Quantitative Analysis of therapeutic antibodies at the pg/mL range from serum samples via affinity enrichment coupled to LC-MS/MS

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How to quantitate an Antibody ?

Technologies used up to now

LIGAND BINDING ASSAYS

- ELISA (e.g. w Biotin - POD)
- ECL (Electrochemiluminescence)

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

Mass-spectrometry?

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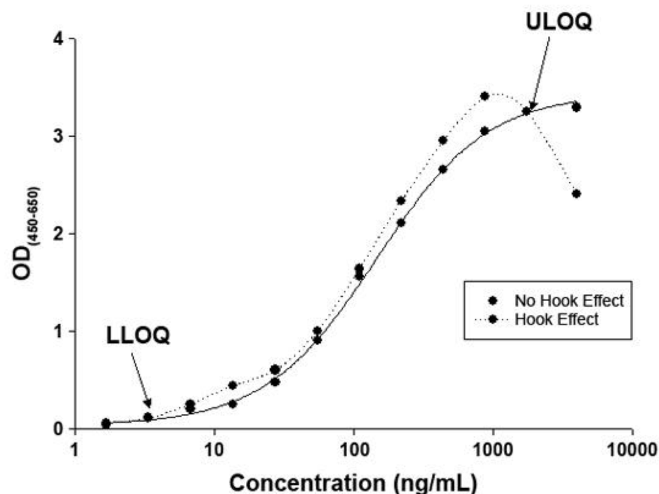
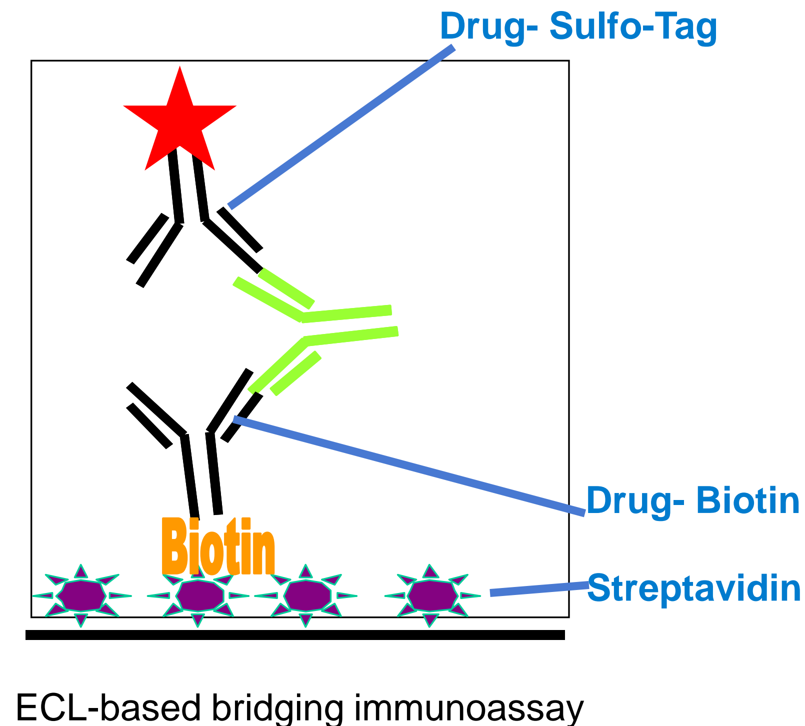
Ligand binding assays (LBA)

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

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

- Assay range, 4PL/5PL curve fit
- Matrix effects
- Non-linearity of response/ Dilution linearity





ECL-based bridging immunoassay

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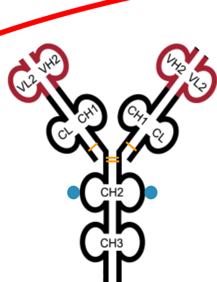
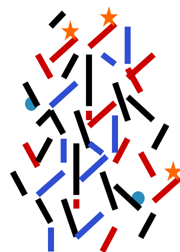
Theoretical approaches for MS based analysis

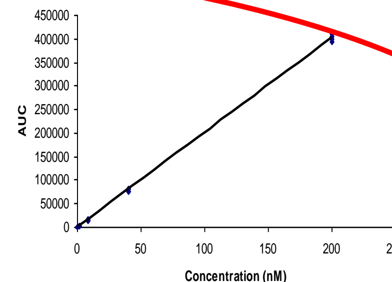
- Bottom-up (tryptic peptides)



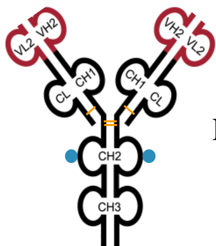
Trypsin-
Digestion
+Isotope-labeled
peptide



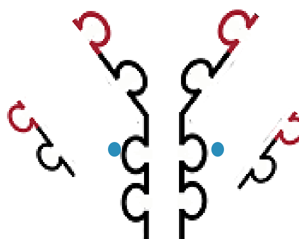
Analysis
Quantification



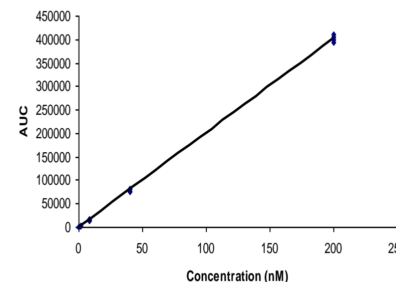
- Top-down (intact Antibody)



Denature
Reduce and Alkylate Cys



Analysis
Quantification



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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

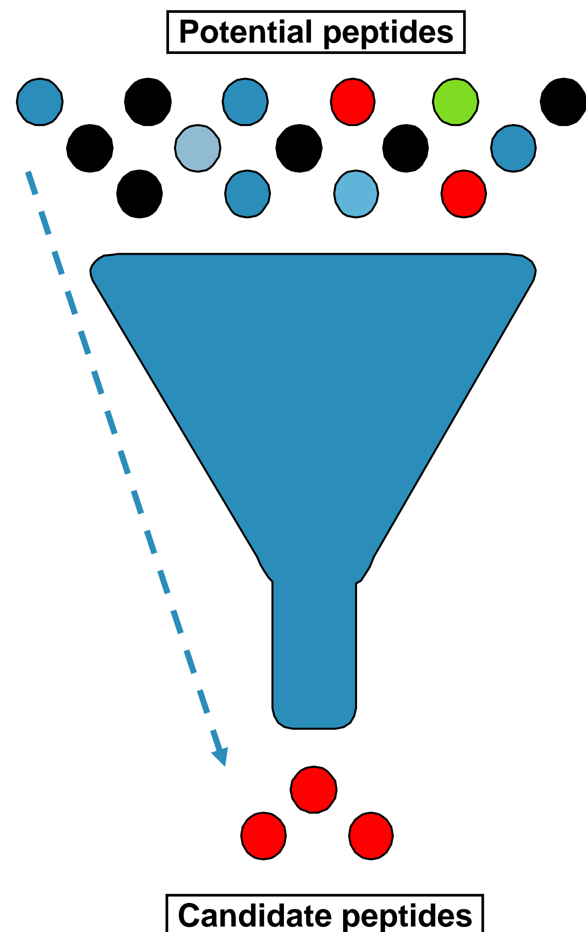
- Are the peptides species-specific?
- Will sample prep remove duplicate peptides?

Protein Digestion

- Confirm that expected peptides are produced
- Incomplete cleavage minimized

MS evaluation

- Select peptides that ionize and fragment appropriately



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Database Searching – are the Peptides Unique?

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**

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

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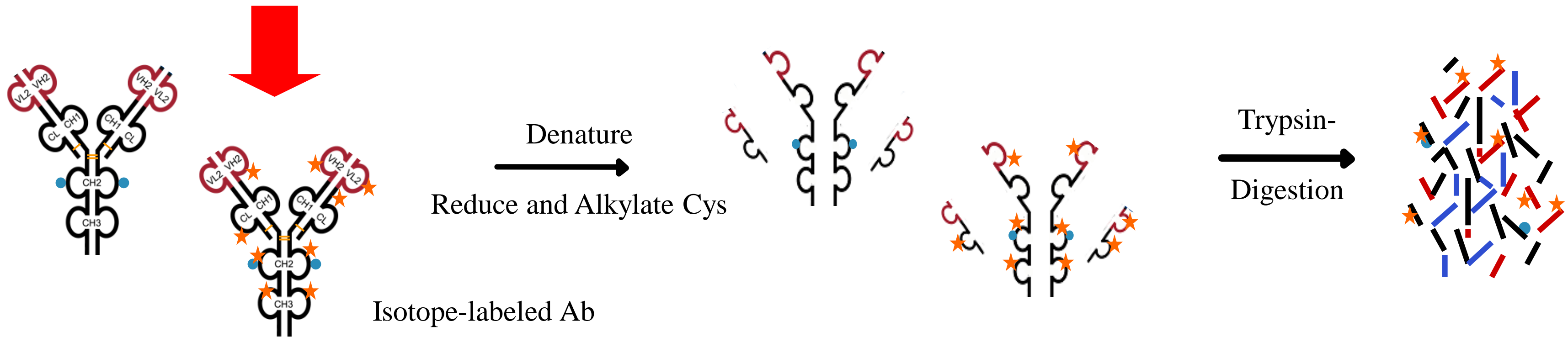
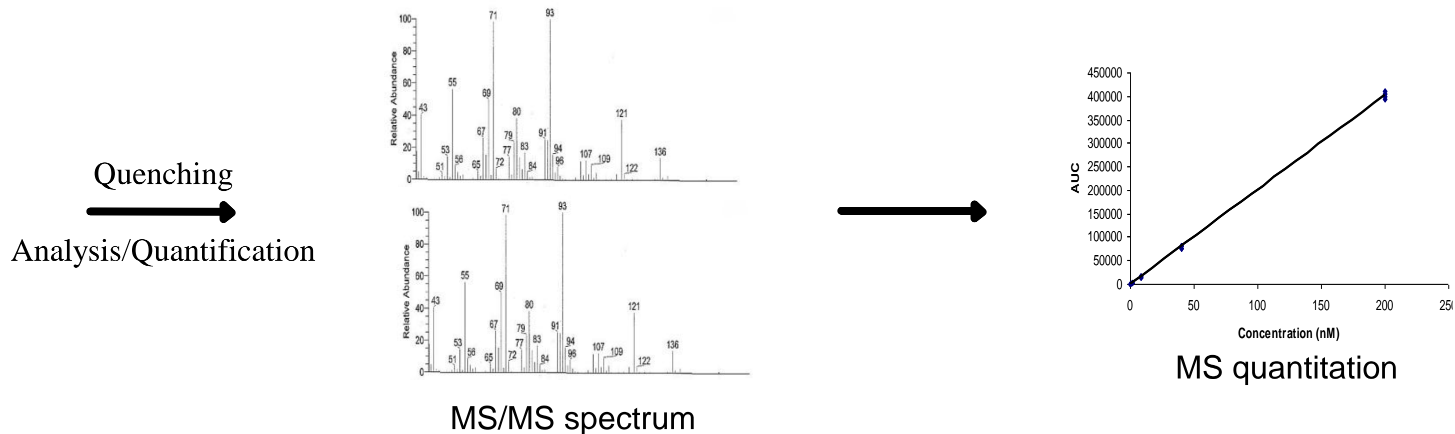
MS Approach for Absolute Antibody Quantitation

Antibody digestion



Analysis of peptides by LC-MS and quantitation



MS/MS spectrum

MS quantitation

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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

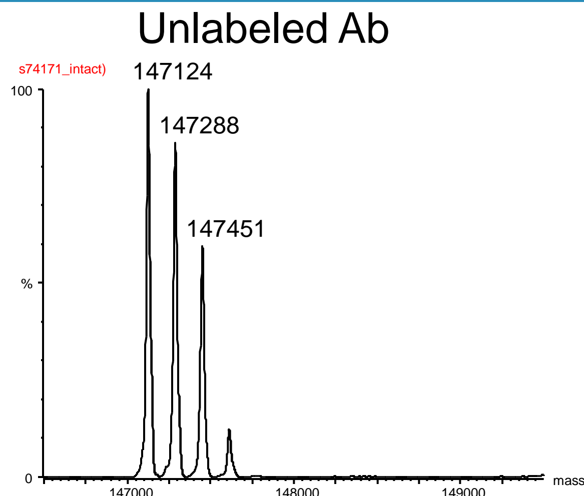
- SILAC Advanced DMEM-F/12 Flex medium (Invitrogen) supplemented as above. This medium lacks the amino acids lysine and arginine, and was supplemented with [U-$^{13}C_6$]-L-lysine and [U-$^{13}C_6$, $^{15}N_4$]-L-arginine

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

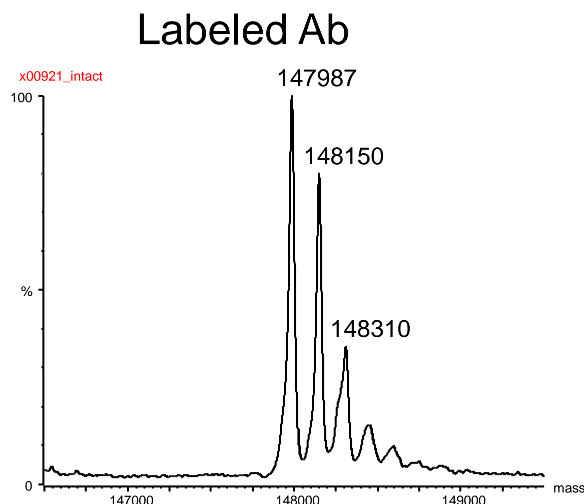
MS showed uniform incorporation of labeled amino acids in greater than 98% of the sample

★ - **Isotope labeled Lys or Arg**

Unlabeled Ab



Labeled Ab



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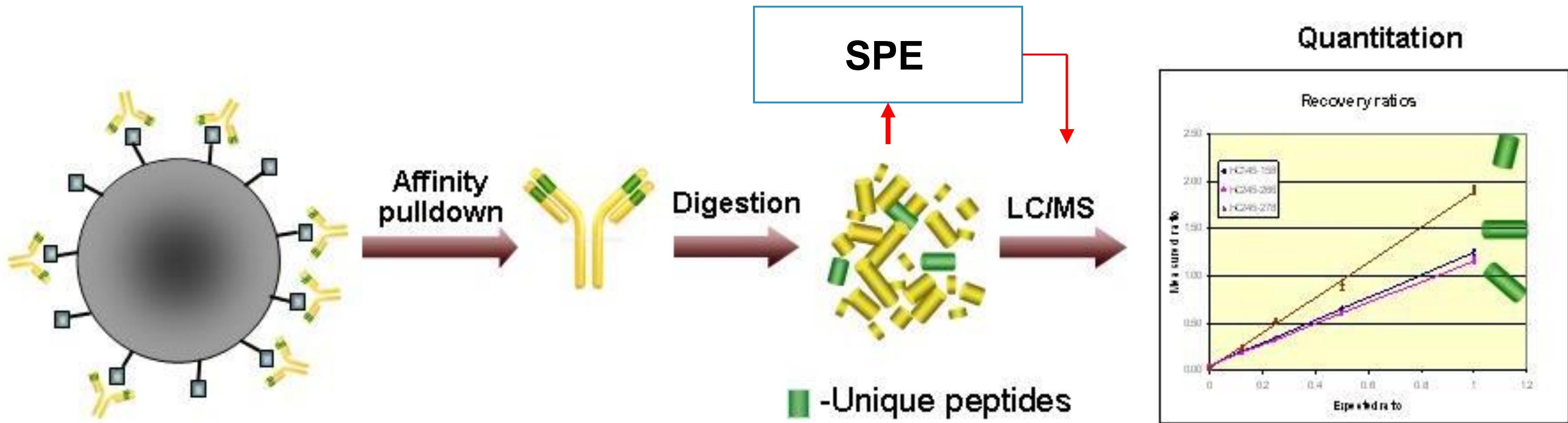
Method scheme

monitored by internal Standard



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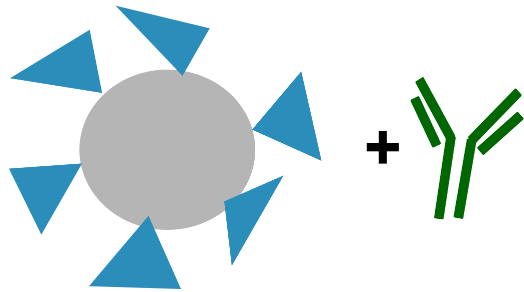
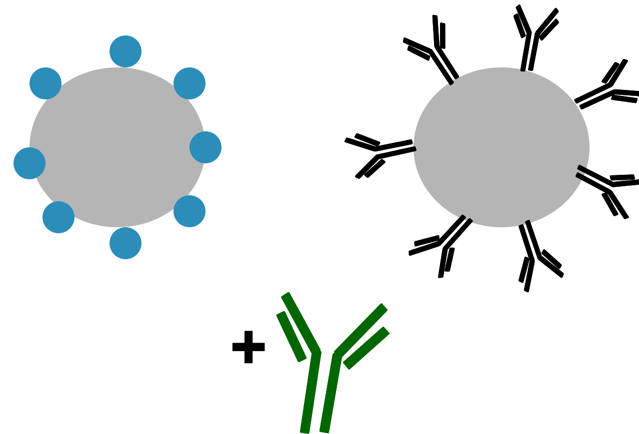
Evaluate affinity enrichment

Generic approach



Protein G-sepharose

Project specific approach



ligand or anti-idiotypic-sepharose

Therapeutic Ab captured → Matrix washed off → Therapeutic Ab digested

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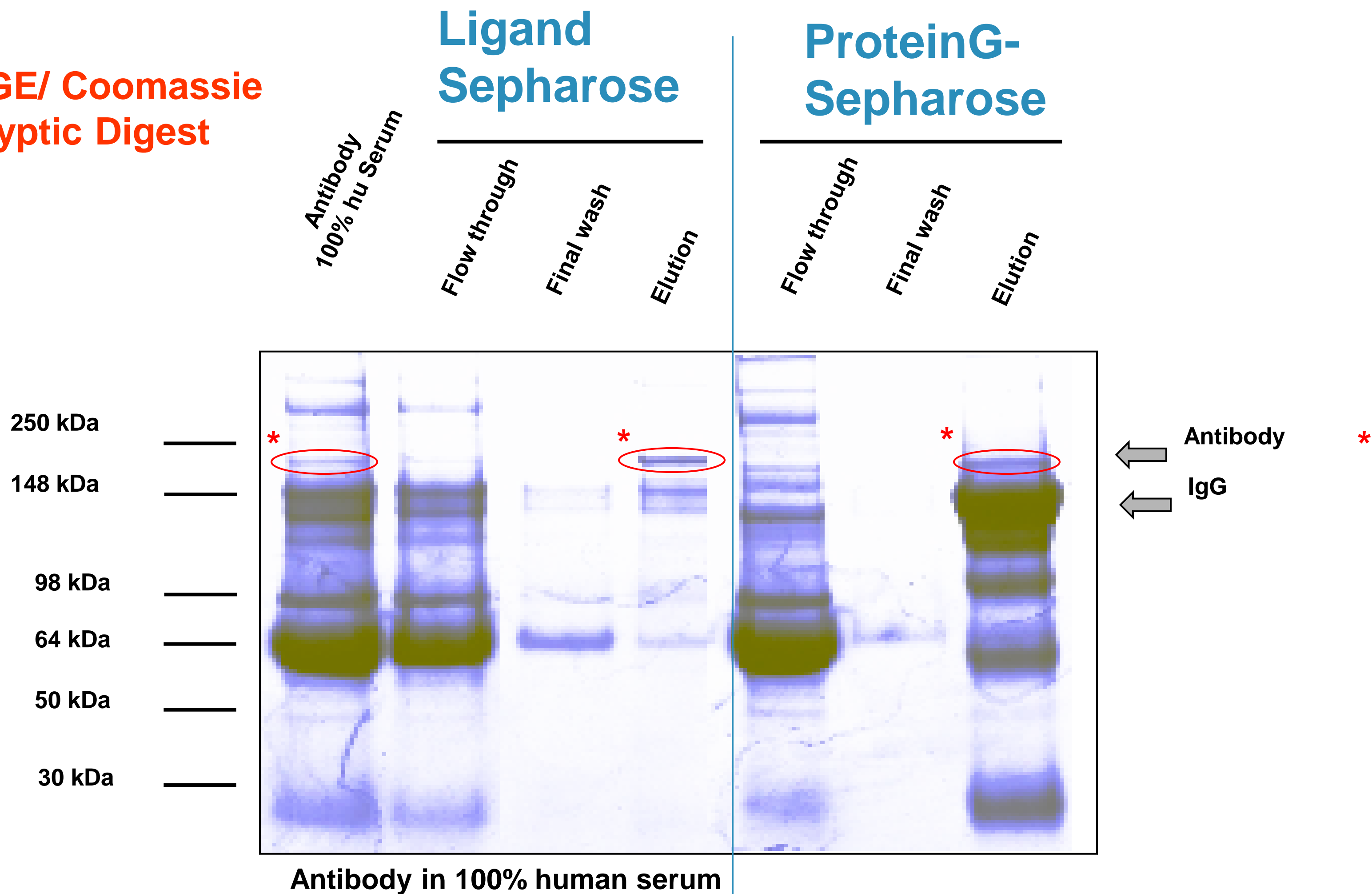
Peptide Recovery after affinity enrichment



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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

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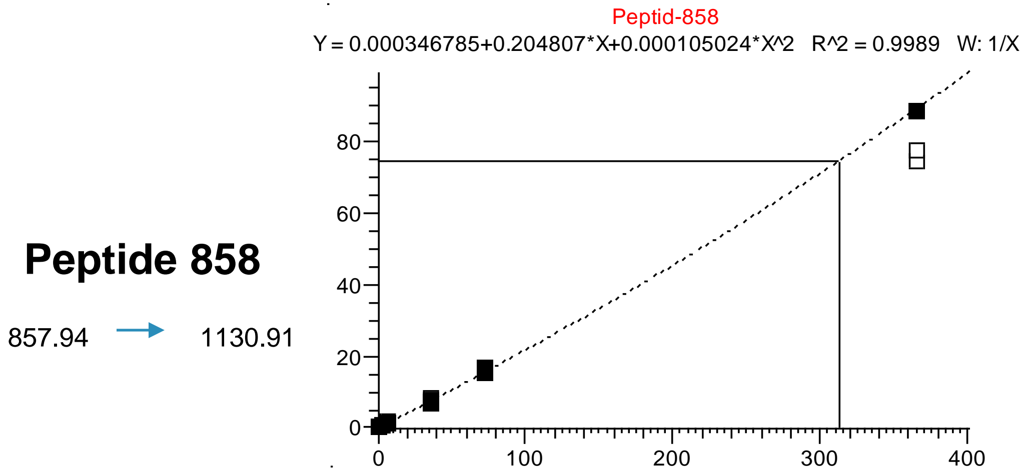
Quantitation via Tryptic Peptides – Quantitation is achievable at low ng/mL levels

- Antibody was purified from serum using Anti-idiotypic Ab coupled to sepharose
- After tryptic digestion, samples were analyzed using MS/MS (Orbitrap LTQ XL)
- MRM for each peptide as indicated were used for quantitation

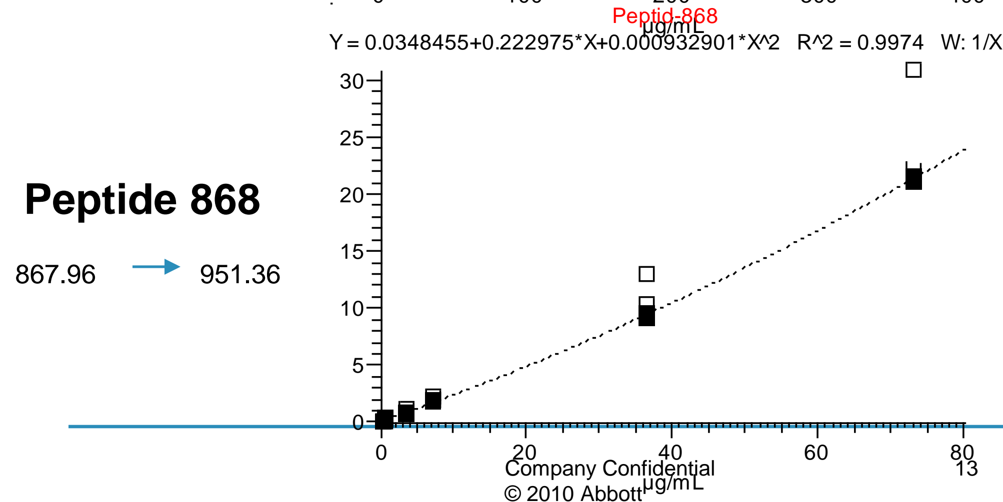
Peptide 858

857.94 → 1130.91



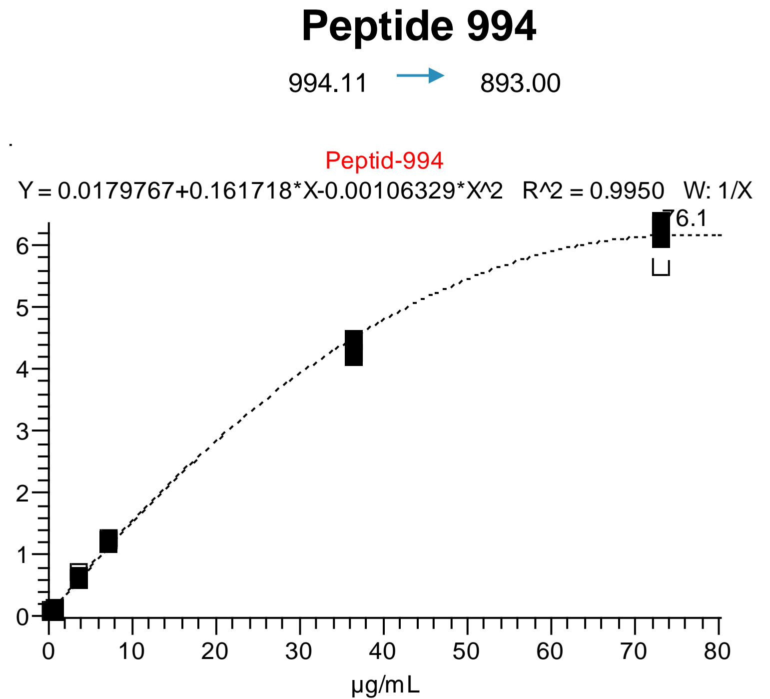
Peptide 868

867.96 → 951.36



Peptide 994

994.11 → 893.00



range: 0.365 - 365 µg/mL

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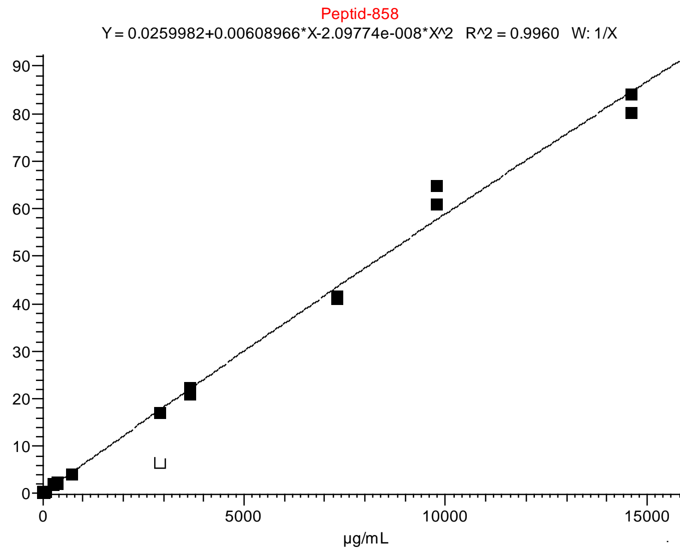
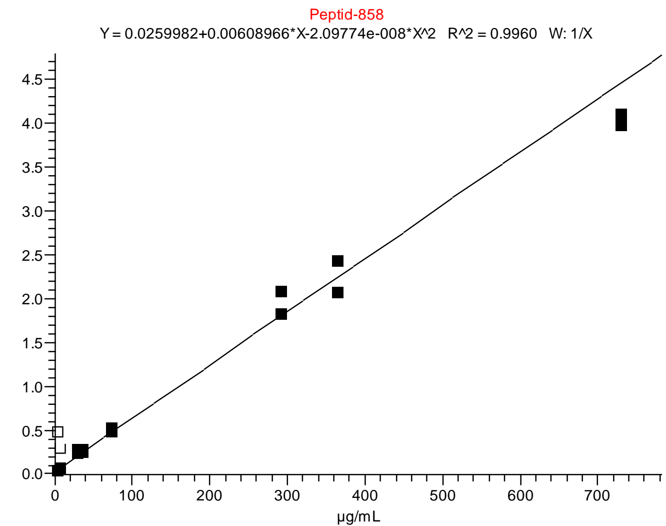
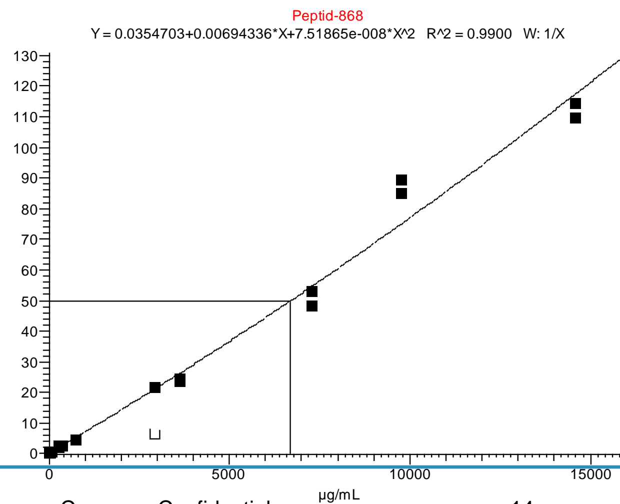
Quantitation over a broad dynamic range: up to 15,000 µg/mL as highest level

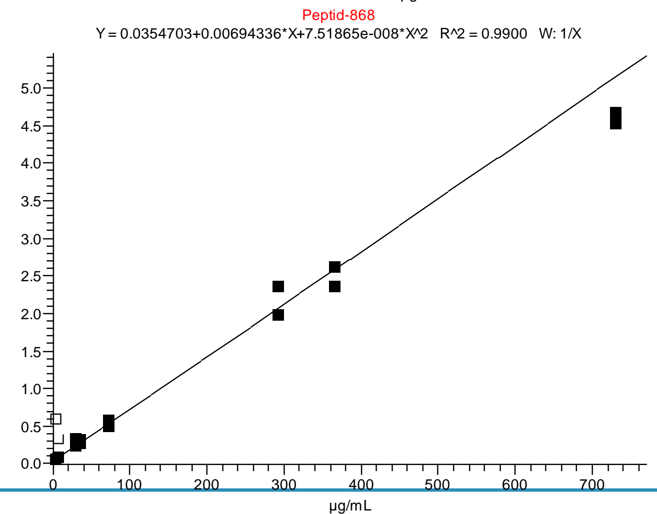
Peptide 858





Peptide 868





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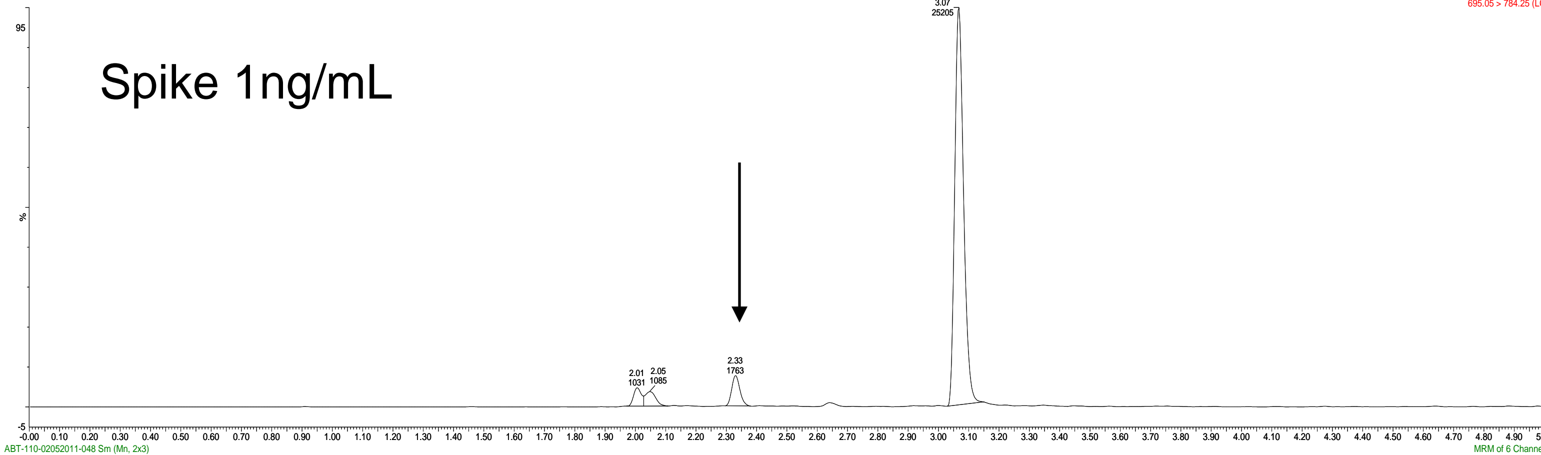
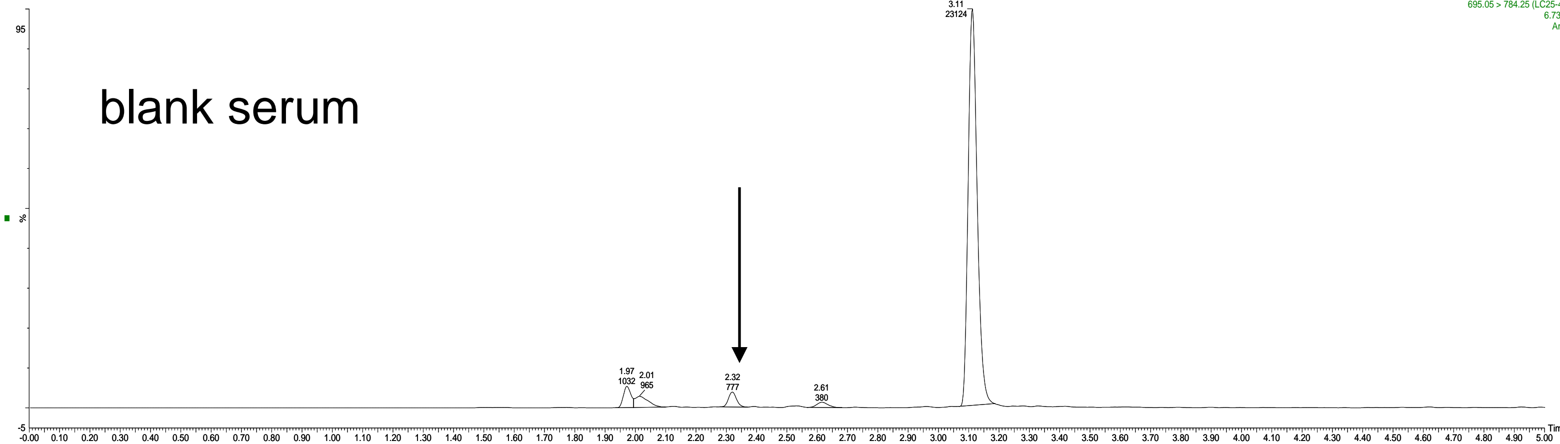
Outlook for excellent sensitivity





LLOQ
1 ng/mL

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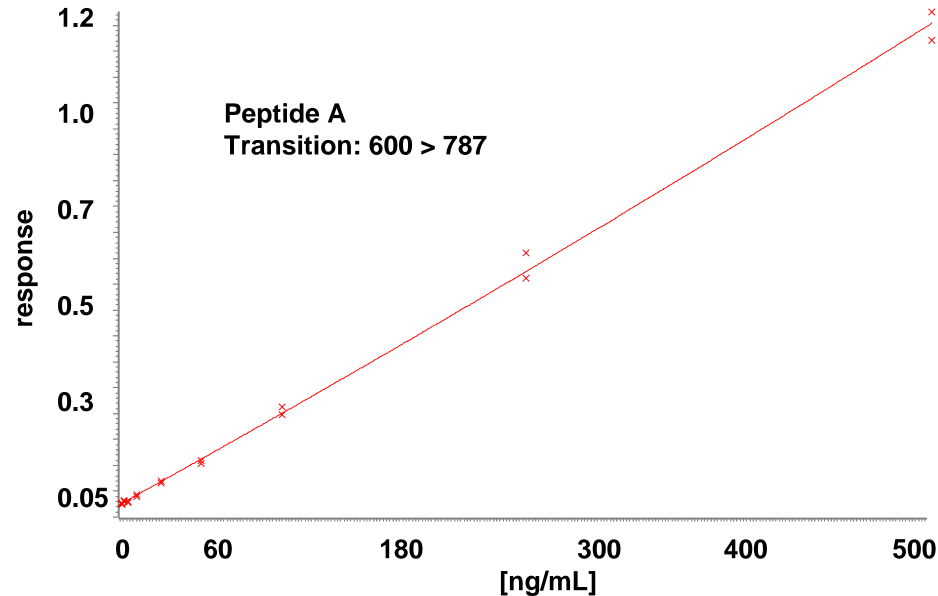
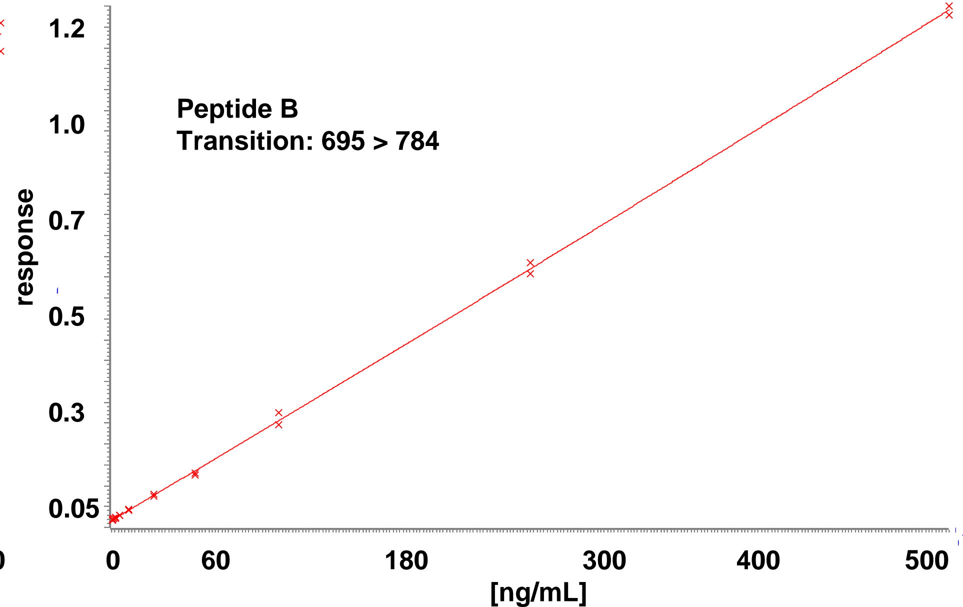
Outlook for excellent sensitivity





For support of micro-dosing studies:

- requires sensitivities in the pg/mL range
- low background (S/N)
- „enrichment“ by large sample volumes (e.g. 300 µL human serum)

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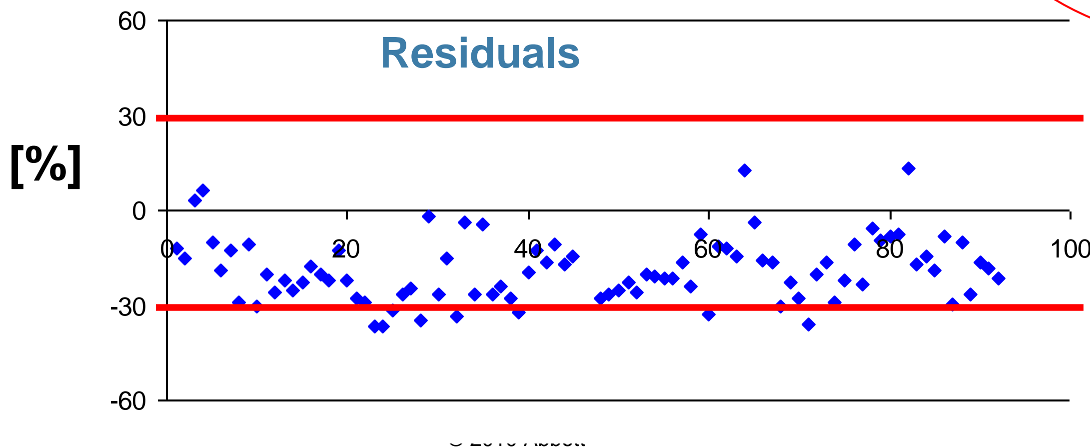
Cross-validation to Ligand Binding Assay

- **design**:
- therapeutic mAb IV at two doses: 20 and 100 mg/kg
- 90 serum samples from CD1 mice were analyzed
- 2 full PK profiles (male)

outcome:
- 89% samples passed ISR acceptance

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



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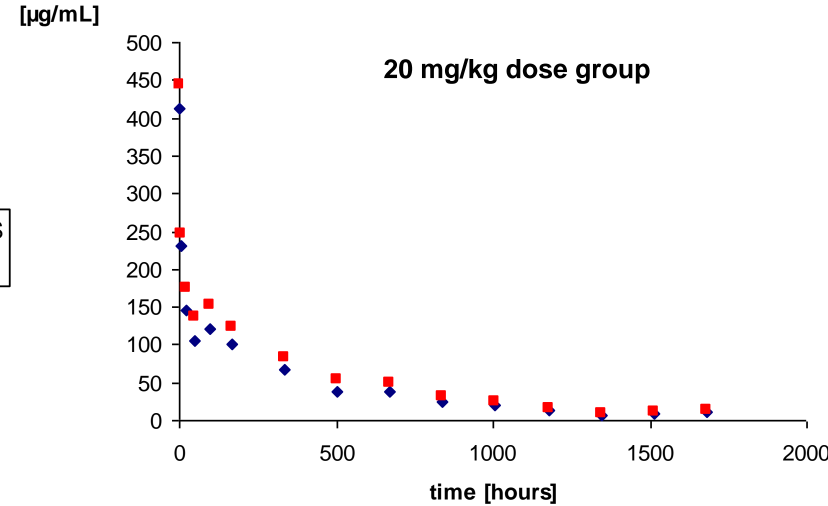
Results of cross-validation

20 mg/kg dose group

mAb [µg/mL]

1000
100
10

◆ LC-MS
■ ECL



100 mg/kg dose group

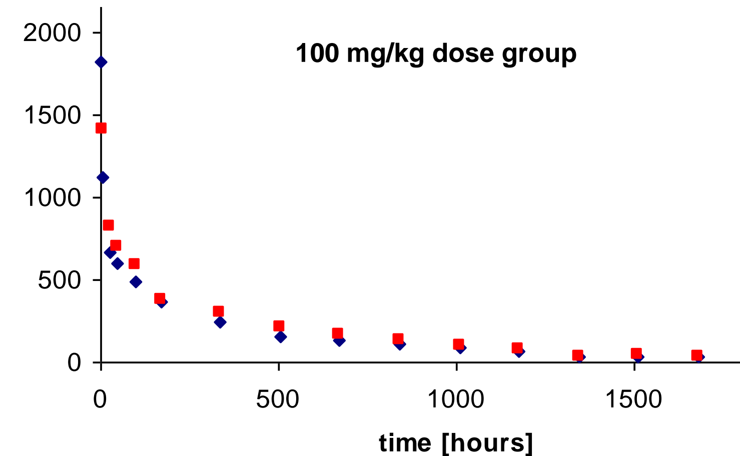
mAb [µg/mL]

10000
1000
100
10
1

0
500
1000
1500
2000

time [hours]

◆ LC-MS
■ ECL



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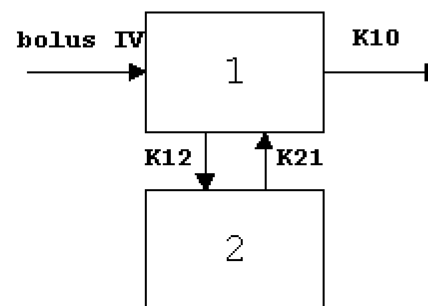
PK analysis of results from both platforms

	AUC [h* mg* mL]	Cmax [mg/mL]	MRT [h]	Cl [L/h/kg]
ECL 20 mg/kg	84.6	0.443	440	0.22
LC-MS 20 mg/kg	67.8	0.412	438	0.28
ECL 100 mg/kg	326	2.38	429	0.29
LC-MS 100 mg/kg	276	1.82	429	0.34

Non-compartmental PK Evaluation

Compartmental PK Evaluation

2 compartment IV-Bolus, micro-constants, no lag time, 1st order elimination



C(T)=A*EXP(-ALPHA*T)+B*EXP(-BETA*T)

<u>Outcome</u>:

only 17% difference

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LC-MS/MS method: Throughput and performance

- **Throughput**: ~ **1000 - 1400 samples per week**

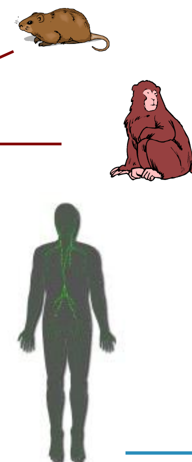
- **Sensitivity**: ~ 1- 2 µg/mL (for tox studies)
  ~ 0.5 -1 ng/mL (for clinical studies)

- **Dynamic range**: minimum three orders of magnitude

- **Short development times**

  method applicable across species

Single assay



- **Low samples volume**: 10 µL or less for Tox studies

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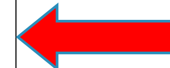
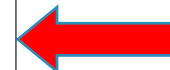
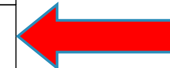
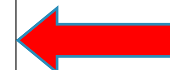
Challenges of LBA vs. reasons for MS method

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** 
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

potentially 0.1 ng/mL

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Thank you team

Volker Berweck and Kathrina Jäger, Dietmar Seemann, Gregor Schaffar
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Backup slides

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Current papers published

Anal. Chem. 2008, 80, 4200–4207

Towards Absolute Quantification of Therapeutic Monoclonal Antibody in Serum by LC–MS/MS Using Isotope-Labeled Antibody Standard and Protein Cleavage Isotope Dilution Mass Spectrometry

Olivier Heudi,[*,†] Samuel Barteau,[†] Dieter Zimmer,[†] Joerg Schmidt,[‡] Kurt Bill,[‡] Natalie Lehmann,[‡] Christian Bauer,[‡] and Olivier Kretz[†]

DMPK/Bioanalytics, and Biotechnology Development, Novartis Pharma AG, CH-4002 Basel Switzerland

Absolute Quantification of Monoclonal Antibodies in Biofluids by Liquid Chromatography–Tandem Mass Spectrometry

Charlotte Hagman,[†] Darrell Ricke,[‡] Stefan Ewert,[§] Stephan Bek,[||] Rocco Falchetto,[†] and Francis Bitsch[*,†]

BioAnalytical Sciences, Discovery Technologies, Novartis Institutes for BioMedical Research, Novartis, Basel, CH-4002 Basel, Switzerland, Bioinformatics, BioMedical Informatics, Novartis Institutes for BioMedicalResearch, Inc., Cambridge, Massachusetts 02139, Biomolecules Production, Antibody Center, Novartis Institutes for BioMedical Research, Novartis, Basel, CH-4002 Basel, Switzerland, and Soluble Biomarkers, Novartis Institutes for BioMedical Research, Novartis, Basel, CH-4002 Basel

Poster from National Biotech Conference 2010

Beyond Immunoassays: Comparative pharmacokinetics of Monoclonal Antibodies using LCMS and ELISA

S. Bilic1, S. Kakar1, S. Ettenberg2, M. Daley2, B. Granda2, Z. Yang2, B. Jaitner3, D. Stoellner3, H. Schran1, A. Skerjanec1
1Novartis Oncology, 2Novartis Institute of Biomedical Research, 3Novartis Biologics

Purpose.
To compare the pharmacokinetics of three different monoclonal antibodies (mAbs) in mouse, rat or cynomolgus monkeys (cyno) based on serum drug concentration using LC-MS and ELISA.
Methods.
Single doses of the three Novartis monoclonal antibodies were administered and 5 to 15 serum samples were obtained up to 21 days post i.v. dose. MAb 1 was tested in cyno at doses of 20 and 100 mg/kg, mAb 2 was tested in rat at a dose of 10mg/kg and mAb 3 was tested in mouse at doses of 2, 10, and 50 mg/kg. The serum samples were split and analyzed using Enzymed Linked Immunosorbant Assay (ELISA) **or** Liquid Chromatography - Mass Spectroscopy (LC-MS). One LC-MS assay was developed for each mAb and was used to analyze sera across all species. An ELISA method was developed for each mAb and species. Serum samples were analyzed in duplicate or triplicate; the average was used as the final result. Pharmacokinetic (PK) parameters were estimated using noncompartmental and compartmental methods.
Results.
In general, there was good agreement between the two methods. Mean concentration ratios (SD) of ELISA vs. LC-MS were 1.02 (0.52), 1.02 (0.09), and 0.88 (0.23), respectively for the three antibodies. Ratios were similar across antibody doses and species. Differences between the pharmacokinetic parameters (AUC, Cmax, t1/2) derived from LC-MS and ELISA were less than 15%.
Conclusion.
This preliminary analysis **suggests that LC-MS can be applied across species**, as it is not compound or matrix specific. However, since the LLOQ of the LC-MS is up to 25-fold lower, it potentially limits the utility of LC-MS in analyzing concentrations following lower MAb doses. Based on the comparable results between ELISA and LC-MS, the two assays **can be considered interchangeable for the purpose of estimating the PK characteristics of MAbs**.

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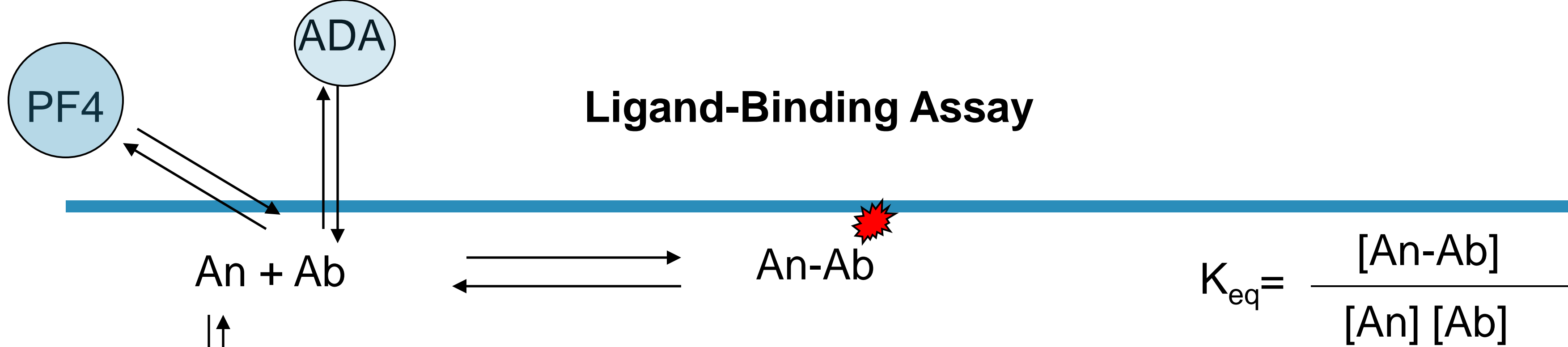
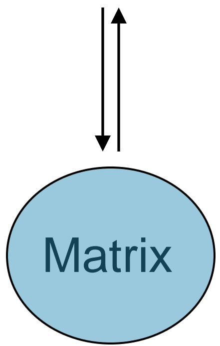
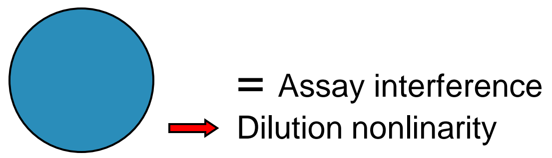
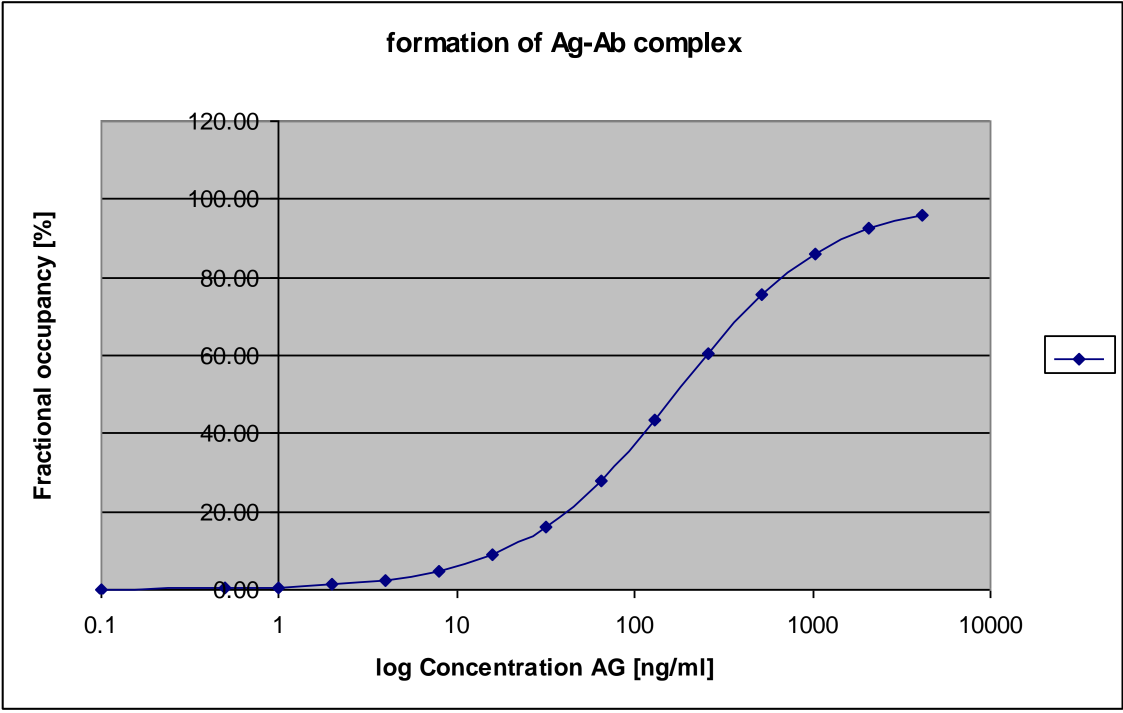
Ligand-Binding Assay









5 Parameter-logistic fitting: F(x) = A + (D/(1+(X/C)^B)^E)

Evaluation of ADA interference

LC-MS Method for ABT-xxx

	Spike 2		
nominal conc.	[105 µg/mL]		
	ratio	conc.	%Difference
QC1 c=14.4 µg/mL	0.1	0.38	-97.4
QC2 c=78.84 µg/mL	0.8	44.15	-44.0
QC3 c=788.4 µg/mL	7.5	619.0	-21.4
QC4 c=3942 µg/mL	37.5	3462	-12.2

LBA for ABT-yyy ECL PK Assay

	Spike 1		
nominal conc.	[100 ng/mL]		
	ratio	conc.	%Difference
QC1 c=2000 ng/mL	20	2148	3.1
QC2 c=1000 ng/mL	10	905.2	-4.9
QC3 c=500 ng/mL	5	359.6	-26.1
QC4 c=125 ng/mL	1.25	76.45	-37.8

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