LC-MS/MS quantification of proteins and peptides in biological fluids : towards a generic approach

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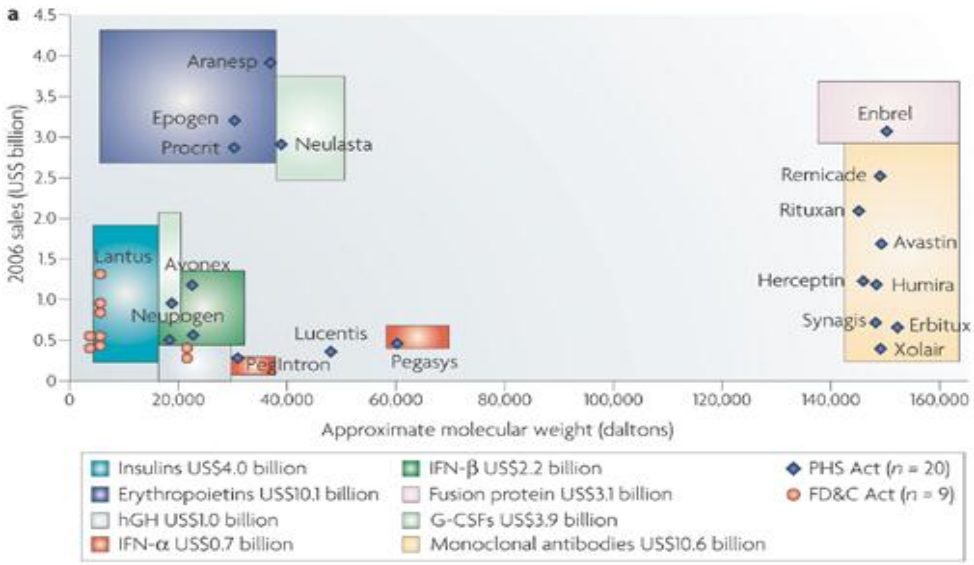
Outline

- Introduction
  - Monoclonal antibodies (mAbs) as therapeutic proteins
  - Strategies for LC-MS/MS assay development
- Methods development and validation
  - Protein cleavage with labeled peptide as ISTD (AQUA)
  - Protein cleavage with analog protein as ISTD (QconCAT)
  - Protein cleavage with analog labeled protein as ISTD (PSAQ)
- Assay Development Time and Throughput
- Conclusions

Economic figures for therapeutic protein products



Lanthier et al. Nature Reviews Drug Discovery (2008); 7: 733-738

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Strategies for LC-MS/MS assay development

MQALVLLLCIGALLGHSSCQNPASPPEEGSPDPDSTGALVEEEDPFFKV
PVNKLAAAVSNFGYDLYRVRSSMSPTTNVLLSPLSVATALSALSLGAEQ
RTESIIHRALYYDLISSPDIHGTYKELLDTVTAPQKNLKSASRIVFEKKLRIK
SSFVAPLEKSYGTRPRVLTGNPRLDLQEINNWVQAQMKGKLARSTKEIP
DEISILLLGVAHFKGQWVTKFDSRKTSLEDFYLDEERTVRVPMMSDPKA
VLRYGLDSDLSCKIAQLPLTGSMSIIFFLPLKVTQNLTLIEESLTSEFIHDID
RELKTVQAVLTVPKLKLSYEGEVTKSLQEMKLQSLFDSPDFSKITGKPIKL
TQVEHRAGFEWNEDGAGTTPSPGLQPAHLTFPLDYHLNQPFIFVLRDTD
TGALLFIGKILDPRGP

Enzymatic Digestion

[H]VPVNK[OH]
[H]VPVNK[OH]
[H]LAAAVSNFGYDLYR[OH]
[H]LAAAVSNFGYDLYR[OH]
[H]LAAAVSNFGYDLYR[OH]
[H]TESIIHR[OH]
[H]ALYYDLISSPDIHGTYK[OH]
[H]ELLDTVTAPQK[OH]
[H]SASR[OH]
[H]IVFEK[OH]
[H]SSFVAPLEK[OH]
[H]SYGTR[OH]
[H]LDLQEINNWVQAQMK[OH]
[H]FDSR[OH]
[H]TSLEDFYLDEER[OH]
[H]YGLDSDLSCK[OH]
[H]LSYEGEVTK[OH]
[H]LQSLFDSPDFSK[OH]
[H]DTDTGALLFIGK[OH]

Targeted Peptide Quantitation using SRM's

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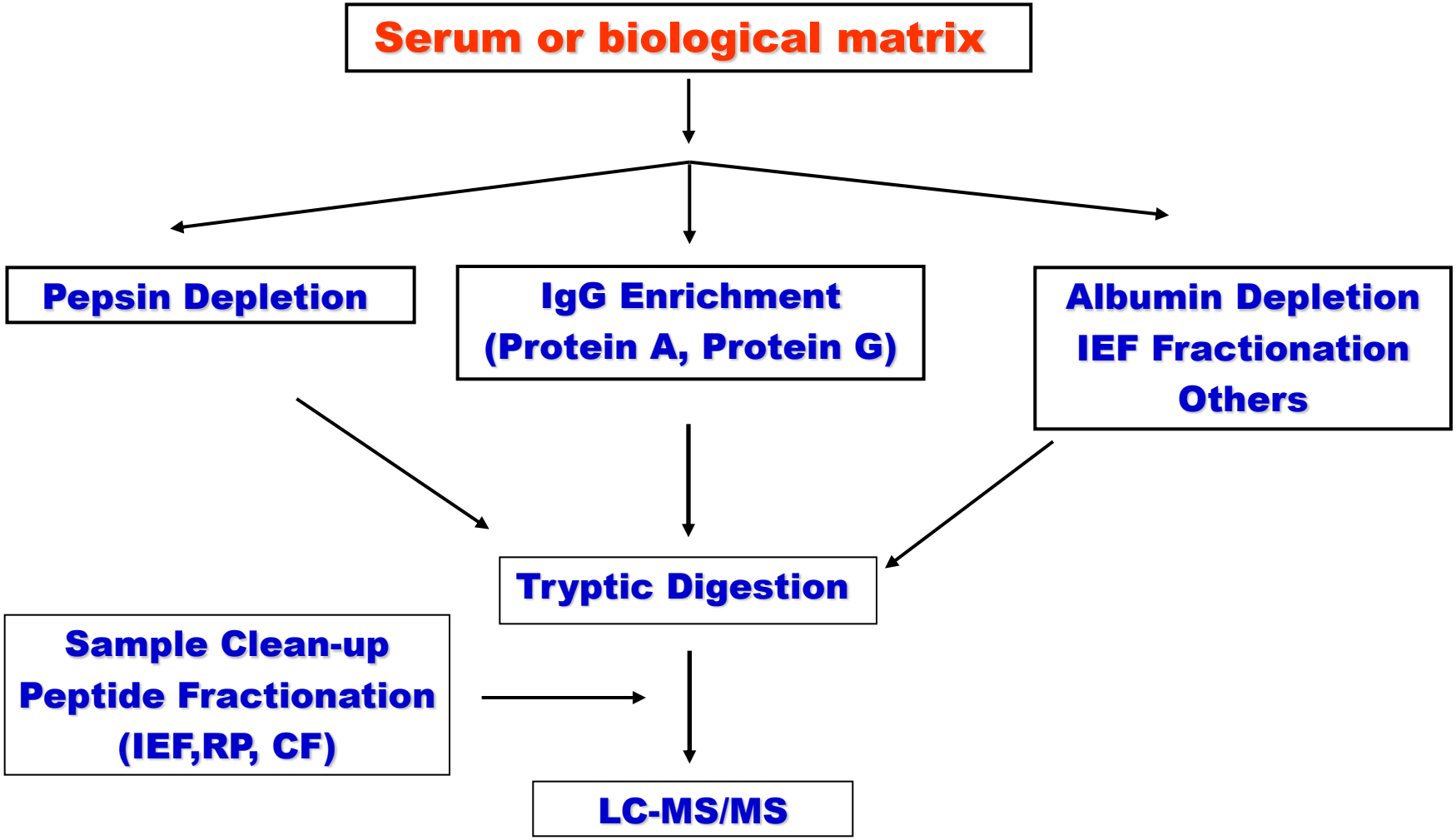
Sample preparation strategies



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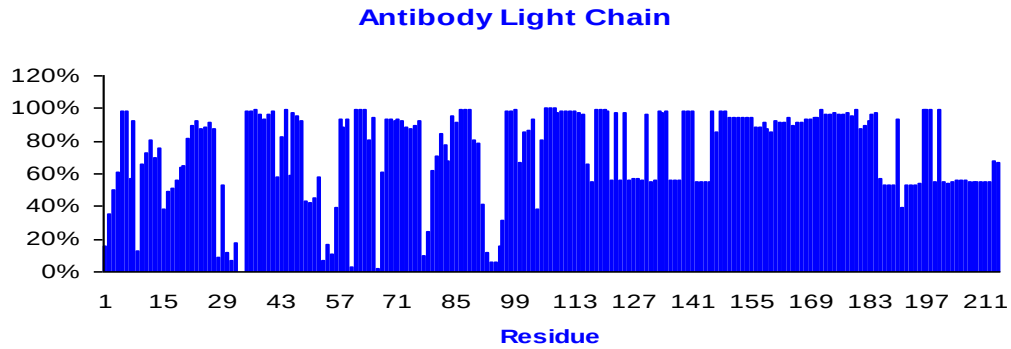
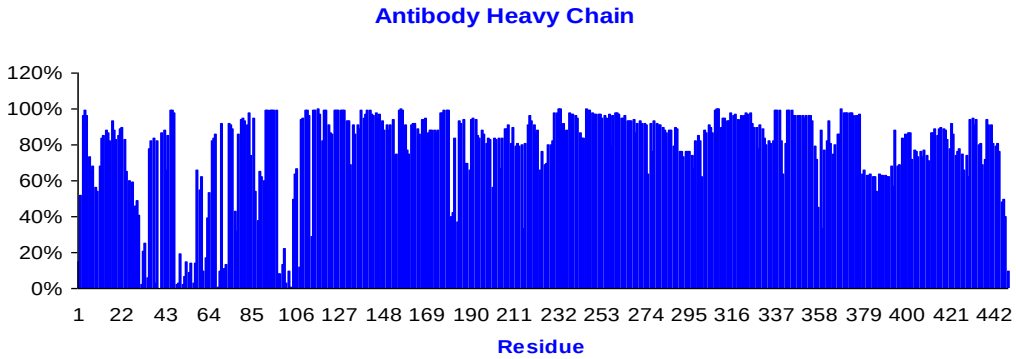
In-silico prediction of the most unique peptide

A bio-informatics tool was constructed based on sequence alignment with germline and mAb.



25 AAAAAAAAAAAAAAAA 39

50 AAAAAAAAAAAAA 61



39 AAAAAAAAAAAAAAAAAAAAAA 59

99 AAAAAAAAAAAAAAAAAAAAAAAAA 122

- **An isotopic variant of one of the unique peptide was synthesized**
- **In the absolute quantification study of the anti-ILβ mAb's PK, this peptide will be used as internal standard.**

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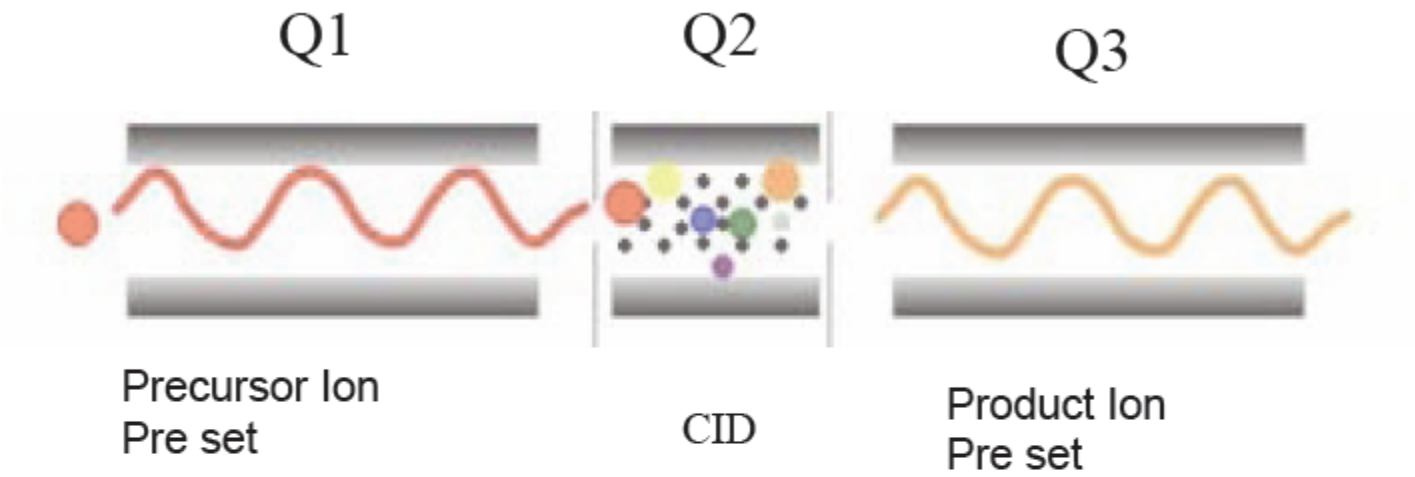
Schematic of Reaction Monitoring High Selectivity and Sensitivity



A “proteotypic” peptide is selected as being representative of a targeted protein. Q1 is set to transmit only the parent m/z of the selected peptide and Q3 is set to transmit only a selected fragment ion. Quantum has a very fast SRM dwell time (2 ms), so that multiple SRM (MRM) analyses can be carried out during each scan of an LC/MS/MS run. It is practical to monitor up to 300 SRM transitions/sec.

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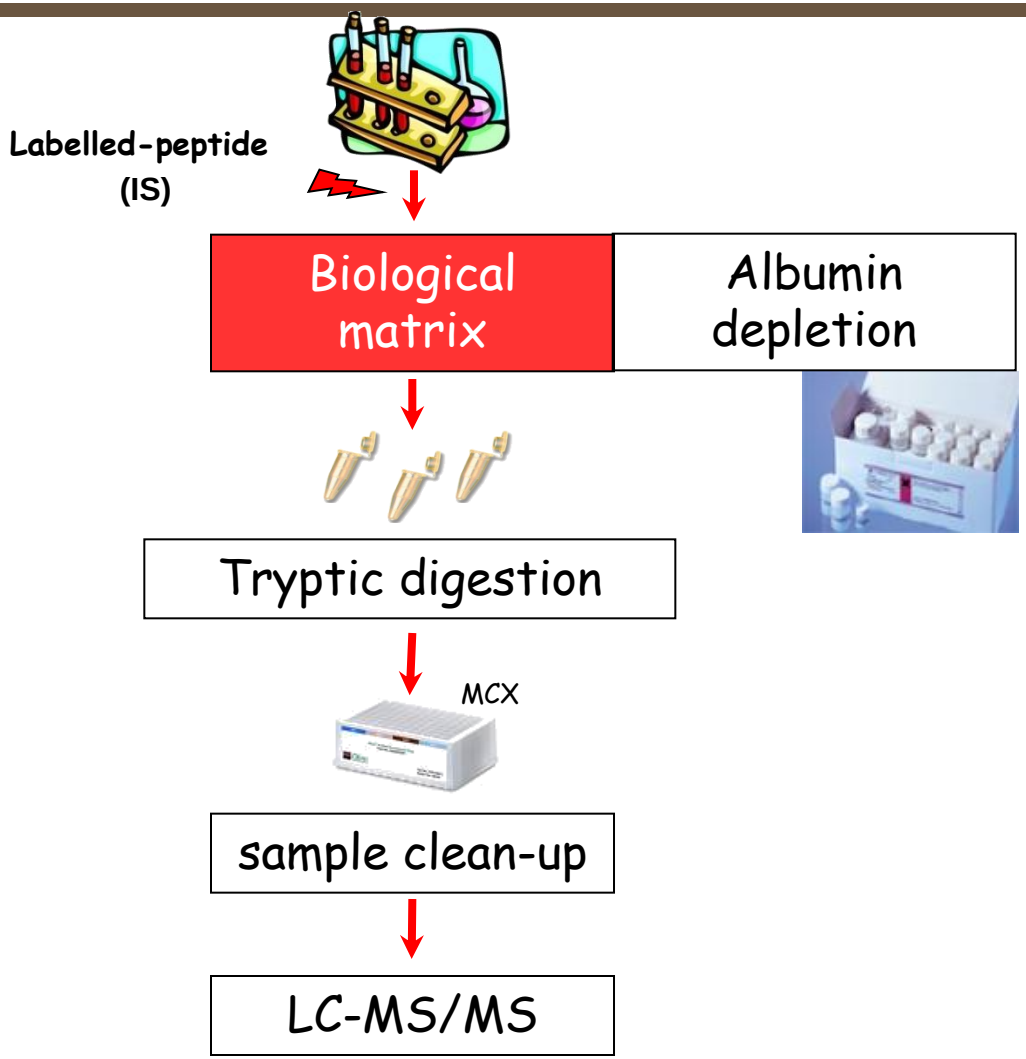
Analytical assay workflow (AQUA)



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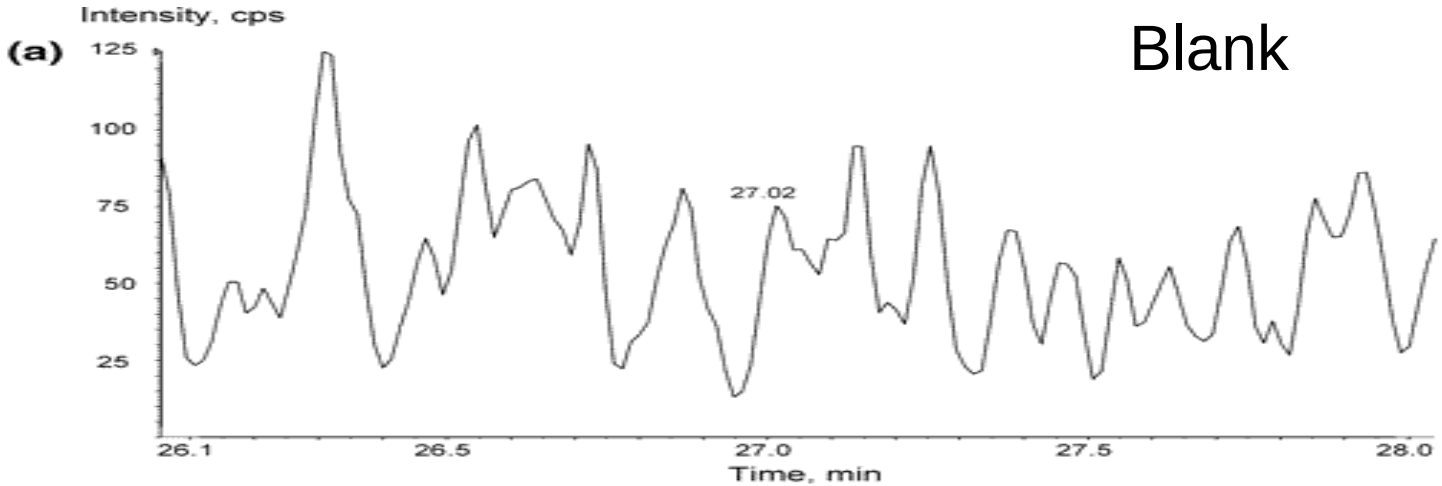
Albumin depletion and trypsin digestion



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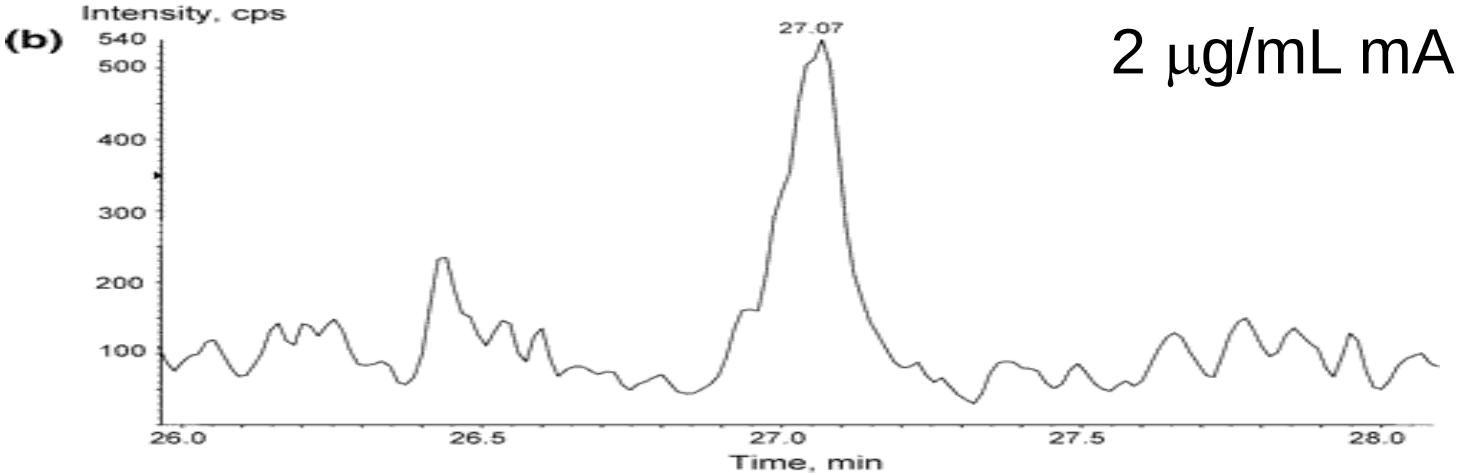

2 μg/mL mAb

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Preliminary validation data

- Good S:N (LLOQ 2 µg/mL), no background in monkey serum
- Linear from 2-1000 µg/mL, R^2 ≥0.998

Precision CV (%)	Intra-day (n=3 replicates)	≤ 9.0%
	Inter-day (n=3 assays)	≤ 9.9%
Accuracy (%)	Assay	99.0-111%
	Overall	96.9-111%

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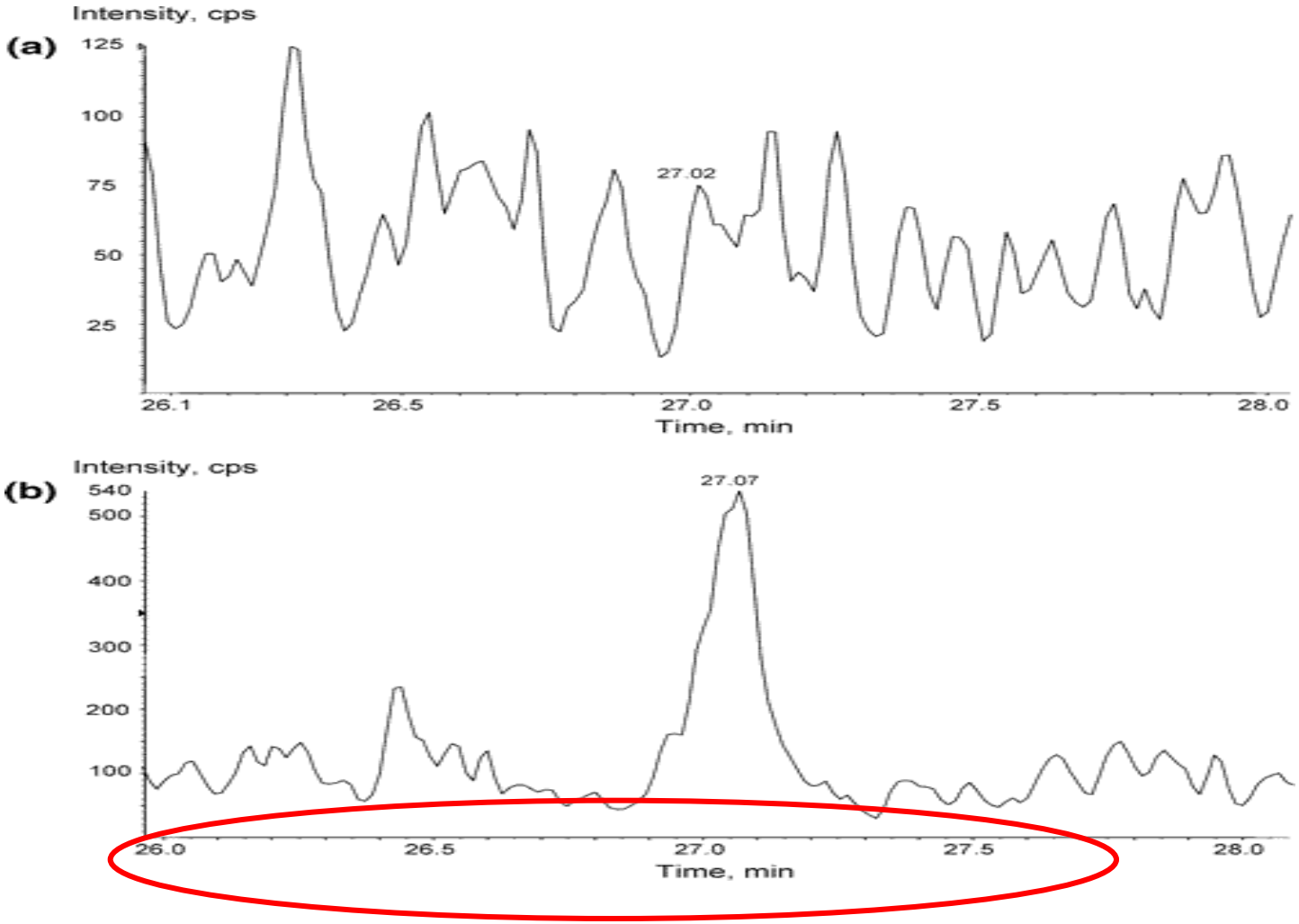
Major drawback of the method



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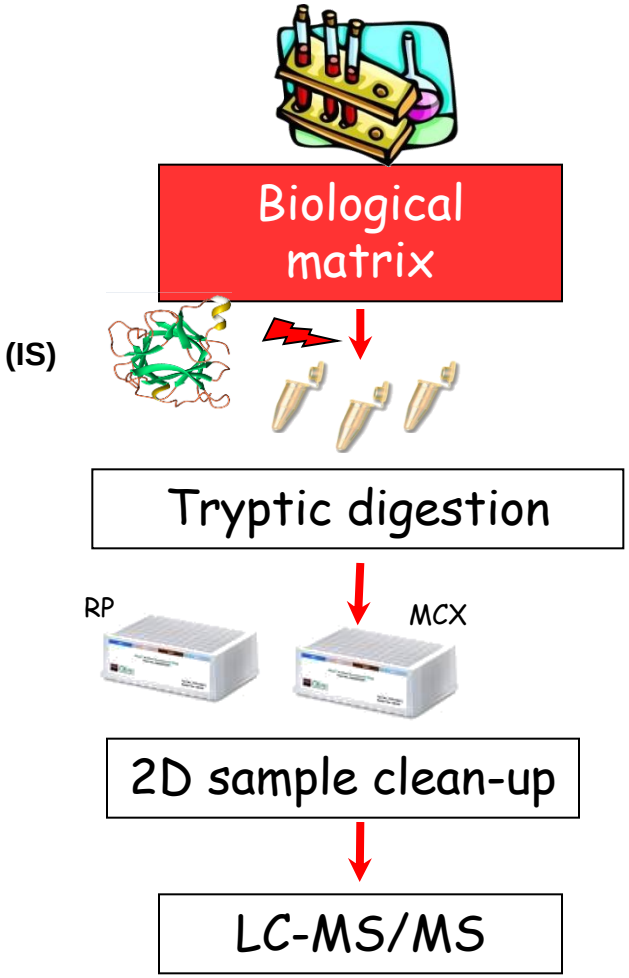
Analytical assay workflow (QconCAT)



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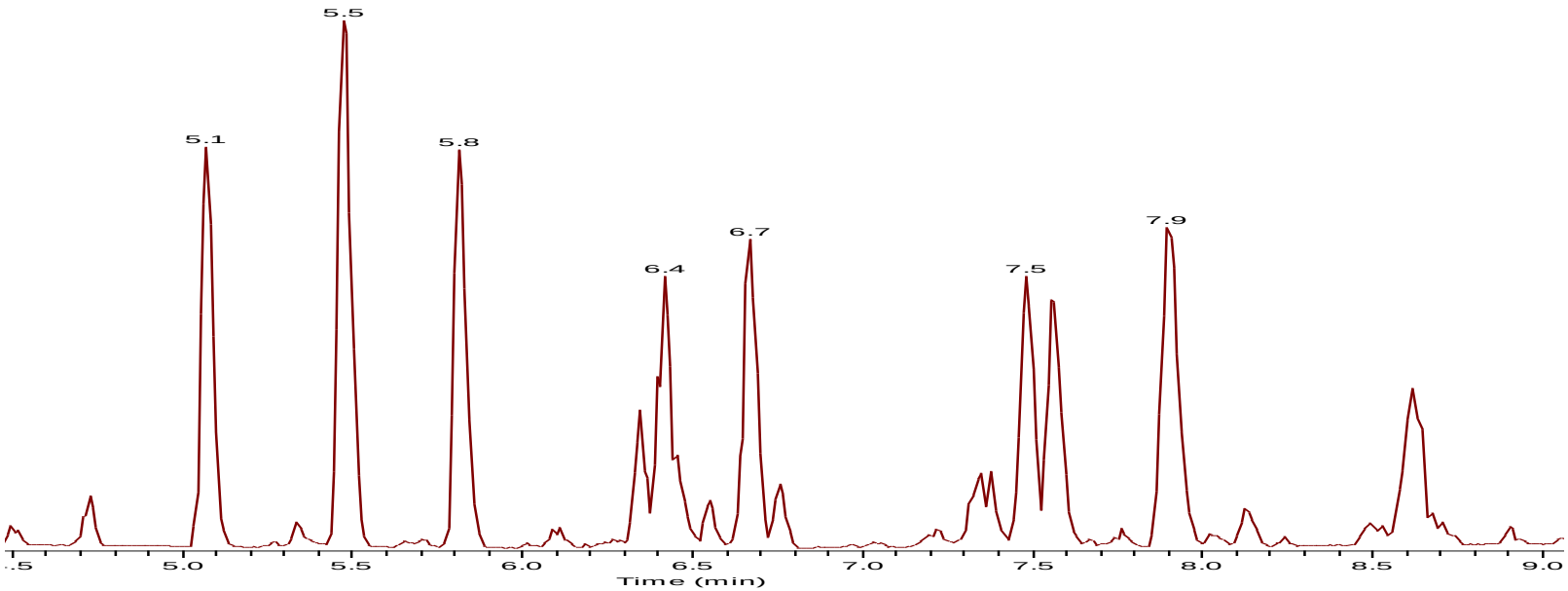
Protein Internal Standard

- Bovine fetuin

  Protein size: 38 KD

  Many tryptic peptides are unique from serums of common study species



- Spiked into samples at the beginning of the assay

  Monitor whole process of the assay, including protein digest step

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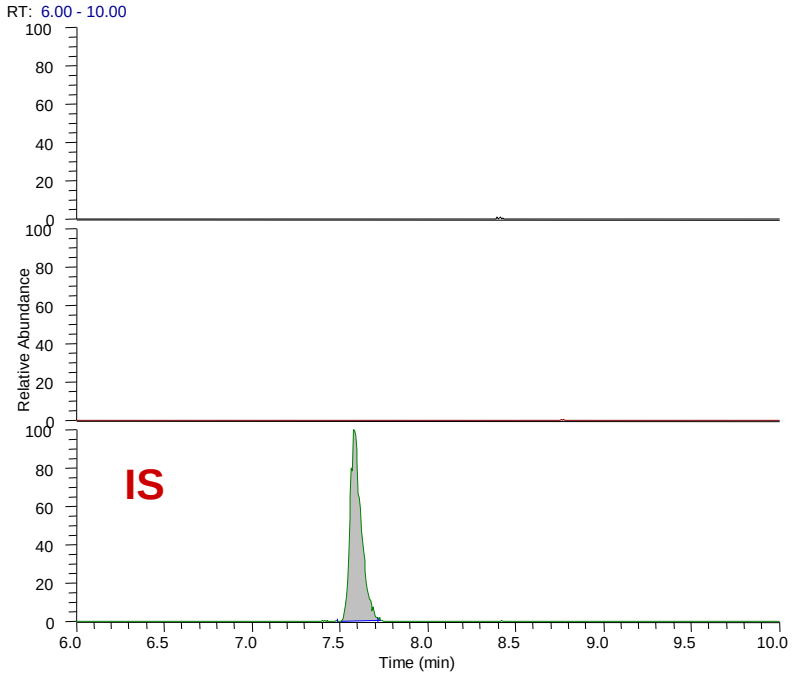
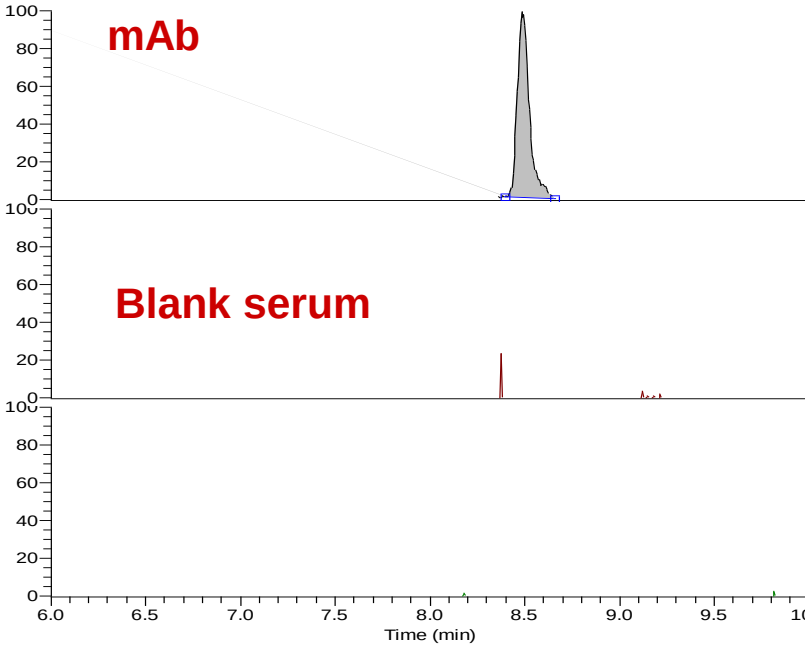
LC-MS/MS assay selectivity

- **Peptide ion of IS (bovine fetuin): TPIVGQPSIPGGPVR and mAb**

IS MS/MS transition
738.09 / 879.50



mAb MS/MS transition
786.71 / 886.94



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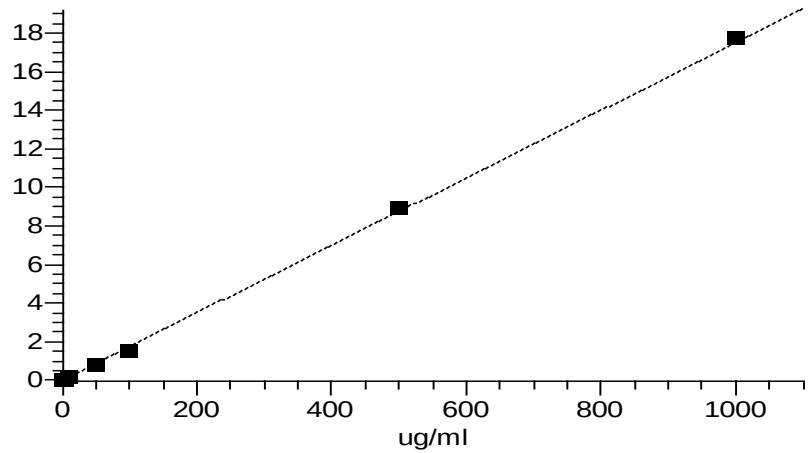
Calibration and precision data

Assay calibration curve



Linear range: 0.5-1000 µg/mL
R^2: 0.9943

Normalized by Protein

Accuracy and Precision

QC (µg/mL) (4 replicates)	Accuracy (%)	Precision CV (%)
1	96	9
3	102	9
100	94	6

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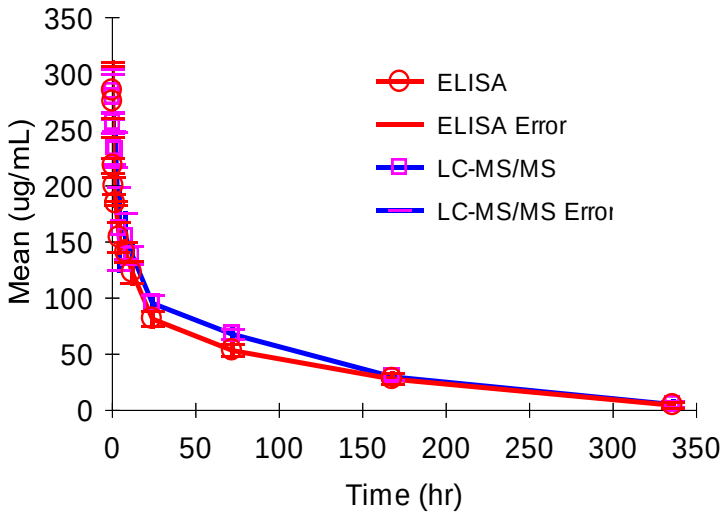
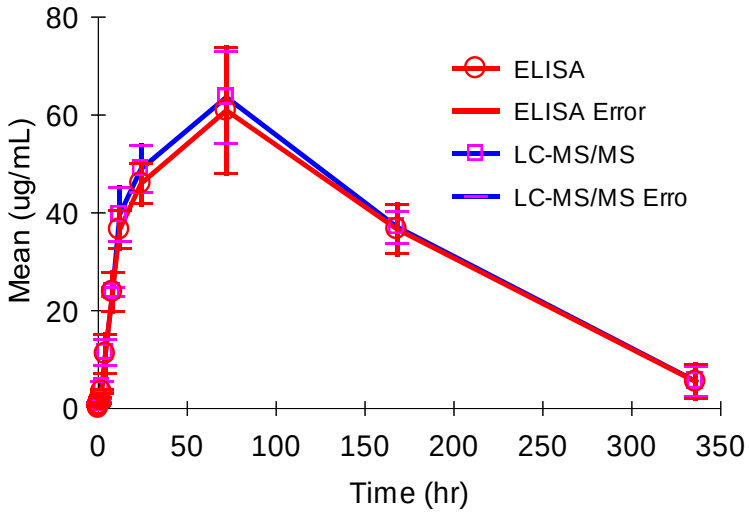
PK study results: LC-MS/MS vs ELISA

mAb administrated i.v. in normal rats (Average of 3 rats)



mAb administrated i.p. in normal rats (Average of 3 rats)



	IV				IP		
	AUC_{inf} hr*mg/mL	Cl mL/hr/kg	Vss mL/kg	$t_{1/2}$ hr	AUC_{inf} hr*mg/mL	Cl/F mL/hr/kg	C_{max} µg/mL
ELISA	13.5±3.2	0.77±0.16	71.2±2.1	70.5±25.2	12.2±4.1	0.88±0.25	62.8±20.6
LCMS	15.7±2.0	0.64±0.08	60.3±8.9	71.4±23.4	12.6±2.3	0.82±0.16	68.0±8.8

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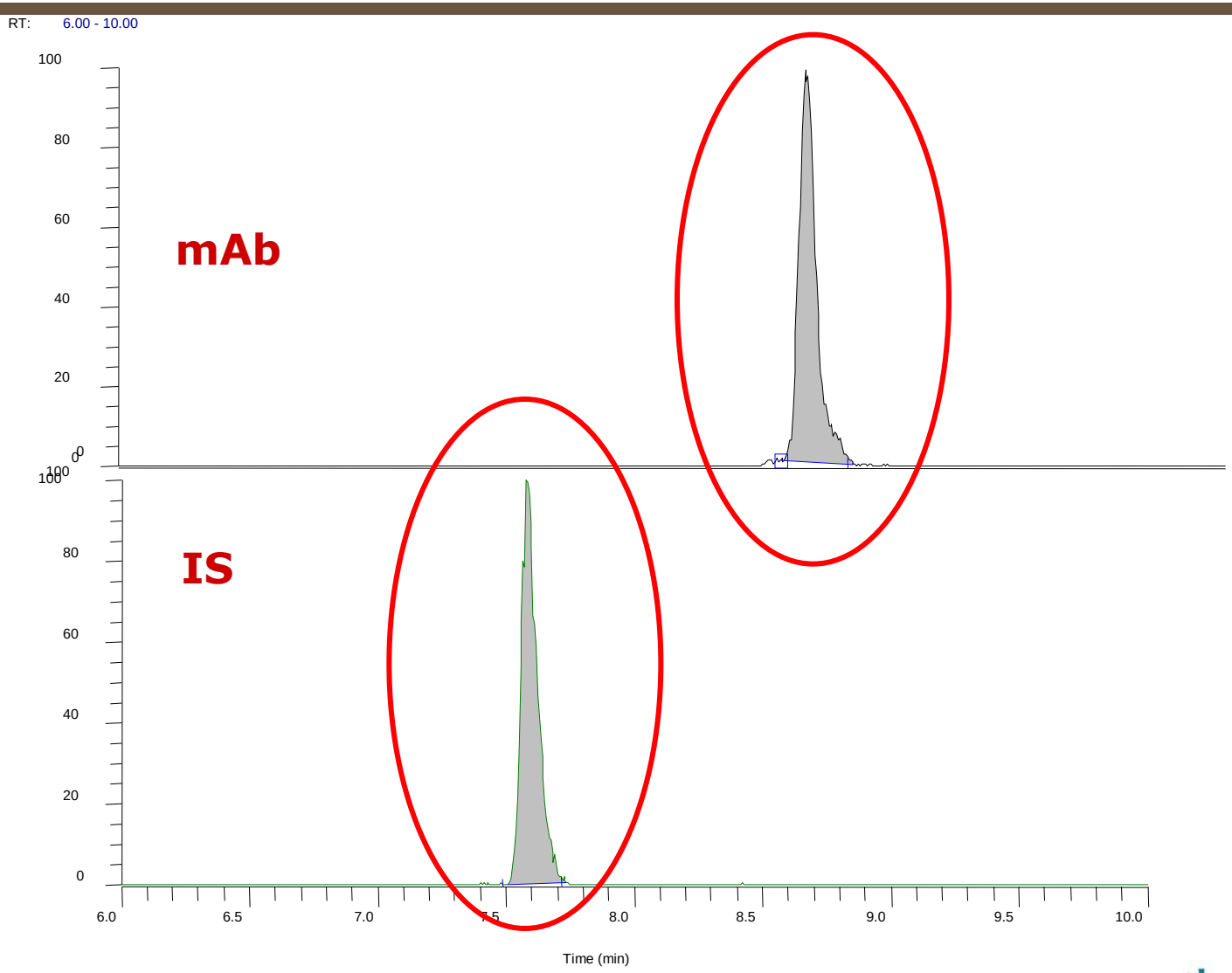
One major drawback



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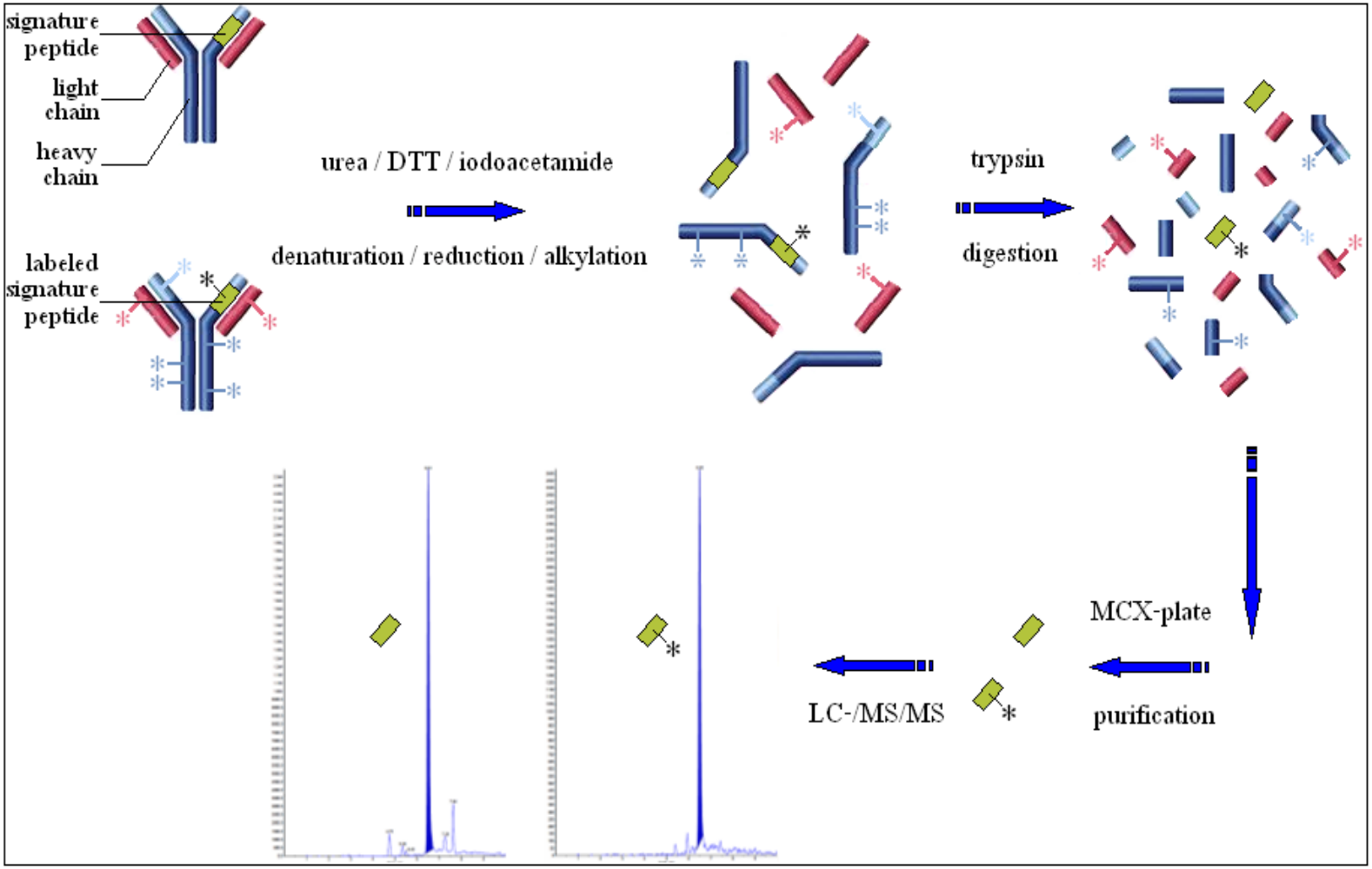
Method workflow (PSAQ)



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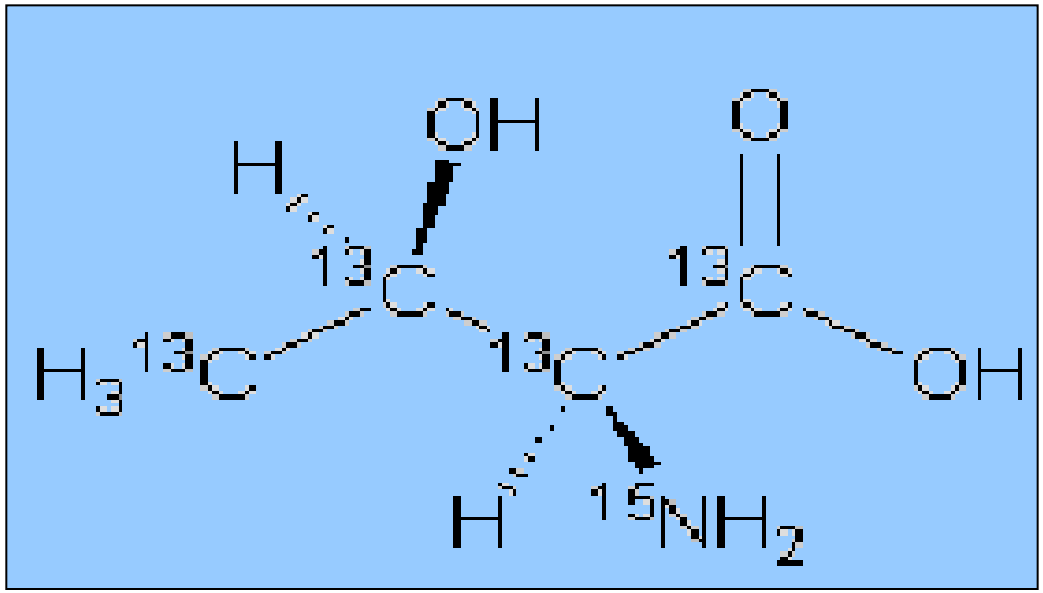
Strategies for IS labeling

T – G – P – F – D – T – W – G – Q – G – T – L – V – T – V – S – S – A – S – T – K

$^{13}C_4$, ^{15}N-Threonine



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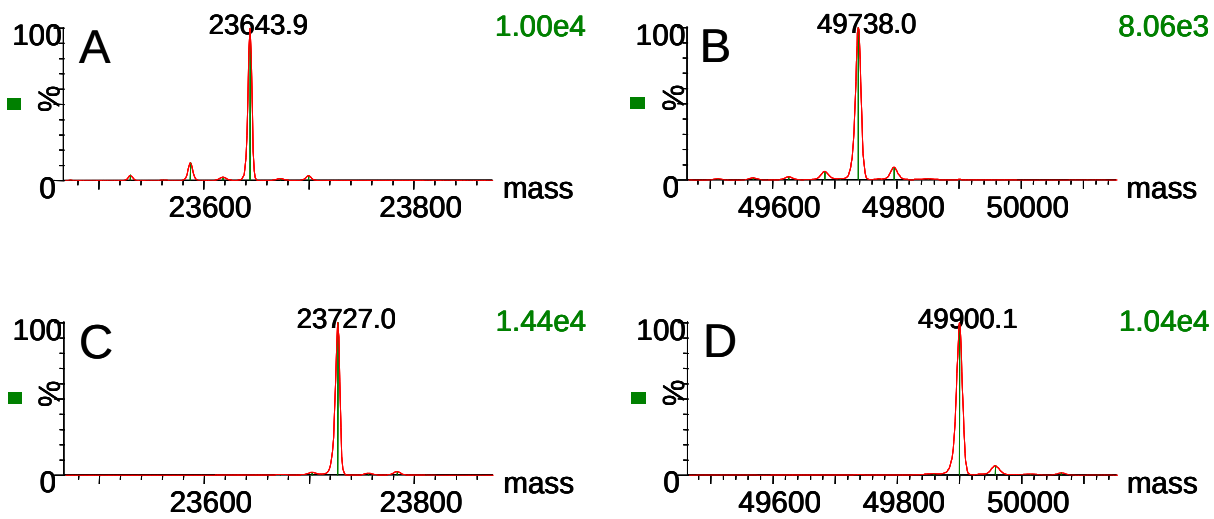
ESI-MS TOF spectra of light and heavy chains

	light chain [Da]	heavy chain [Da]
non-labeled mAb	**23643.26**	**49737.08**
number of threonines	17 threonines	33 threonines
mass difference per isotope-labeled threonine	4.96	4.96
mass increase of mAb by labeled threonine	84.32	163.68
isotope-labeled mAb	**23727.58**	**49900.76**



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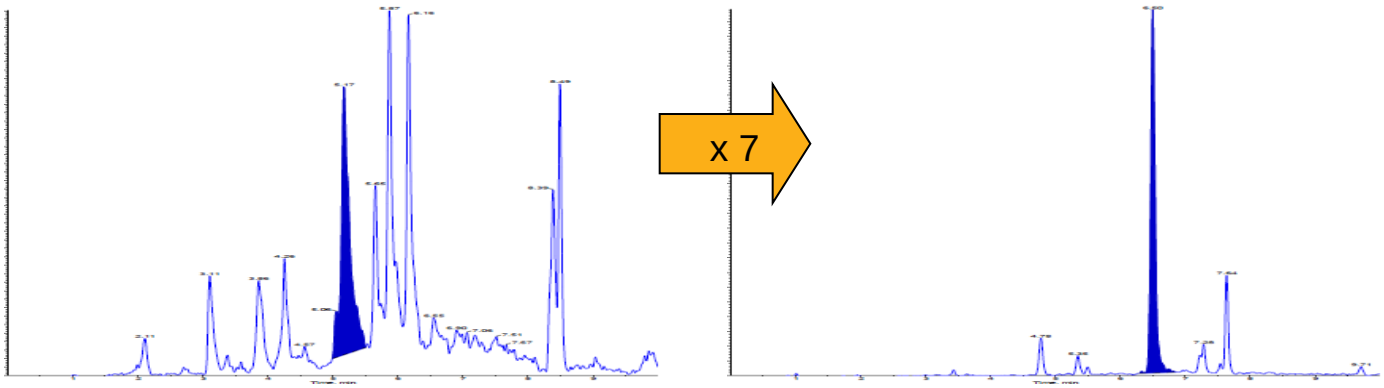
LC-optimization

	Condition 1	Condition 2
Column	AGILENT ZORBAX ECLIPSE XDB-C_8 2.1 x 50 – 3.5µm	ACE3 C_{18} 4.6 x 150 – 3.0µm
Flow rate (mL/min)	0.5	1
Gradient	10% ⇨ 95% B in 7‘	30% ⇨ 95% B in 7‘
LC run time (min)	10	10

STD @ 50 µg/mL mAb



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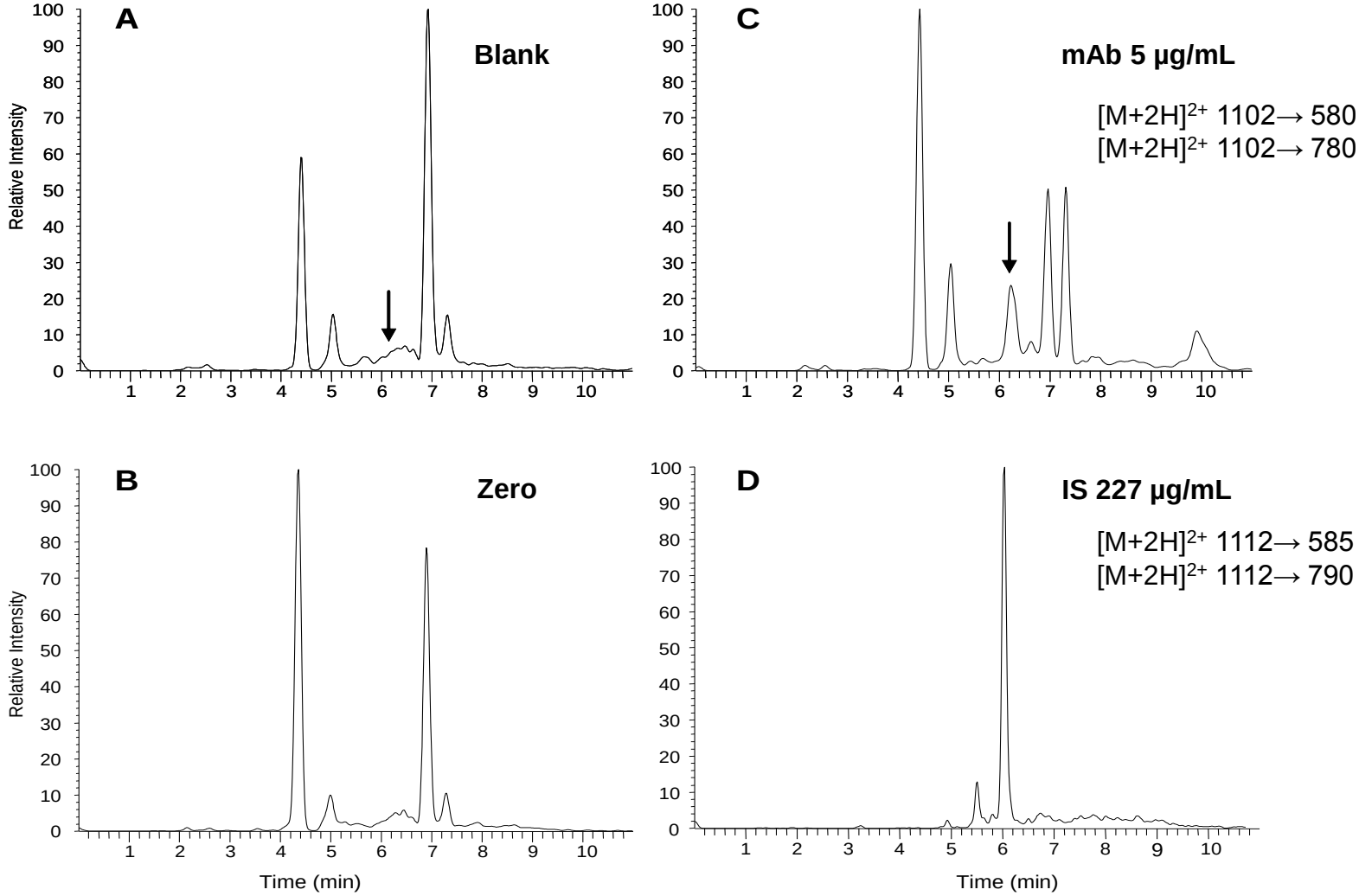
Assay selectivity



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Accuracy and precision data

Concentration (µg/mL)		5.00	15.0	500	750
	n	5	5	5	5
Intra-run1 CV (%)		16.4	9.17	8.29	2.35
Intrarun1 Bias (%)		5.56	0.05	-8.48	-4.81
	n	5	5	5	5
Intra-run2 CV (%)		17.2	12.8	6.54	7.51
Intra-run2 Bias (%)		8.41	5.16	-8.21	-10.3
	n	10	10	10	10
Inter-run CV (%)		15.9	10.9	6.87	6.01
Inter-run Bias (%)		6.99	2.61	-8.33	-7.57

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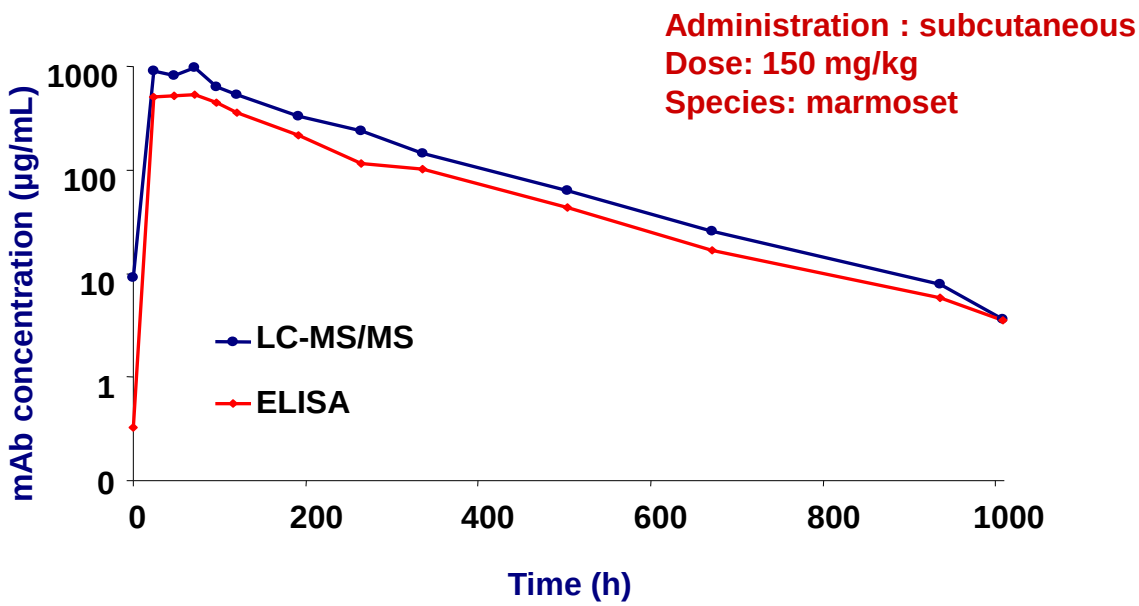
PK study results: LC-MS/MS vs ELISA



Parameters	Units	ELISA	LC-MS/MS
Dose	mg/kg	150	150
T_{max}	h	48 to 72	24
C_{max}	µg/mL	553	906
C_{max}/Dose	(µg/mL)/(mg/kg)	3.69	6.04
AUC	µg*hours/mL	113502	181443
AUC/dose	(µg*hours/mL)/(mg/kg)	757	1210
AUC interval	h	0-1008	0-1008

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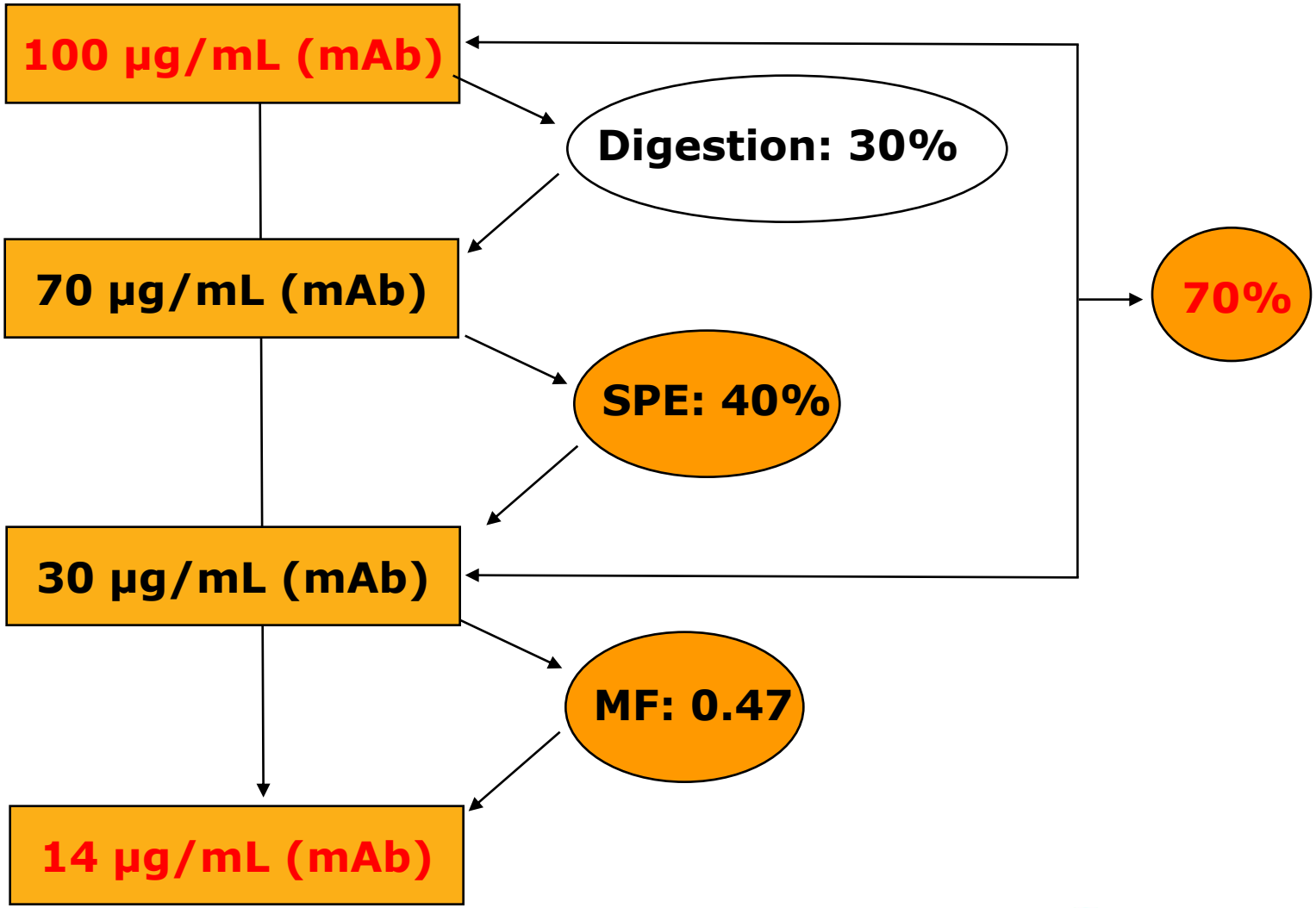
Where de we lose our mAb



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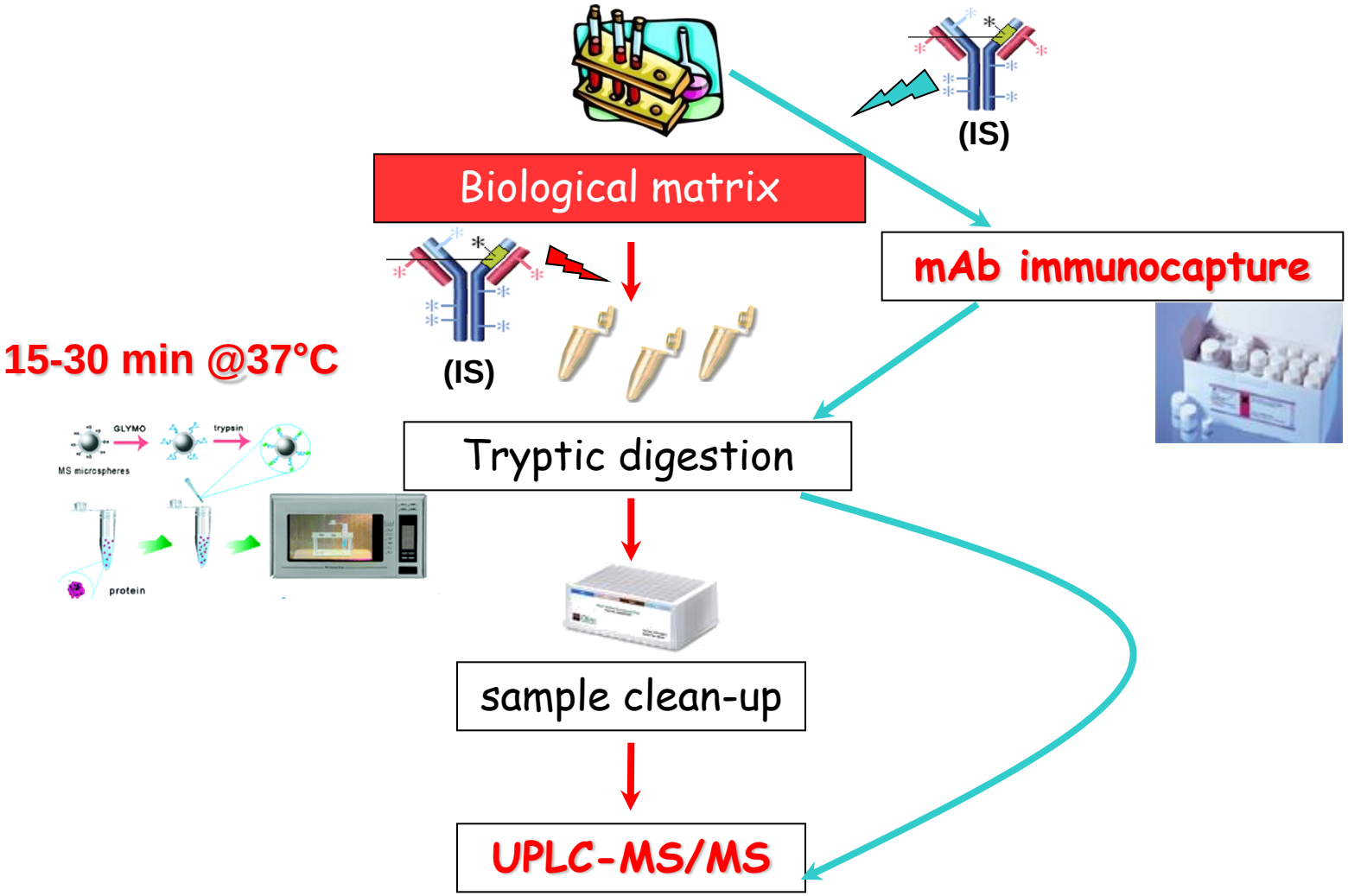
Recommendation



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Assay development time and throughput

Assay development

Peptide selection
LC-MS/MS optimization
Sample cleanup optimization → 1 week

Preliminary validation → 1 week

Development time
2 weeks

Work Flow --- 96-well plate format

Serum sample
+
IS addition → Day 1

↓

Trypsin Digest → Over night

↓

Sample cleanup → Day 2

↓

LC-MS/MS analysis → Over night, 6-10 min/sample

Analysis time 1 batch
2 days

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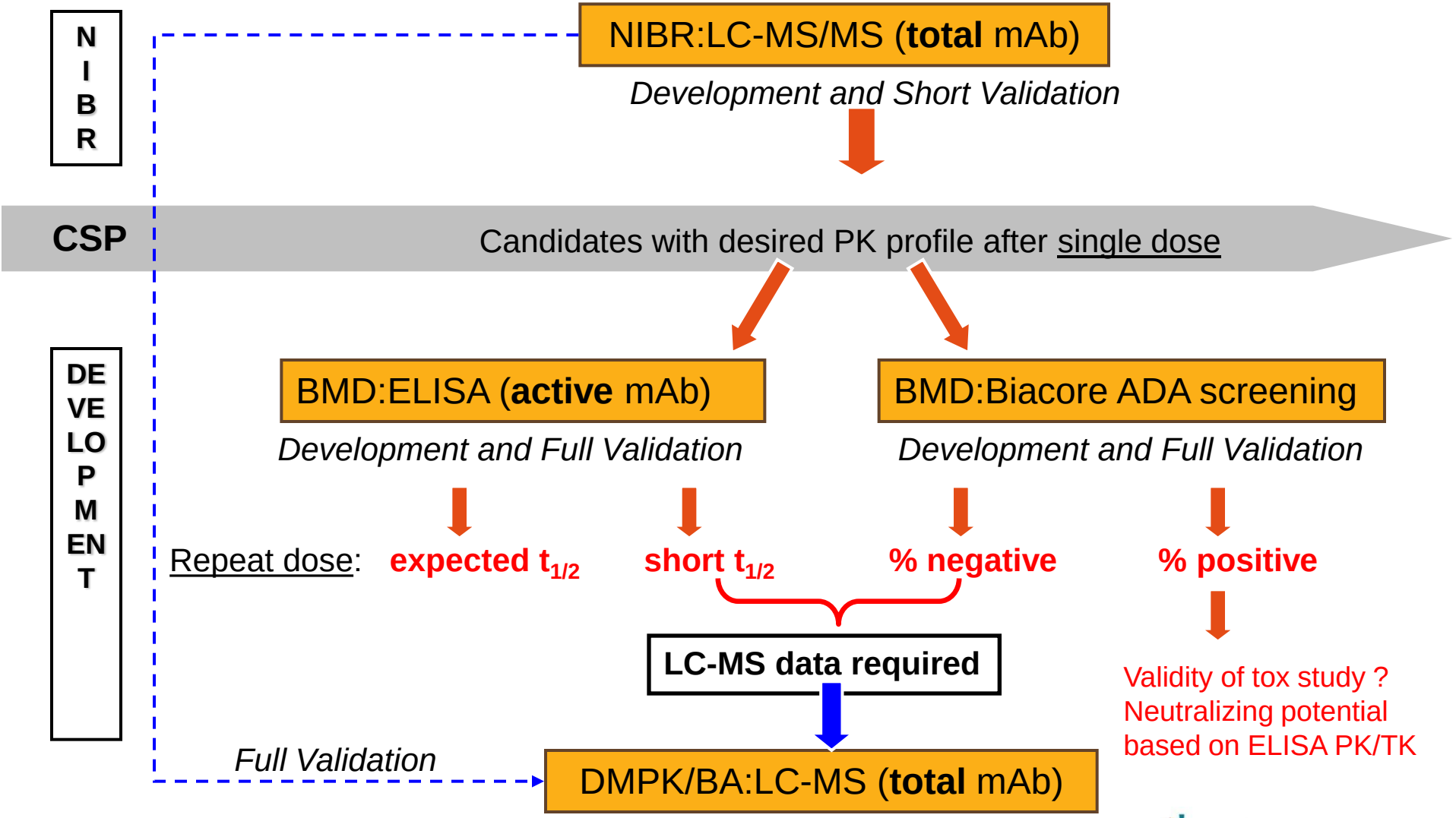
Serial Approach: 1. ELISA, 2. LC-MS/MS



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SWOT Analysis

Strengths

Quicker assay development
Good accuracy and precision
Multi-mAbs quantification
Comparable throughput to small molecule
Generic approach (smart IS)
Applicable to several species
Applicability to other fields

Weaknesses

Sensibility to be improved
Choice of the signature peptide
Choice of the internal standard
The trypsin digestion time
Measurement of free or total mAb?

Opportunities

Speed up mAbs candidate selection
Expertise available in DMPK
Participation in the MS working groups
Visibility inside and outside Novartis

Microsoft
owerPoint Presentatic

Threats

New approach only little background
Validation and authorities acceptance
Different method formats exist
Comparison with reference methods

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Acknowledgements

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BA group members

Basel, Switzerland

Soluble Biomarkers

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Anette ZAAR

Cambridge, MA, USA

Protein analytics

Karen WANG

Brian GRANDA

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DMPK

Francis TSE

Ziping YANG

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