365 µg/mL

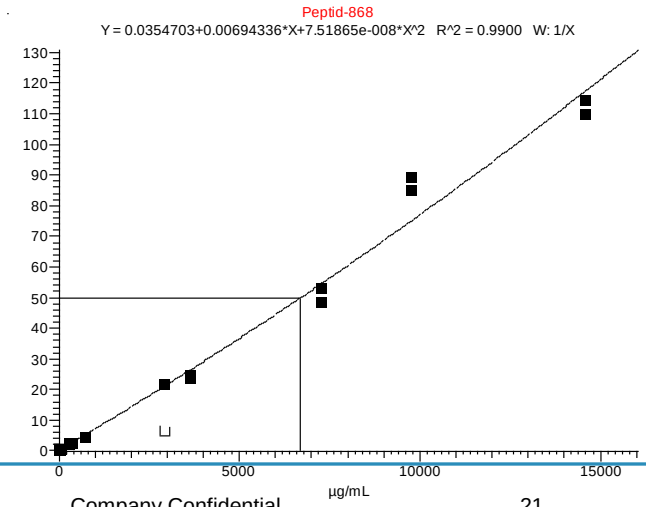
Quantitation over a broad dynamic range: up to 15,000 µg/mL as highest level

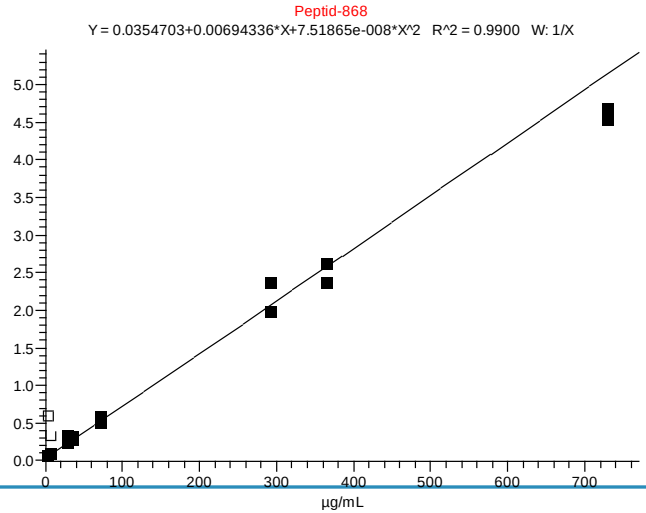
Peptide 858





Peptide 868





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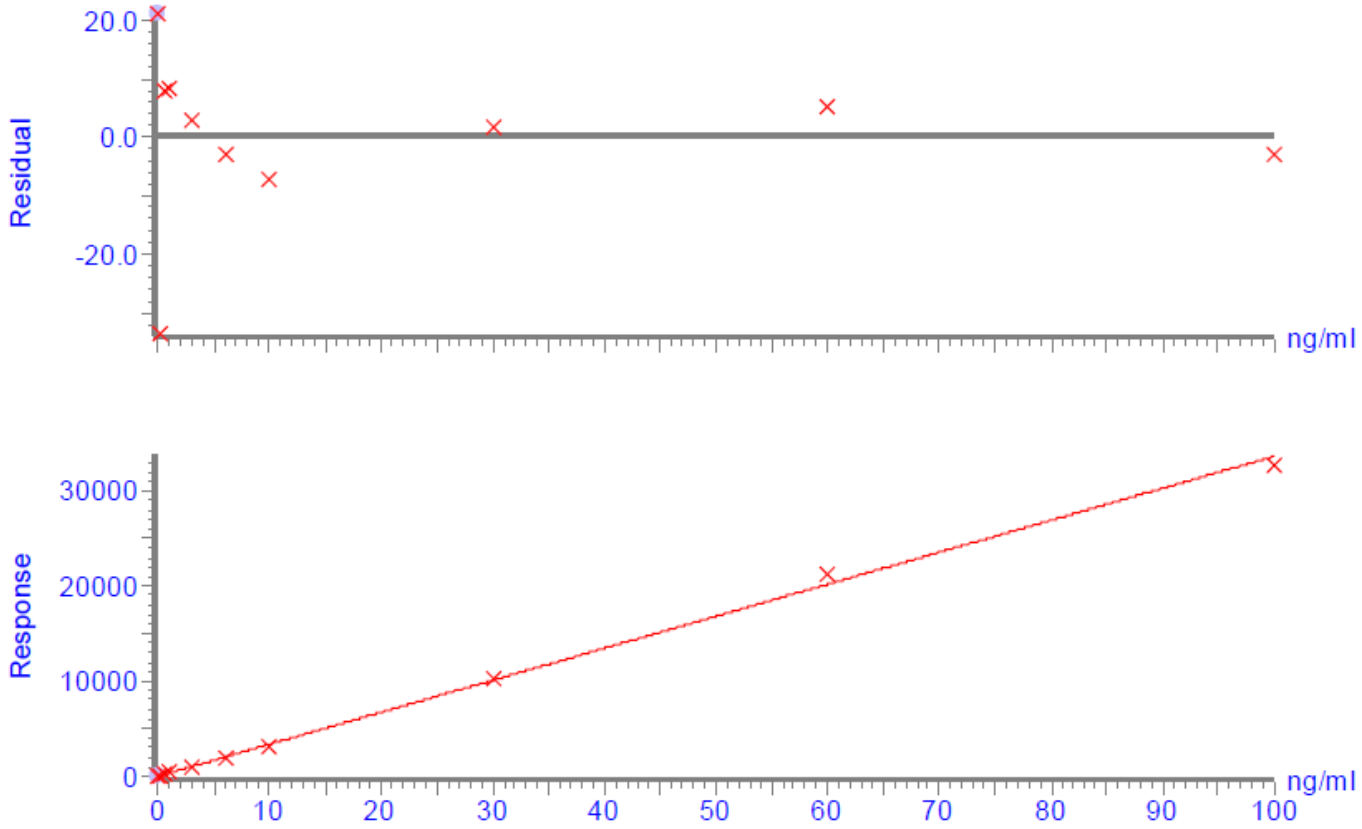
Outlook for excellent sensitivity

Correlation coefficient: r = 0.999111, r^2 = 0.998223
Calibration curve: 336.55 * x + -11.2272
Response type: External Std, Area
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None



Purity of internal standard 98 %

Only external calibration

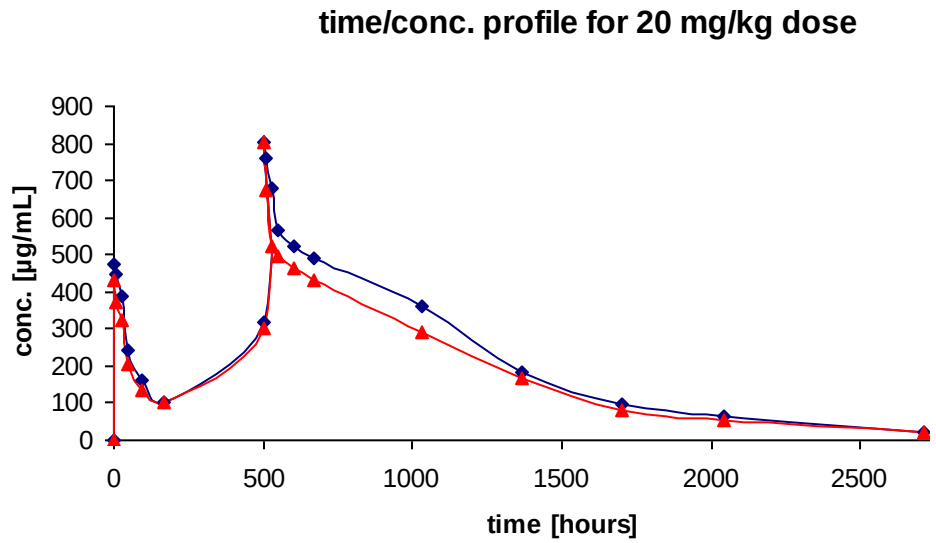
Expected LLOQ 0.1 ng/mL

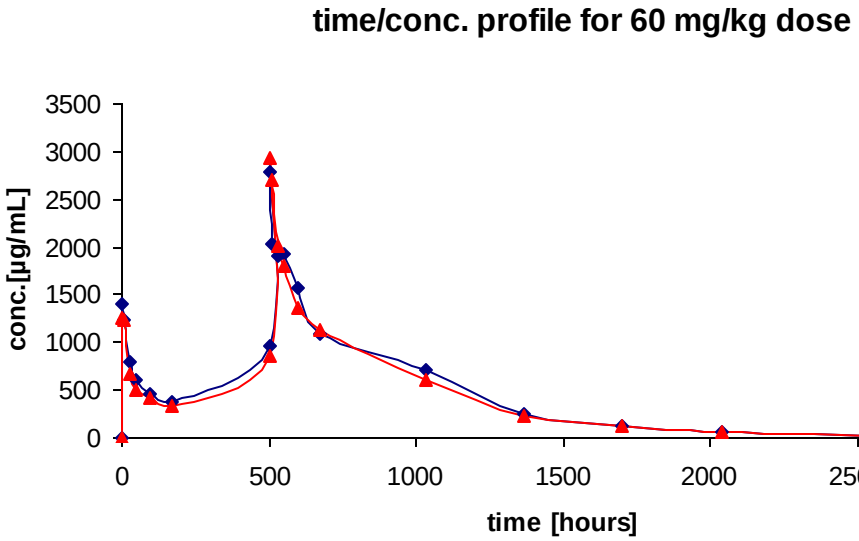
Cross-validation to Ligand Binding Assay

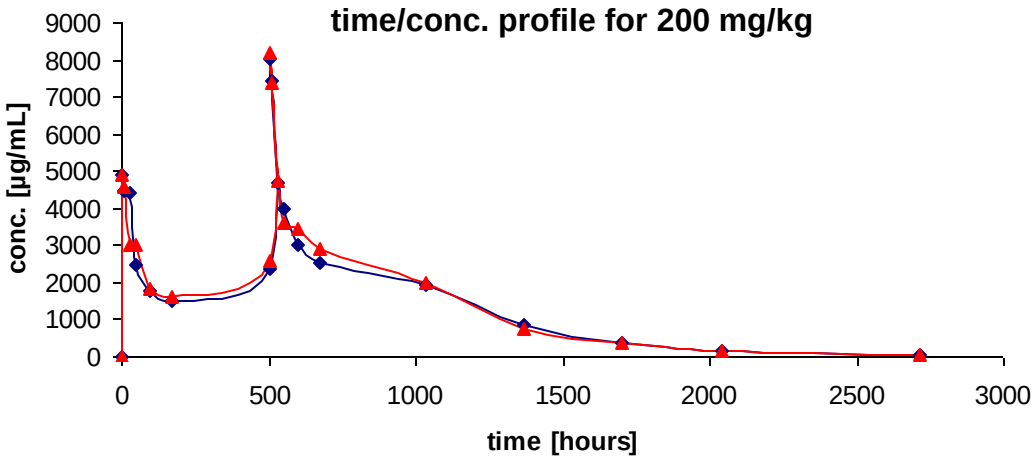
- **design**:
- 57 serum samples from three dosing groups were analyzed
- 6 full PK profiles (male/female)

- **outcome**:
- 91% samples passed ISR acceptance for MS-Methods
- 98% samples passed ISR acceptance for LBA

Results of cross-validation







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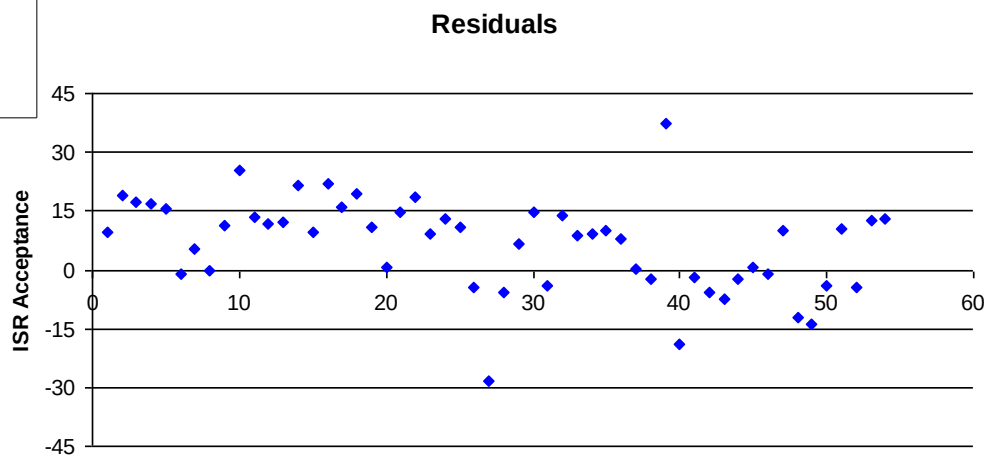
Statistical analysis of cross-validation results

$$\frac{\text{(ISR value - original value)}}{\text{mean}} * 100\%$$

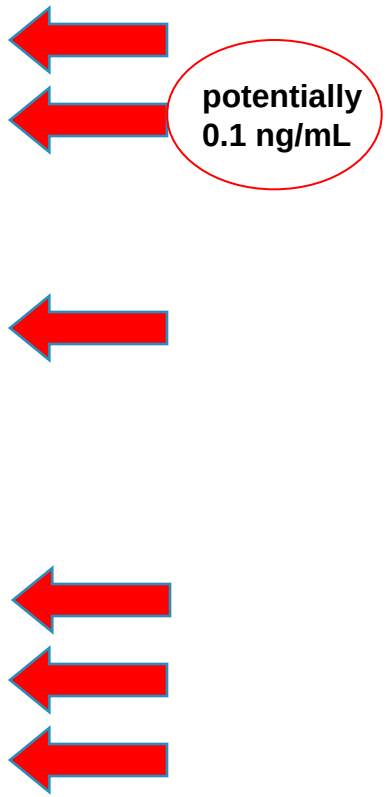
2% outside < 30%

9% outside < 20%



Challenges of LBA vs. reasons for MS method

Ligand Binding Assays	**MS of Proteins**
well established for Proteins (discovery, clinic, CRO)	Established in Protein world for relative small molecules only
tool kits commercially available	Tool kits commercially available
high specifity - epitope	High specificity (unique peptide)
highly sensitive (ng/mL)	Sensitivity ~1-5 ng/mL 10 fold more sensitive than LBA
measures free Ab (total Ab)	measures total Ab only different picture w affinity step
relative cheap consumables (ECL plates)	Relative cheap consumables (Trypsin)
Parallel measurement on MT-plates	Sequential measurement, but full automation established
Indirect assay principle	**Direct assay principle**
relies on high affinity detection Abs	relies on mass/charge separation
Produces nonlinear Calibration Curves = robustness, small dynamic range	Produces linear calibration curves, wide dynamic range
Assay interference = dilution nonlinearity,	no interference after cleanup procedure
Careful optimization and validation of assays = time critical	Fast optimization and validation



Thank you team

Volker Berweck and Kathrina Jäger, Dietmar Seemann, Gregor Schaffar
Protein Bioanalysis-Ludwigshafen

Laura J. Miesbauer, Melanie J. Patterson, Steven P. Cepa, and Robert W. Johnson
Structural Chemistry, Advanced Technology, Chicago

Marc R. Lake, Jeanne Cabel and Karl Walter
Protein Biochemistry, Advanced Technology, Chicago

Paul L. Richardson
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