Bioanalysis of Protein Therapeutics and Antibody-Drug Conjugates by LC-MS
液质联用在蛋白制品和抗体-药物偶合药物生物分析中的应用

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2018 Nanjing International DMPK Symposium – Workshop
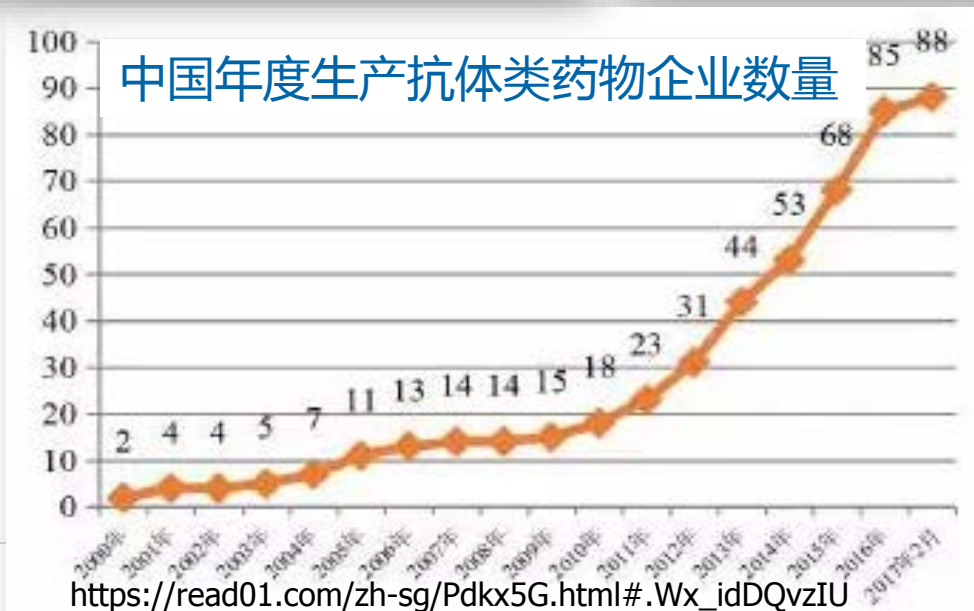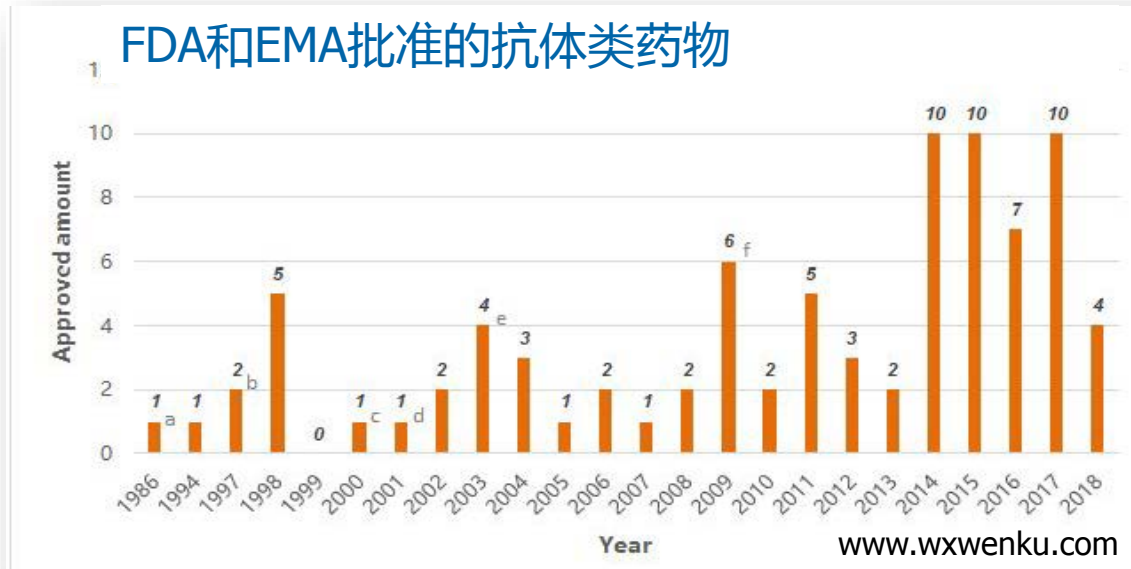
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June 29, 2018 / 中国· 南京

Bristol-Myers Squibb

1. Introduction: Current status, Challenges and Solutions

概述：业界现状，挑战，解决方案

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Therapeutic Antibody and Proteins Market

FDA和EMA批准的抗体类药物

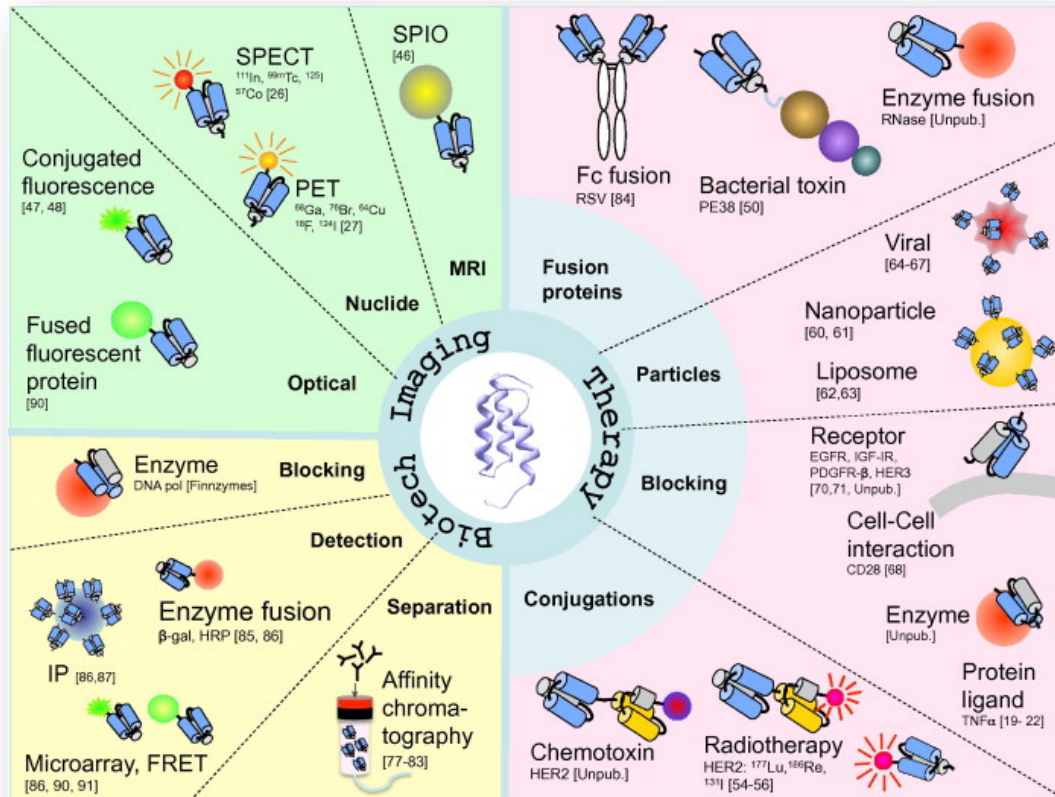www.wxwenku.com

抗体类药物市场规模，亿美元

@前瞻经济学人APP

中国年度生产抗体类药物企业数量

https://read01.com/zh-sg/Pdkx5G.html#.Wx_idDQvzIU

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3

Antibody and fragments

Glycoengineered Abs

PEGylation or Fc conjugation to stabilize
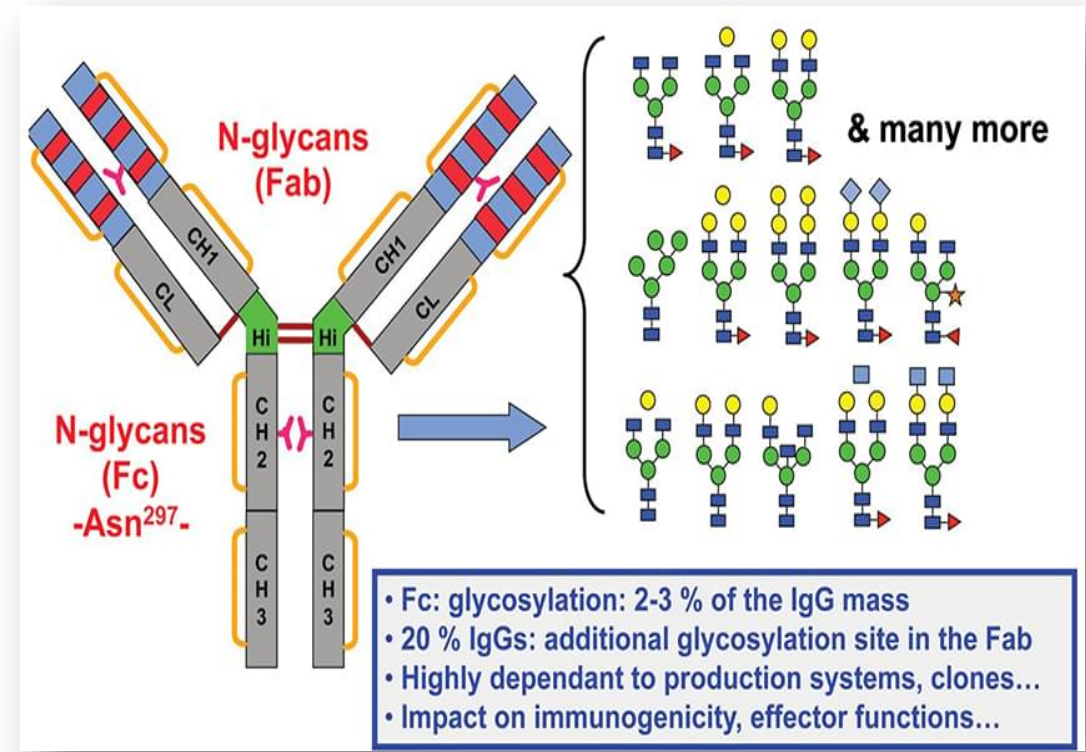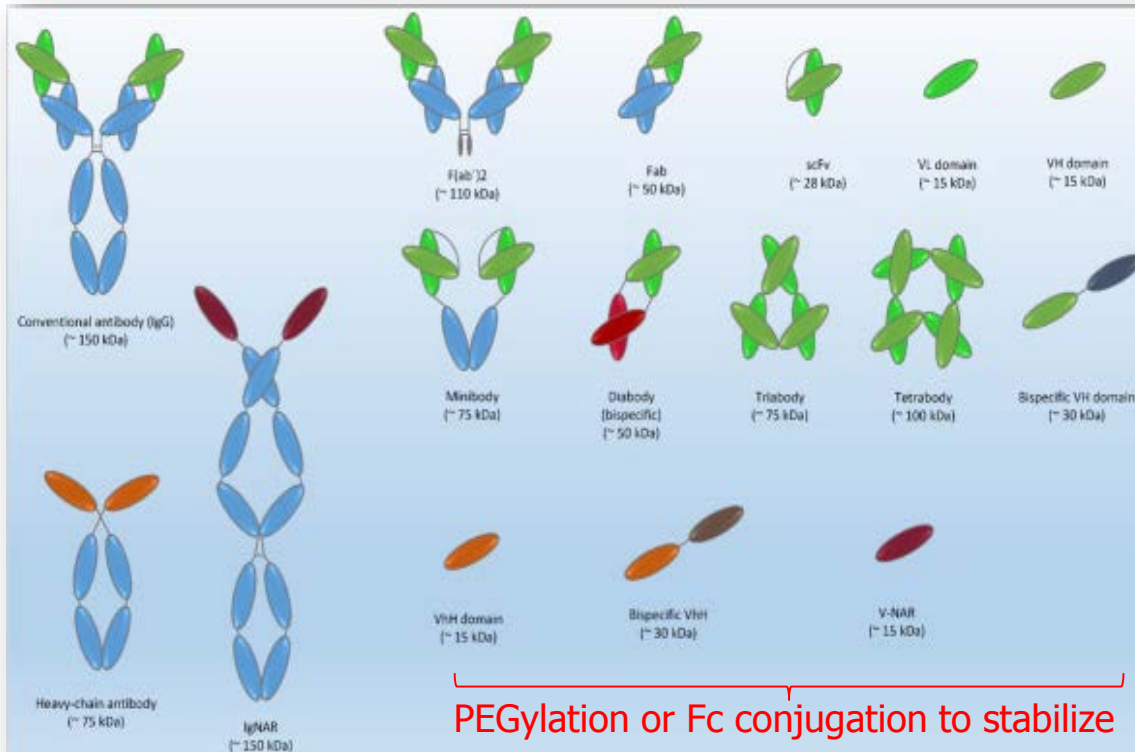
Journal of Controlled Release, 268: 323

- Fc: glycosylation: 2-3 % of the IgG mass
- 20 % IgGs: additional glycosylation site in the Fab
- Highly dependant to production systems, clones...
- Impact on immunogenicity, effector functions...

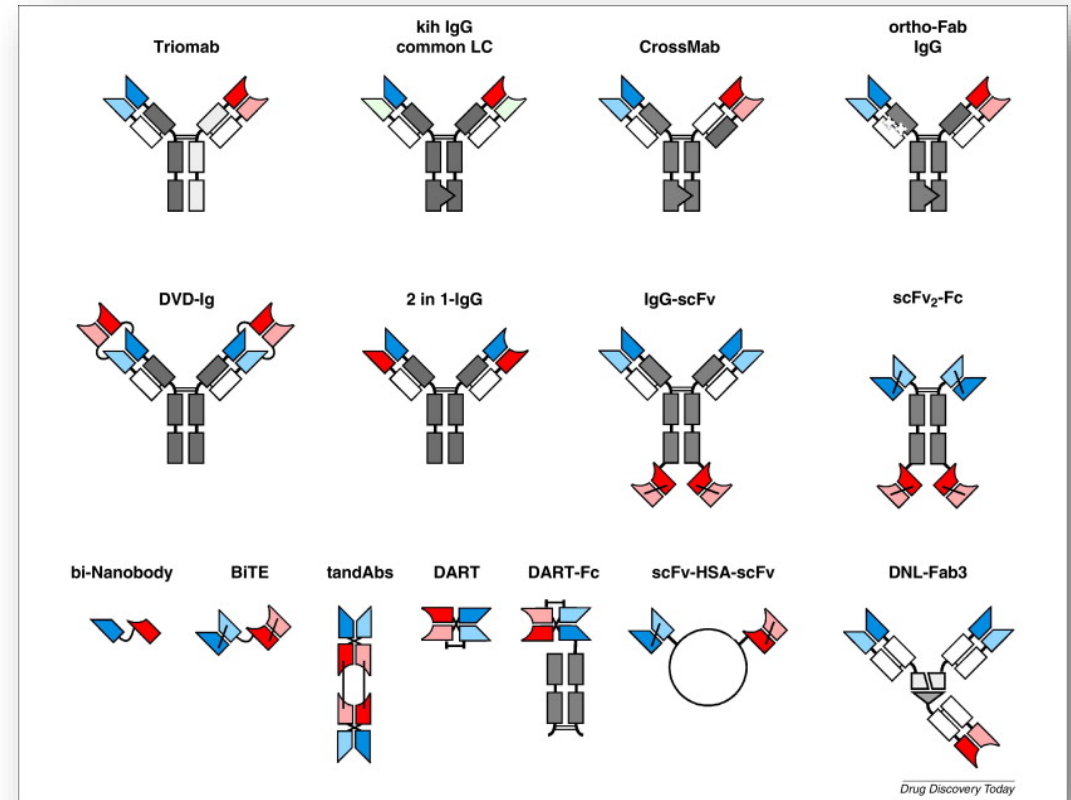https://www.creativebiolabs.net/antibody-glycoengineering.htm

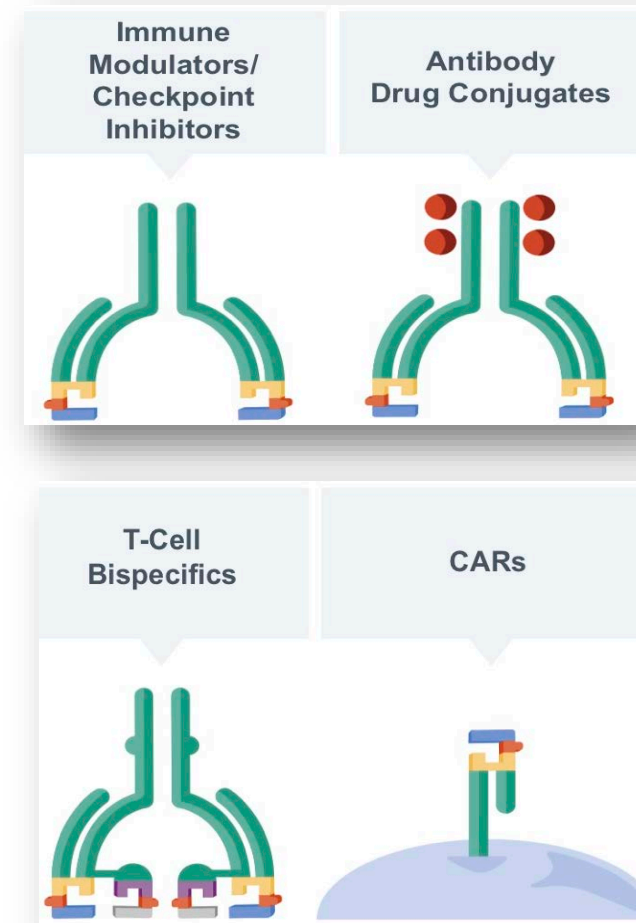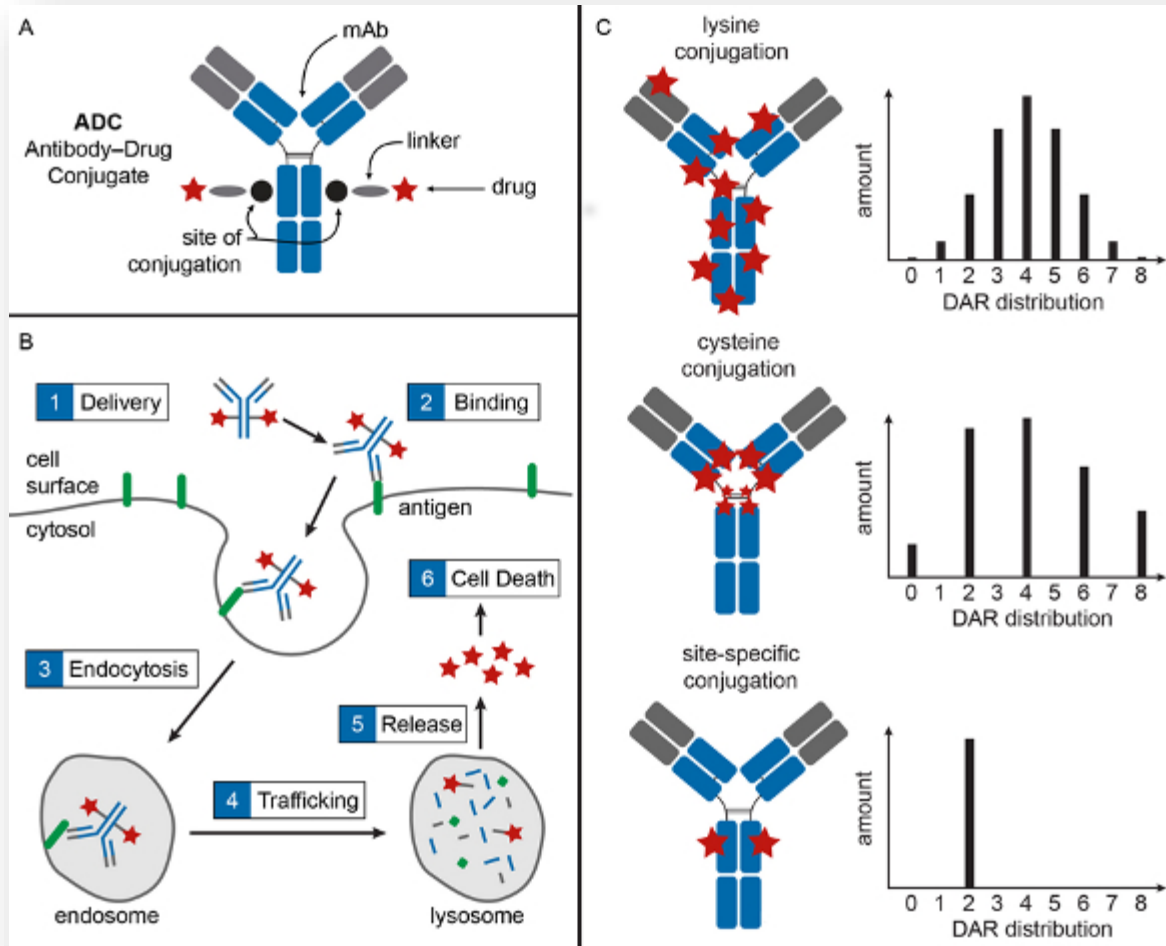# Fusion Proteins

Bispecific Abs

FEBS Letters, 2010, 584: 2670

Drug Discovery Today, 2015, 20: 7

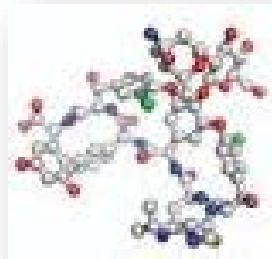# Antibody Drug Conjugates

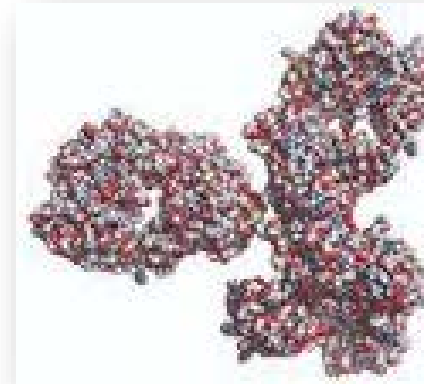# Probody and Conjugates

www.americanpharmaceuticalreview.com/

www.cytomx.com/

Complexity of Protein Mass Spectra

Small molecule
<1k Da
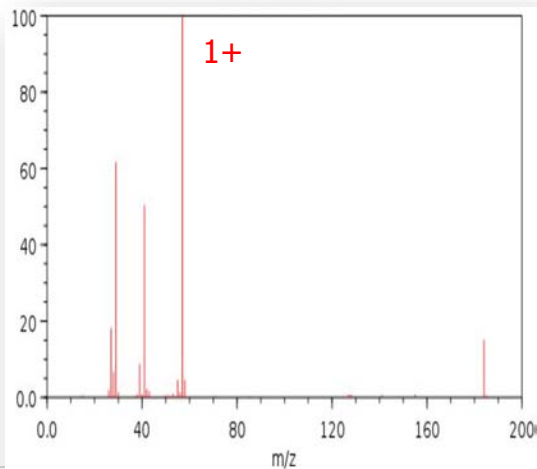
Macrocycle
1-3 kDa
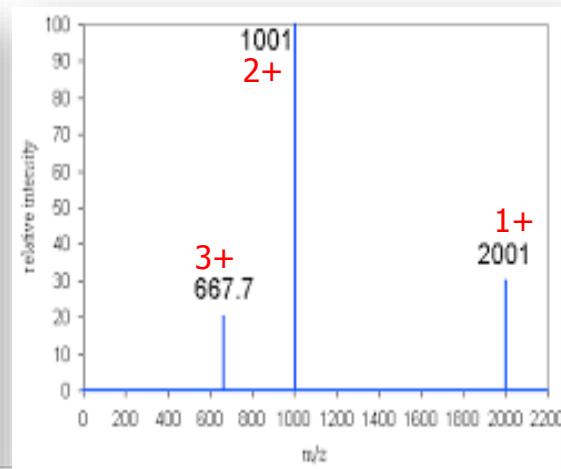
Monoclonal Antibody
150 kDa
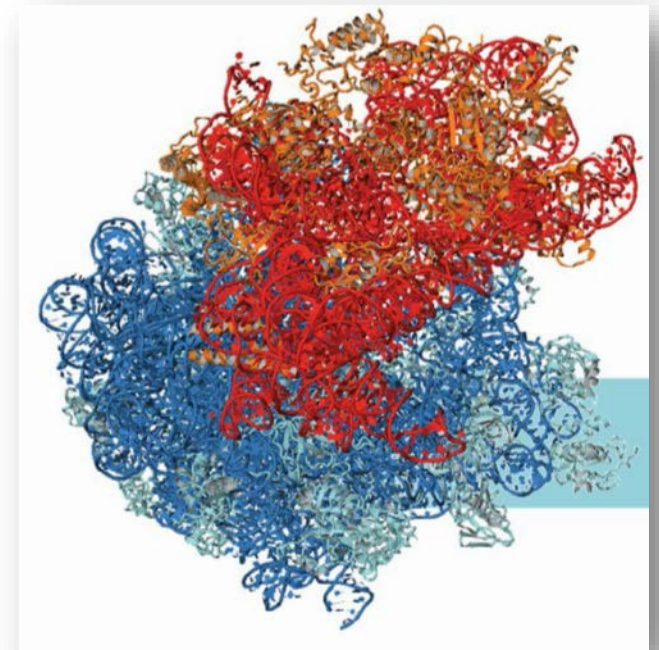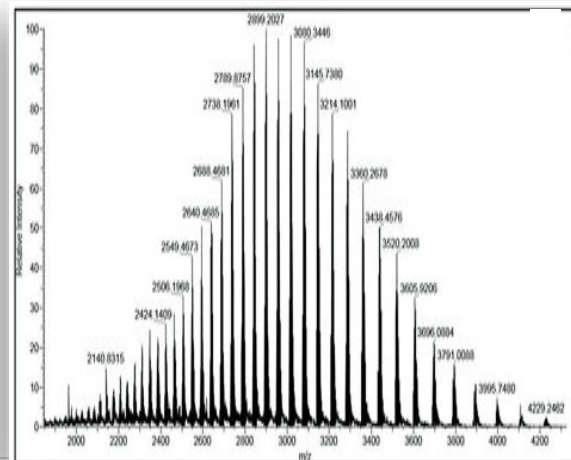
Ribosome (70S)
2,300 kDa
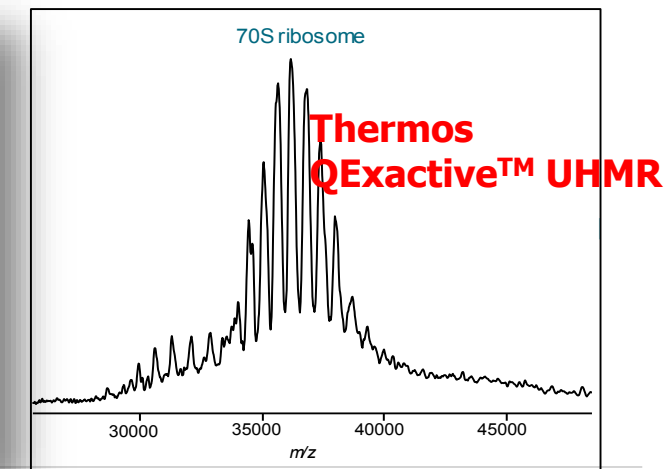
The First Scientist to Apply LC-MS for Protein Analysis

John Bennett Fenn

2002 Nobel Prize in Chemistry

Born	June 15, 1917 New York City, New York, U.S.
Died	December 10, 2010 (aged 93) Richmond, Virginia, U.S.
Residence	United States
Nationality	United States
Alma mater	Berea College Yale University
Known for	Electrospray ionization
Awards	Nobel Prize in Chemistry (2002)
Scientific career	
Fields	Chemistry
Institutions	Princeton University Yale University Virginia Commonwealth University

Bristol-Myers Squibb

Electrospray Ionization (ESI)

- Introduced by Chapman in 1930
- John Fenn applied it to study large biomolecules
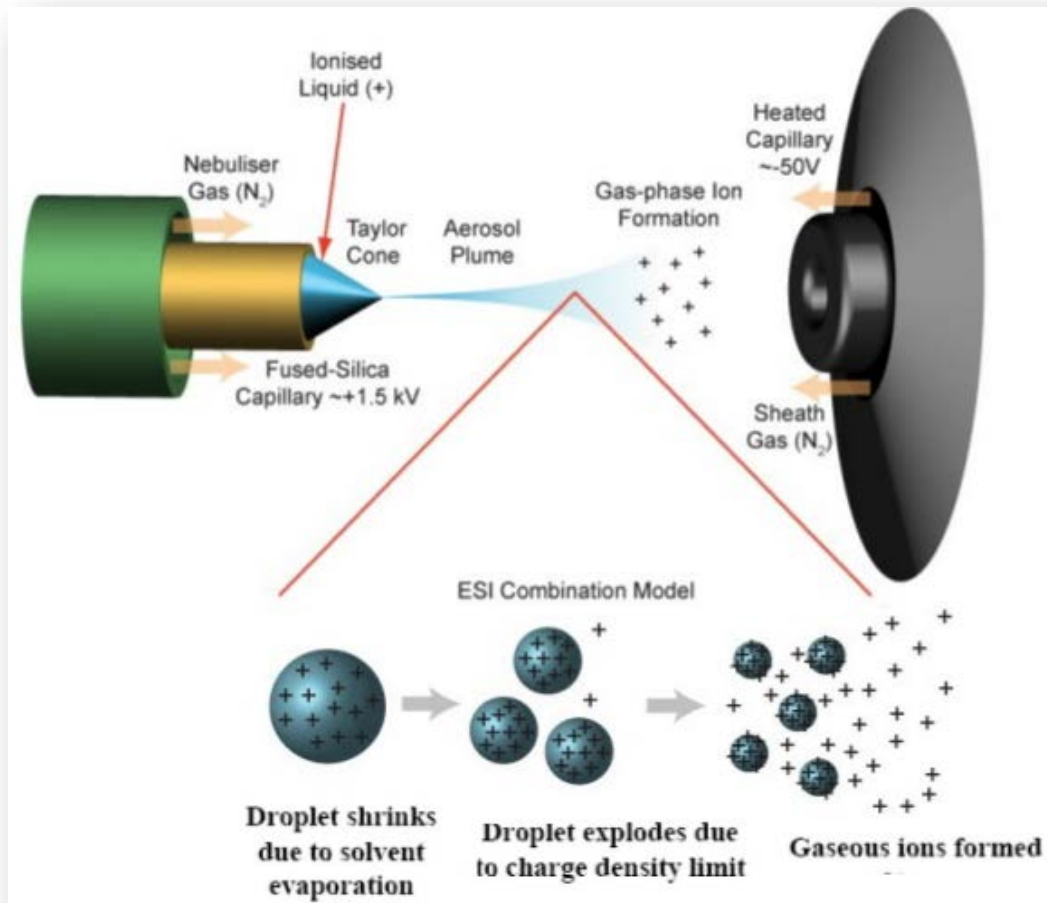
Publication Trend of LC-MS Protein Analysis

Source: www.ncbi.nlm.nih.gov/pubmed/
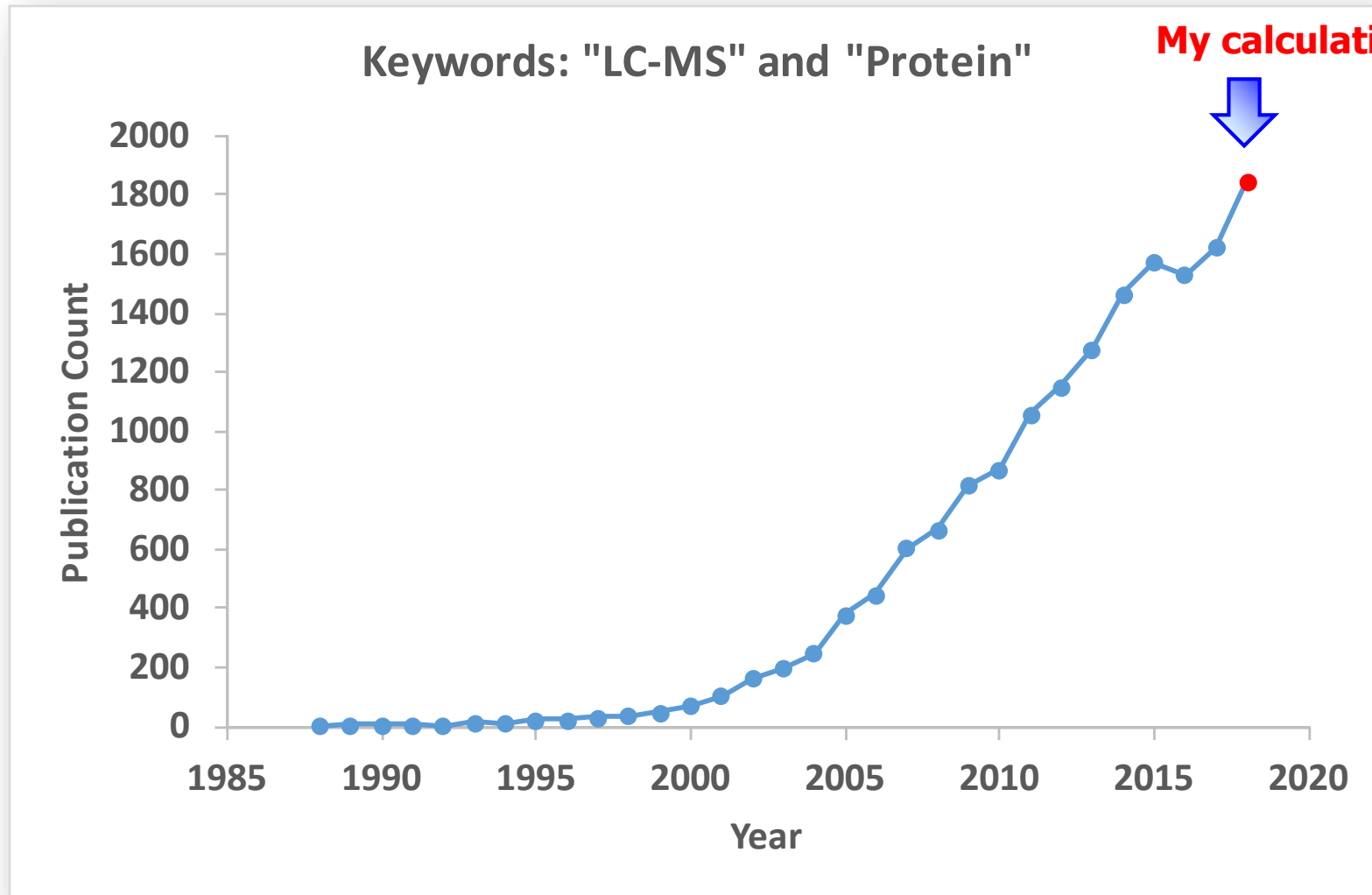
LC-MS Applications and Bioanalysis

- Analytics of protein therapeutics in drug discovery and development is quite different from small molecules

  - ✓ Chemistry, Manufacturing & Controls (CMC)

  - ✓ Bioanalysis (pharmacokinetics, immunogenicity, biomarkers)

- The quality of bioanalytical work is critical to pharmacokinetic (PK) and pharmacodynamics (PD) assessments to guide drug development

  - ✓ Biological samples (plasma, serum, tissues)

  - ✓ Amount of endogenous proteins (70 mg/mL) >> analyte proteins (10^{-9} mg/mL)

Current Status in Bioanalysis

- Bioanalytical technologies
  - ✓ Ligand binding assays (LBA)
  - ✓ LC-MS (including hybrid LC-MS)

- Assay sensitivity
  - ✓ Low pg/mL level, assay dependent

- Bioanalytical applications
  - ✓ PK (LLOQ $\geq$ 500 ng/mL)
  - ✓ Immunogenicity (LOD $\geq$ 100 ng/mL of control)
  - ✓ Biomarker (LLOQ 1 pg/mL - 10 ng/mL, biomarker dependent)

LC-MS Workflow for Protein Bioanalysis

Sample Pretreatment	Sample Introduction	Ion Source	Mass Spec	Computer Software

- PPT
- Immunocapture (beads, cartridges)

- Direct infusion
- HPLC (C18, C8, C4, HIC, SEC, diphenyl, Superficially porous particles (SPP)
- Micro-LC
- Nano-LC
- Capillary electrophoresis (CE)
- Acoustic delivery

- Electrospray (ESI)
- Micro-LC ESI interface
- Nano-LC ESI interface
- Ion mobility spec (IMS)

- Triple Quadruples
- Ion trap (quadrupole, linear, orbitrap, ion cyclotron resonance)
- Time of flight (TOF)

- Analyst (Sciex)
- Xcalibur (Thermos)
- MassLynx (Waters)
- ExPASy
- Skyline (MacCoss)
- MaxQuant (Jürgen Cox)
- BioPharma Finder™ (Thermos)
- Others (https://en.wikipedia.org/wiki/List_of_mass_spectrometry_software)

White Paper

Recommendations for Validation of LC-MS/MS Bioanalytical Methods for Protein Biotherapeutics

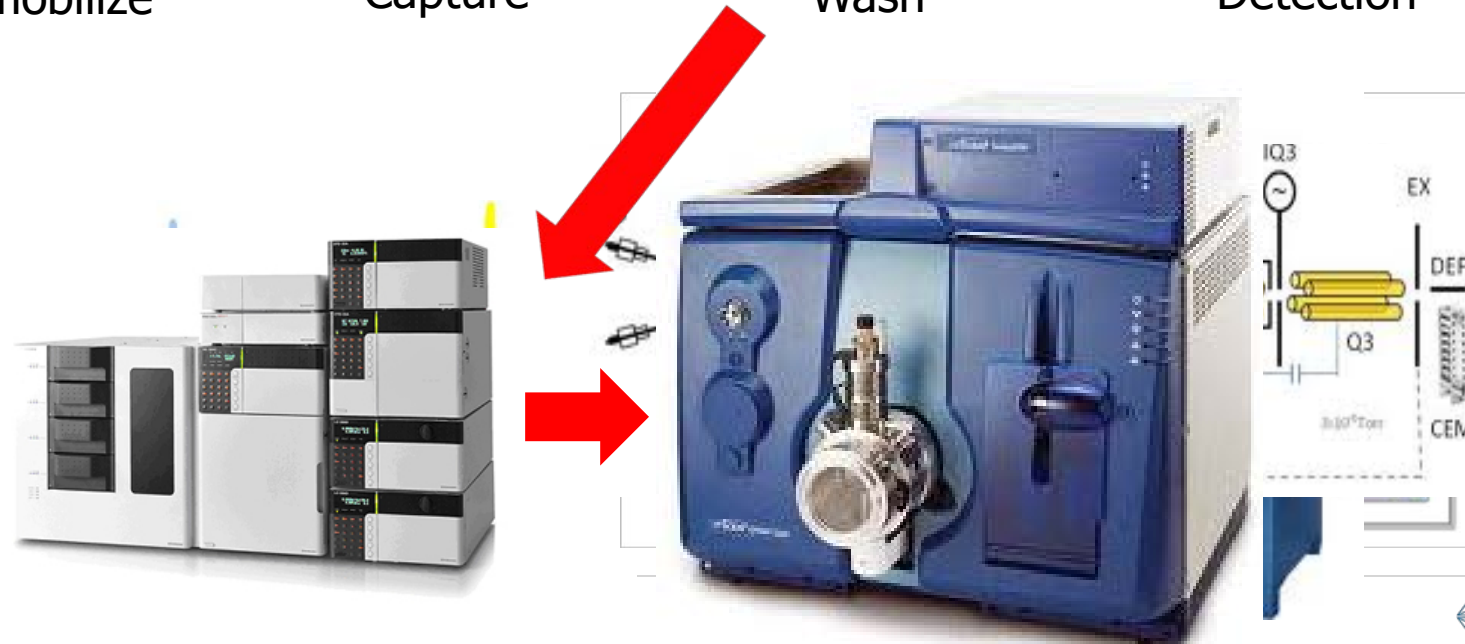Rand Jenkins,[1] Jeffrey X. Duggan,[2] Anne-Françoise Aubry,[3] Jianing Zeng,[3] Jean W. Lee,[4] Laura Cojocaru,[5] Dawn Dufield,[6] Fabio Garofolo,[7] Surinder Kaur,[8] Gary A. Schultz,[9] Keyang Xu,[8] Ziping Yang,[10] John Yu,[2] Yan J. Zhang,[3] and Faye Vazvaei[11,12]
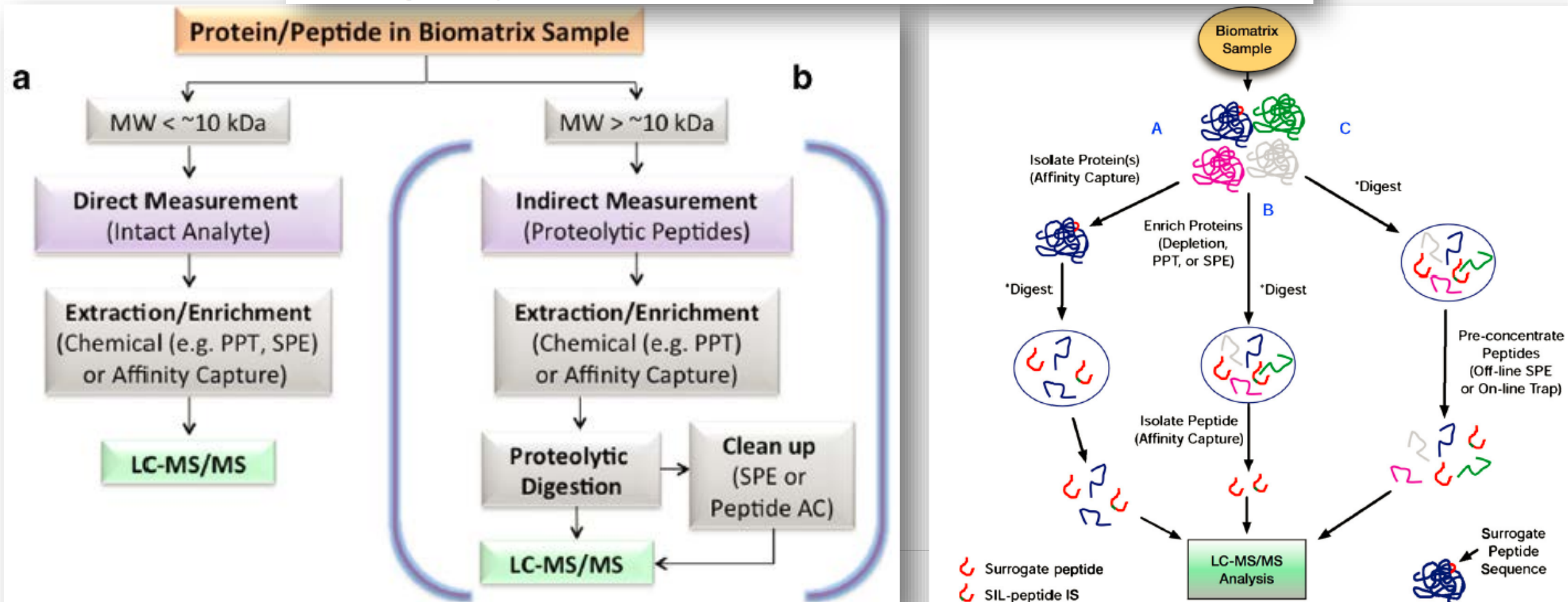
LBA vs. LC-MS in Assay Format

LBA

Immobilize   Capture   Wash   Detection
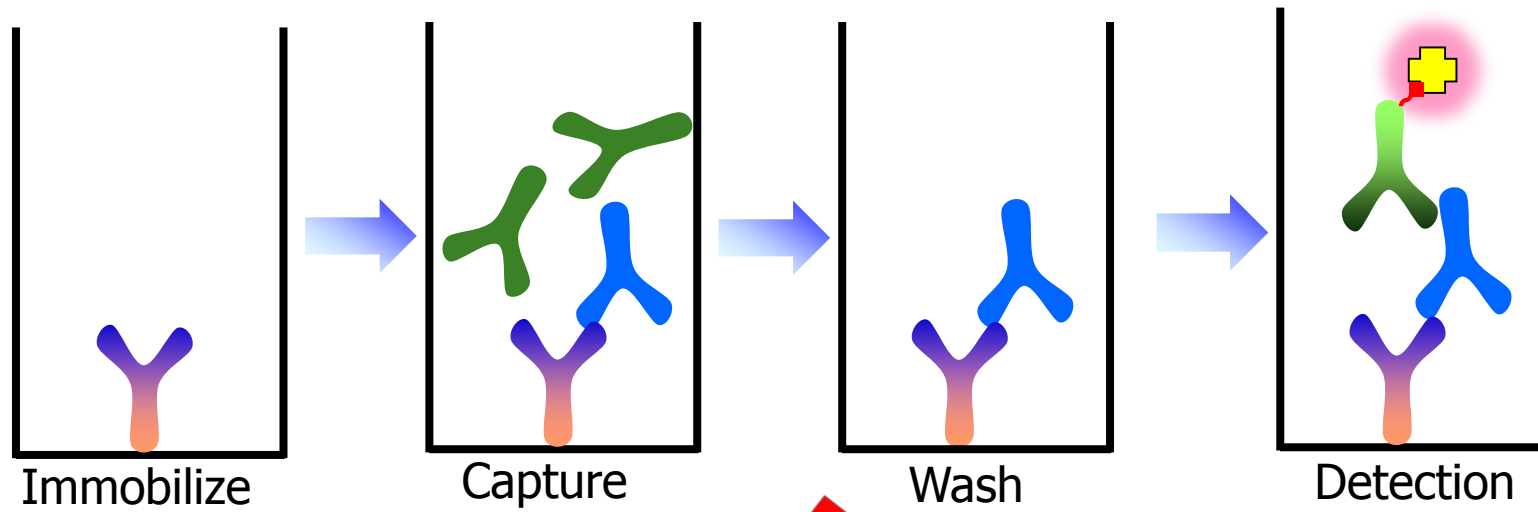
LC-MS

LBA vs. LC-MS in Assay Features

Feature	LBA	LC-MS
Specificity/selectivity	Highly reagent dependent; limitation in differentiating proteins with minor differences	Besides reagents, LC and MS provide additional two levels of specificity/selectivity
Sensitivity	pg/mL and above; majorly dependent upon binding affinity	pg/mL and above depending upon molecular weights; 10-100 times less than LBA
Calibration curve/range	4 Parameter Logistic (4PL) or 5PL; range $10-10^3$ folds	Linear or quadratic; Wider dynamic range 10^2-10^4 folds
Critical reagents	Need Ab pairs for capture and detection; need to label both capture or detection Ab	Need only capture Ab; biotin labeling
Assay throughput	4-8 hours/run (1-2 plates)	10-20 hours/run (1-2 plates)
Measuring "active" or "total"	Either one	Either one

LC-MS Assay Provides Three-Level Specificity

- Level 1: Sample pretreatment
  - ✓ Harsh conditions (protein precipitation, solid phase extraction)
  - ✓ Mild conditions (immunocapture, size exclusion)
  - ✓ Combinations (H × H, M × M, M × H)
  - ✓ Post-extraction treatments (reduction, derivatization, proteolysis, deglycosylation)
- Level 2: Liquid chromatography
  - ✓ Variety of column chemistry (RP, HILIC, SEC, IPC, SCX)
  - ✓ 2 dimension separations (RP x RP, SCX x RP, RP x HILIC, RP x IPC)

- Level 3: Mass spectrometry
  - ✓ QQQ, Q-TOF, Q-orbitrap,
  - ✓ Ion Mobility (SelexION/DMS), additional dimension ion separation

LC-MS Assay Has Unique Features

1) Good specificity/selectivity

Differentiate protein isoforms, modifications, substitutions, isobaric interferences

2) Multiplexing capacity

Drug(s), anti-drug antibody (ADA), disease targets

3) Flexibility

Curve range, sample volume, selection of surrogate peptides

4) Internal standardization

Stable Isotopically Labeled (SIL)-protein > SIL-flanking-peptide > SIL-peptide > analogues

5) Short lead time for method development

2 - 6 weeks if no specific reagent is needed

6) Less dependent on reagents for selectivity

Good selectivity from coupling LC and MS

Challenges of LC-MS in Protein Bioanalysis

1) Less sensitive than LBA, but generally sufficient for PK bioanalysis

- ✓ LLOQ of 5 ng/mL (30 pM, 25 µL sample) for mAb[1]
- ✓ LLOQ of 0.78 pg/mL (0.05 pM, 500 µL sample) for IL-21[2]

2) Requires proteolysis for large proteins (MW > 10 kDa)

- ✓ Enzymatic digestion
- ✓ Acid hydrolysis

3) Sometimes need immunocapture for analyzing active analytes

- ✓ Protein A/G
- ✓ Anti-human Fc
- ✓ Anti-light chain
- ✓ Anti-Idiotypic (blocker and non-blocker)

[1] Jiang H, et al. Anal Chem, 2014, 86:2673
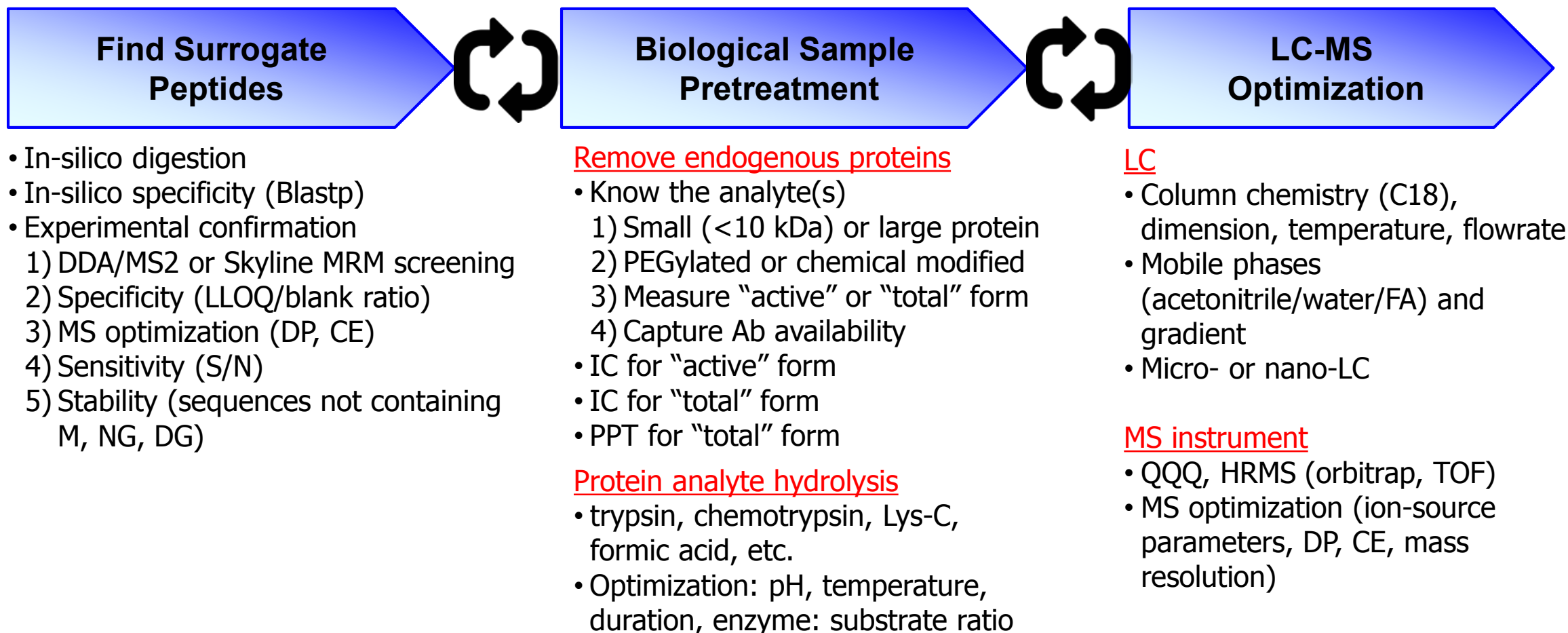[2] Palandra et al, Anal Chem, 2013, 85:5522

2. Method Development and Optimization

方法的建立和优化

Hao Jiang, PhD

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Strategy for Method Development

Find Surrogate Peptides → **Biological Sample Pretreatment** → **LC-MS Optimization**

- In-silico digestion
- In-silico specificity (Blastp)
- Experimental confirmation
  1) DDA/MS2 or Skyline MRM screening
  2) Specificity (LLOQ/blank ratio)
  3) MS optimization (DP, CE)
  4) Sensitivity (S/N)
  5) Stability (sequences not containing M, NG, DG)

Remove endogenous proteins
- Know the analyte(s)
  1) Small (<10 kDa) or large protein
  2) PEGylated or chemical modified
  3) Measure "active" or "total" form
  4) Capture Ab availability
- IC for "active" form
- IC for "total" form
- PPT for "total" form

Protein analyte hydrolysis
- trypsin, chemotrypsin, Lys-C, formic acid, etc.
- Optimization: pH, temperature, duration, enzyme: substrate ratio

LC
- Column chemistry (C18), dimension, temperature, flowrate
- Mobile phases (acetonitrile/water/FA) and gradient
- Micro- or nano-LC

MS instrument
- QQQ, HRMS (orbitrap, TOF)
- MS optimization (ion-source parameters, DP, CE, mass resolution)

Surrogate Peptide Selection and Optimization (Skyline)

Import this list to mass spec for collecting MRM data

Surrogate Peptide Selection and Optimization (Skyline), Cont'd
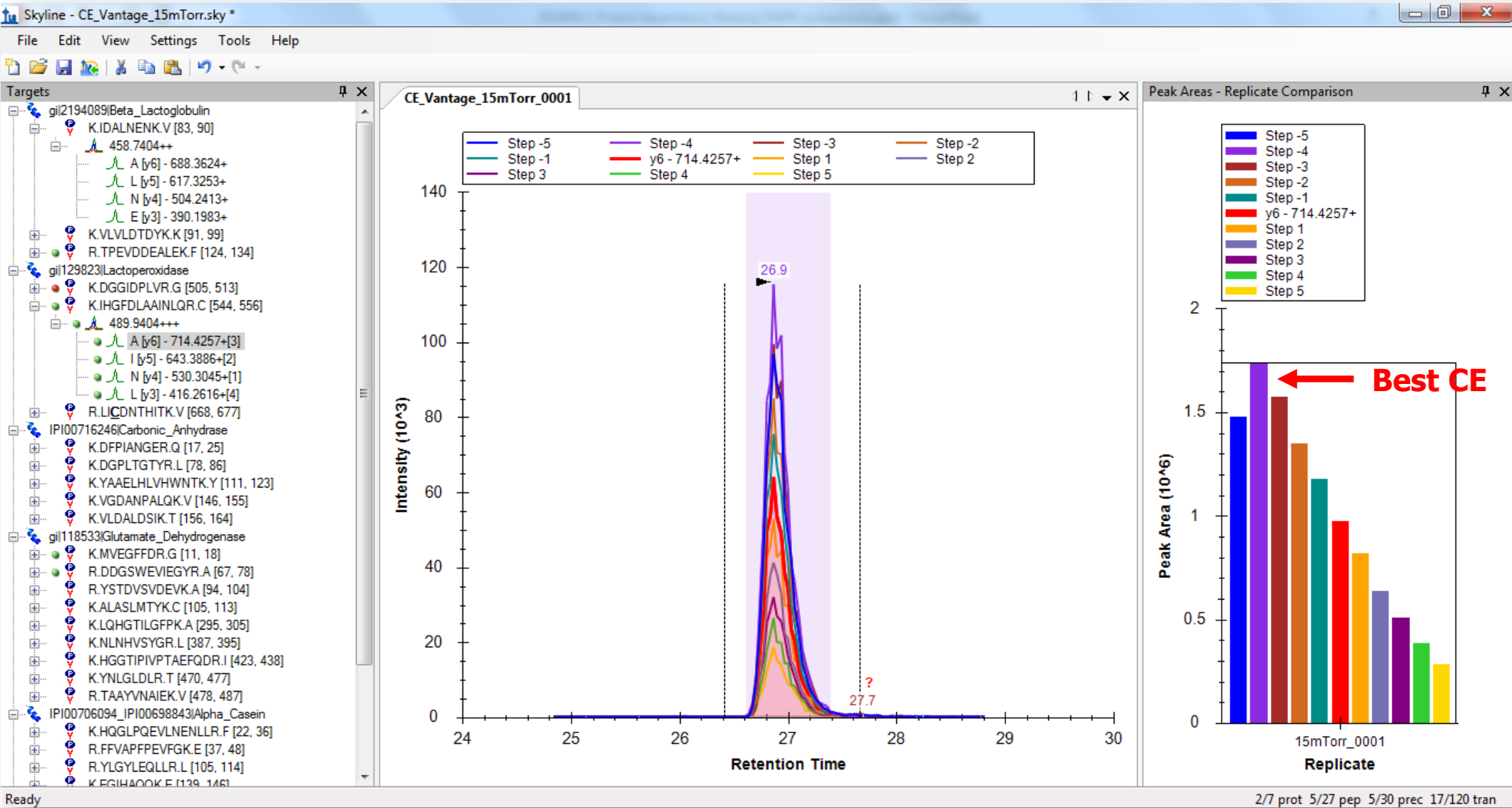
Protein Precipitation (PPT, Lowest Specificity)

- IC-free

- "Total" analyte assay

- Simple and cost-effective

- Proteins are precipitated with water-miscible organic solvents (e.g., ACN, MeOH, IPA).

- The supernatant and the precipitate are separated by centrifugation.

  – Organic soluble proteins in supernatant, e.g. PEGylated proteins; need to dry down prior to digestion

  – Large proteins in protein pellet, e.g., mAbs; need to be fully resuspended in digestion buffer prior to digestion

Organic solvents

Plasma → **Centrifugation** → **Supernatant** → **Organic soluble proteins** → **Trypsin Digestion** → **LC-MS/MS**

Pellets → **Large proteins** → **Trypsin Digestion** → **LC-MS/MS**

Pellet Digestion

Legend:
- Small proteins or PEGlyated protein
- Large proteins (5 kDa or above)
- Serum components
- Surrogate Peptide

Wu ST et al. Rapid Commun Mass Spectrom. **2011**, 25:281
Ouyang Z et al. Bioanalysis. **2012**, 4:17
Jiang H et al., Anal Chem. **2013**, 85:9859

Solid Phase Extraction (SPE, Higher Specificity)

- "Total" assay without IC at the beginning
- "Active" assay with anti-Id IC at the beginning
- Fully automated process
- Extensive effort on method development
- Recovery may be low
- For the extraction of peptides or digested samples based on C18, C8, C4, or ion exchange
  - Reversed-phase (RP)
  - Strong-cation exchange (SCX)
  - Mix cation mode (MCX)

interferent ●   analyte ●★

Add the Sample   Wash the Sample to Remove Interferents   Elute the Analytes

(a)   (b)   (c)

Yuan L et al. Bioanalysis. **2013**, 5:2379
Gong C et al. Chromatogr A. **2015**, 1424:27
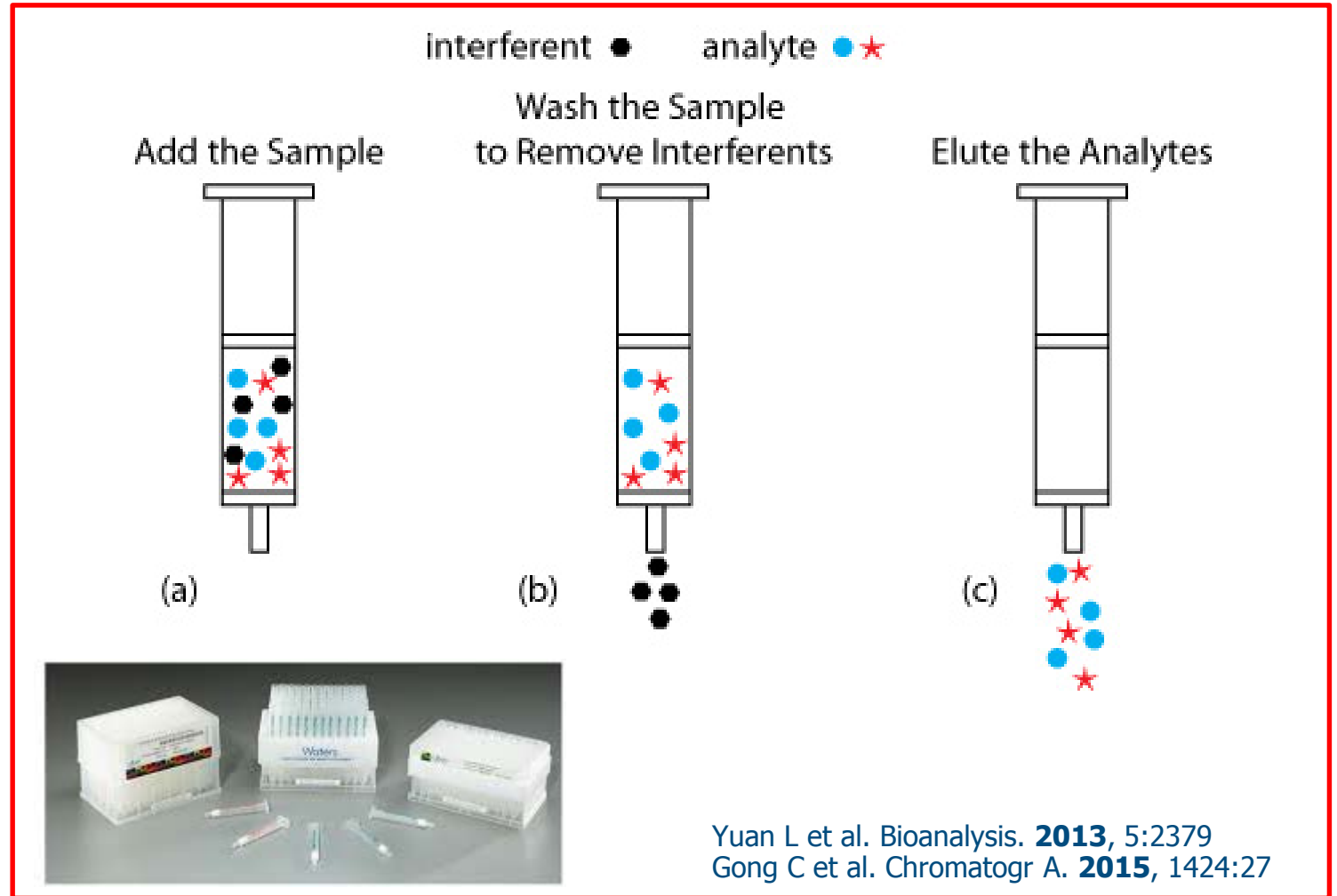
Immunocapture (IC, Highest Specificity)

- Bead- or cartridge-based
- Extracted samples are clean
- Good assay sensitivity
- Reagent-dependent
- Capture antibody immobilized based on biotin-streptavidin binding or direct binding
- Fully automated process
- Analyte dependent capture Ab
  - Generic capture Ab (Protein A/G, anti-Fc)
  - Specific capture Ab (target protein, anti-Id, anti-framework)
- One capture Ab

1   2   3   4
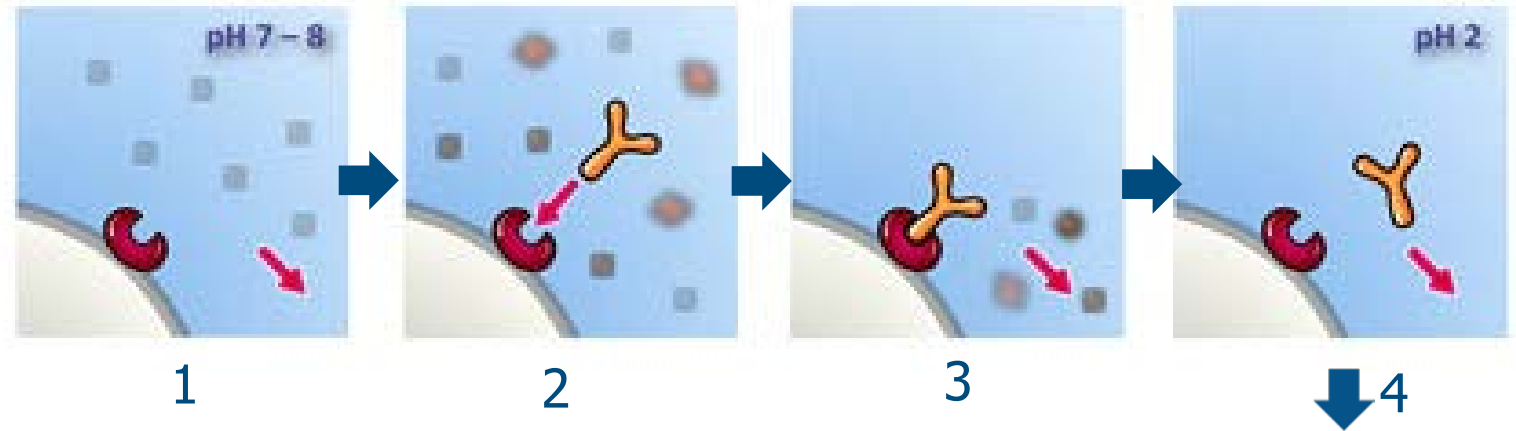
Procedure:
1. Immobilize capture Ab or protein on cartridges/beads
2. Immunocapture the analyte
3. Wash & repeat
4. Elute analyte
5. Trypsin digestion
6. LC-MS analysis

6   5
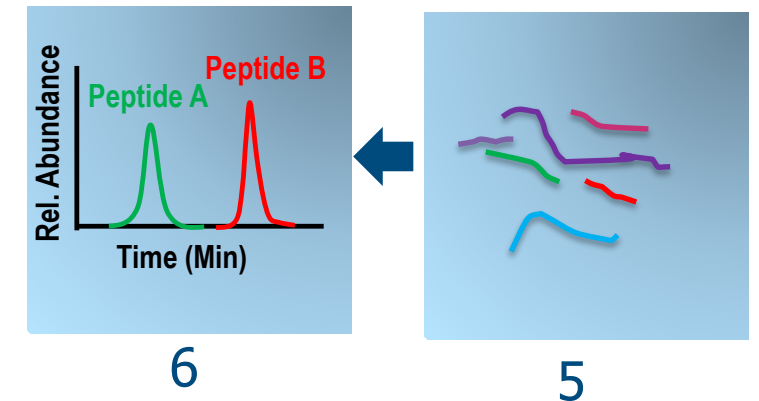
Kaur S *et al. Bioanalysis.* **2016,** 8:1565-1577.
Ackermann BL. *Bioanalysis*, **2016**, 8:1535-1537.

> *"Ligand binding assay (LBA) is simple, sensitive, and high throughput, and LC-MS is a powerful analytical tool to provide a better assay specificity at molecular structure level ."*

Application Case Studies

1. Two mAbs in non-clinical toxicokinetic (TK) study

2. PEG-dAb in clinical pharmacokinetic (PK) study

3. PEG-protein in-vivo biotransformation (BTX)

4. Neutralizing antibody (NAb) assay development

Case Study #1: Two mAbs in Non-Clinical Toxicokinetic (TK) Study

IV infusion to monkeys once weekly for 4 weeks of mAb-A, mAb-B or coadministration

Chemiluminescent NeutrAvidin-HRP

anti-human IgG4 mAb

mAb-A (IgG4)

mAb-A

Anti-mAb-A
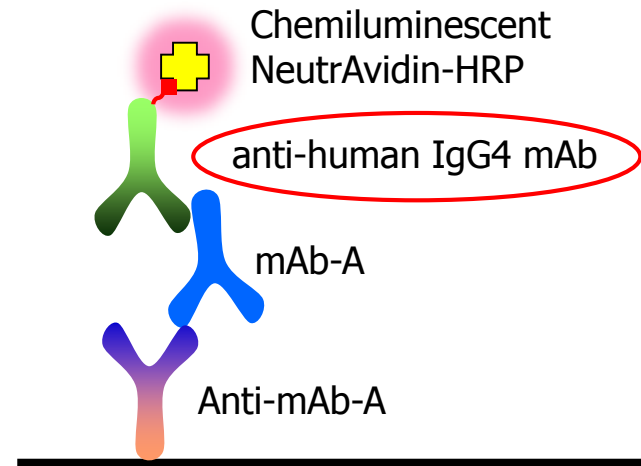
- Cross-reactivity of detection antibody for IgG4
- High detection background with high levels of mAb-B

mAb-B (IgG4)

mAb-B

SULFO-TAG-anti-mAb-B Clone 2

Biotin-anti-mAb-B Clone 1

MSD SA plate
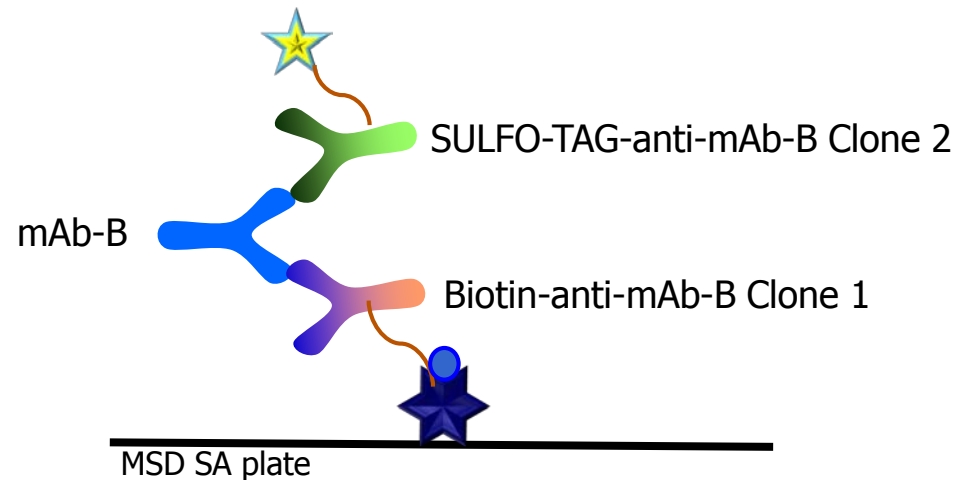
Immunocapture-Free Assay - "Total" Drug Assay

mAb drug
Serum proteins (large)
Serum proteins (small)
Lipids

Protein precipitation
- Addition of methanol
- Centrifugation

Supernatant is discarded

Pellet reconstitution
Digestion buffer

Protein digestion
- Denaturation
- Alkylation
- Trypsin digestion

LC-MS/MS Analysis

Surrogate Peptide

Jiang H, Anal. Chem. 2013, 85: 9859
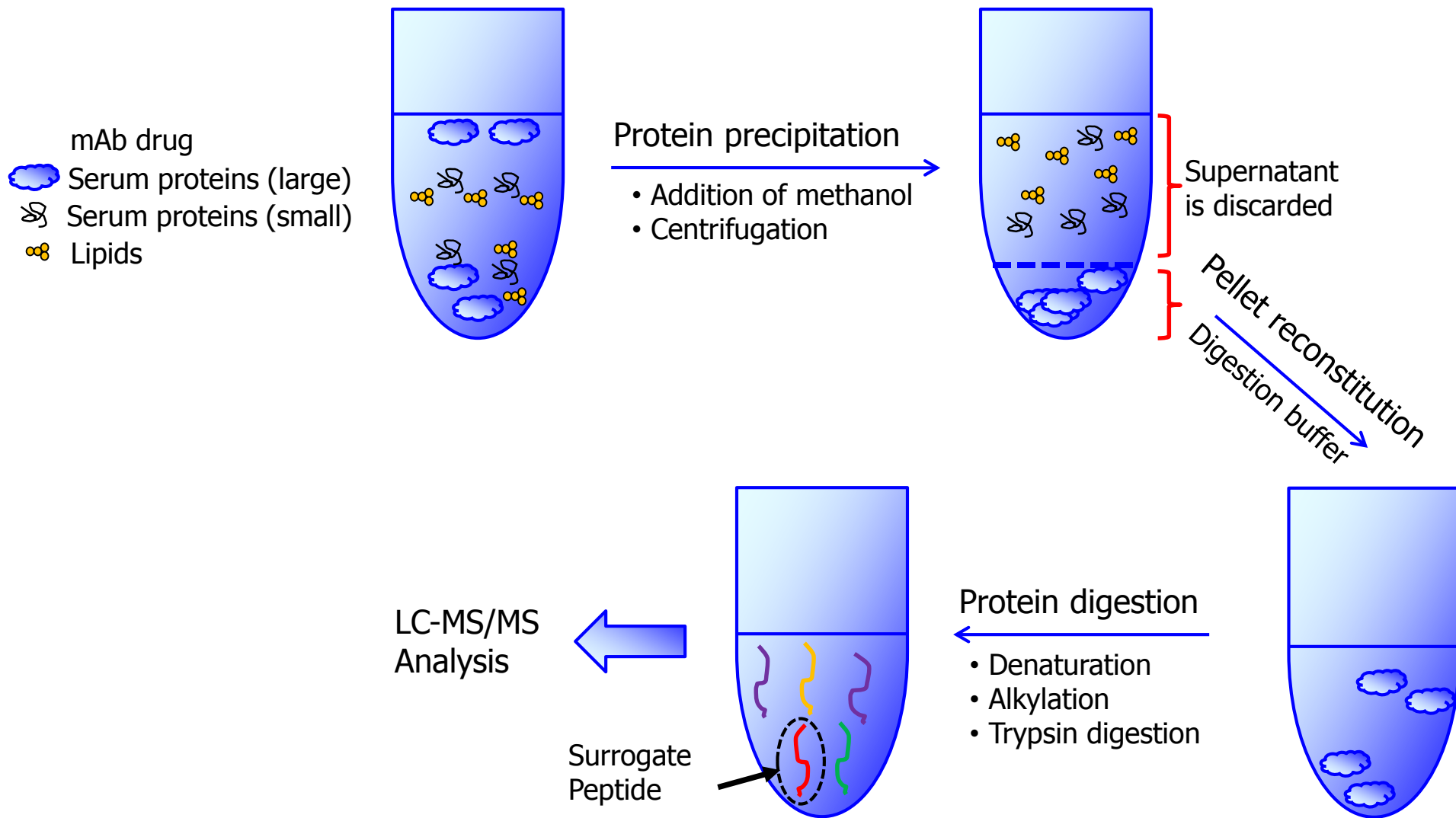
Surrogate Peptides for LC-MS Quantitation
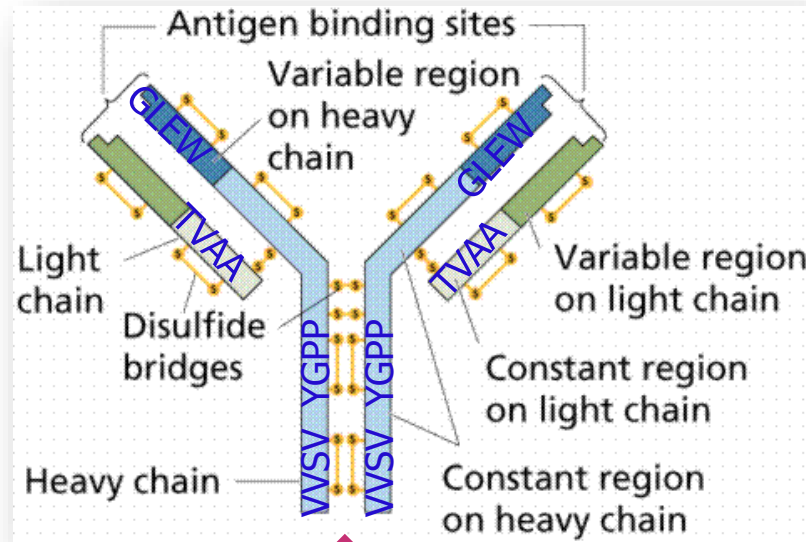
DDA/MS2 scan
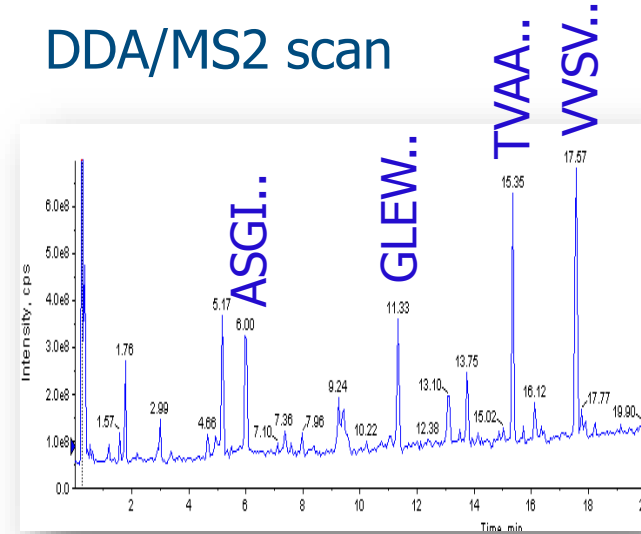
Tryptic Peptide Mapping

Peptides Screening

Sensitivity
Specificity
Accuracy
Precision
Reproducibility

GLEW

VVSV
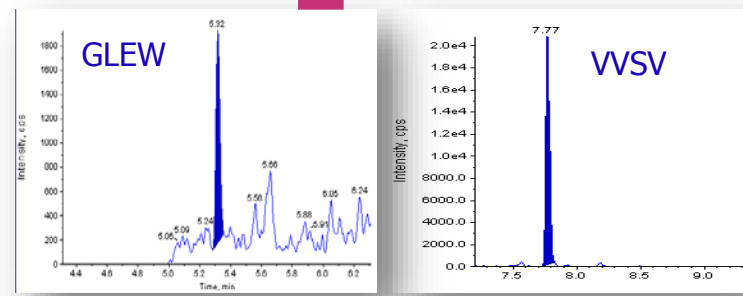
Quantitation peptide

Confirmatory peptide

MRM Quantification

Sample Pretreatment and LC-MS Analysis

- Precipitation with methanol (25 µL serum sample)

- Reduction and alkylation (dithiothreitol and iodoacetamide)

- Internal standardization (with SIL-peptides)

- Trypsin digestion (50°C for 30 min)

- Stop digestion (with 10% formic acid)

- LC-MS/MS analysis

  ✓ 2-µL injection on uHPLC (120 µL of processed samples)

  ✓ C_{18} column with 0.1% formic acid in acetonitrile/water

  ✓ Multiple reaction monitoring (MRM) detection

  ✓ 10 min run time for each sample

Fully Validated LC-MS/MS Assay

- Accuracy and precision runs (n = 3)

- Sensitivity (10 lots of serum)

- Specificity (10 lots of serum, co-dosed drug, Anti-Drug Antibodies (ADA))

- Stability (RT, FT, LTS, RI, PSS)

- Robustness (recovery/matrix effect, run size, autosampler carryover)

- Dilution linearity

- Cross validation between LBA and LC-MS (QCs and study samples)

Jiang H, Anal. Chem. 2013, 85: 9859

Good LC-MS Assay Performance

Surrogate Peptide	Function/Location	Cal. Curve	Regression
GLEWIGEINHR	Quantitation, CDR (mAb-A)	5 – 500 µg/mL	Linear, $1/x^2$
ASGIXXXXXXMHWVR	Quantitation, CDR (mAb-B)	25 – 500 µg/mL	Linear, $1/x^2$
VVSVLTVLHQDWLNGK	Confirmatory, Fc (both)	5 – 500 µg/mL	Linear, $1/x^2$
TVAAPSVFIFPPSDEQLK	Confirmatory, LC (both)	25 – 500 µg/mL	Linear, $1/x^2$

Surrogate Peptide	%Dev	Between-Run (%CV)	Within-Run (%CV)
GLEWIGEINHR	≤ ±2.8	≤ 3.6	≤ 3.8
ASGIXXXXXXMHWVR	≤ ±4.9	≤ 2.1	≤ 6.0
VVSVLTVLHQDWLNGK	≤ ±4.2	≤ 7.5	≤ 9.5
TVAAPSVFIFPPSDEQLK	≤ ±3.2	≤ 2.8	≤ 3.0

- mAb-A and mAb-B in monkey sera were simultaneously quantitated by LC-MS
- 15/20% acceptance criteria
- 'X" stands for BMS proprietary info

No Interference from Co-dosed Drug and ADA

Analyte	Spiked with 2000 µg/mL mAb-B GLEW..	Spiked with 4000 µg/mL mAb-A ASGI..
Nominal (µg/mL)	15.00	60.00
Measured (µg/mL)	14.40	56.47
	14.78	57.56
	15.91	60.04
Mean (µg/mL)	15.03	58.02
%Dev	0.2	-3.3
%CV	5.2	3.2
Nominal (µg/mL)	400.00	400.00
Measured (µg/mL)	407.50	377.87
	405.29	387.13
	389.42	376.07
Mean(µg/mL)	400.74	380.36
%Dev	0.2	-4.9
%CV	2.5	1.6

ADA clone ID	LLOQ (µg/mL)	GLEW.. Mean	%Dev	%CV	VVSV.. Mean	%Dev	%CV
1	5	4.7	-5.3	1.2	5.6	11.3	6.3
2	5	4.8	-4.7	4.4	5.6	11.3	3.7
3	5	4.4	-12.7	5.8	5.3	6.0	3.3
4	5	4.9	-1.3	1.2	5.6	12.0	6.2
5	5	4.9	-2.7	6.3	5.6	11.3	2.7
6	5	5.1	2.7	13.0	6.1	21.3	16.9
7	5	5.0	0.0	6.9	5.7	14.7	4.4
8	5	4.8	-4.0	0.0	5.5	10.7	2.8
9	5	5.0	0.7	4.6	5.5	10.7	6.8
10	5	5.2	3.3	4.0	5.7	13.3	6.2
11	5	4.9	-2.7	4.3	5.6	12.0	5.4
12	5	4.9	-2.7	4.7	5.5	9.3	4.2
13	5	4.7	-6.7	2.5	5.5	9.3	2.8

Comparable PK Profiles from LC-MS and LBA
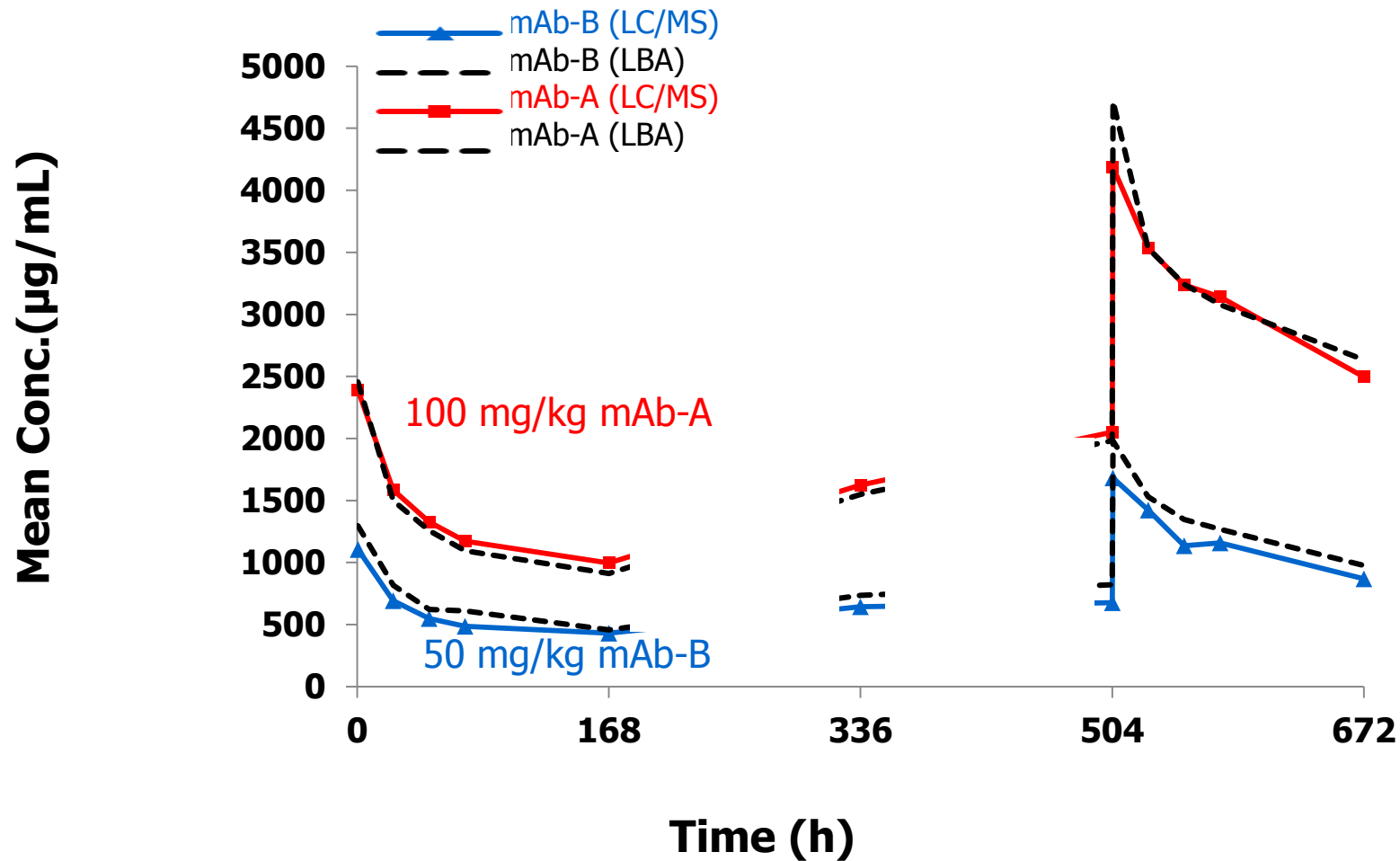
No observable soluble target or ADA interference with LBA
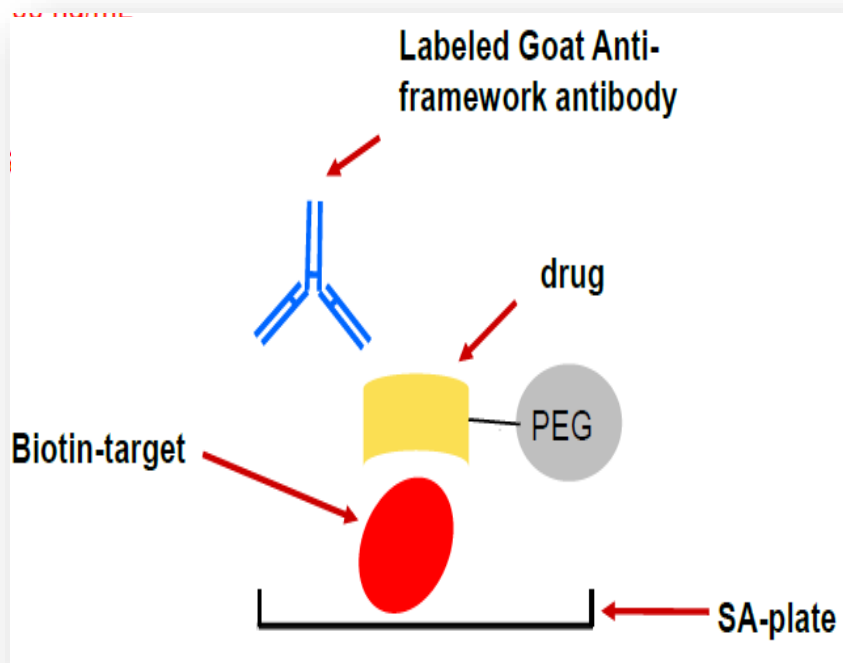
Jiang H, Anal. Chem. 2013, 85: 9859

Good Correlation of PK Data from LC-MS vs. LBA
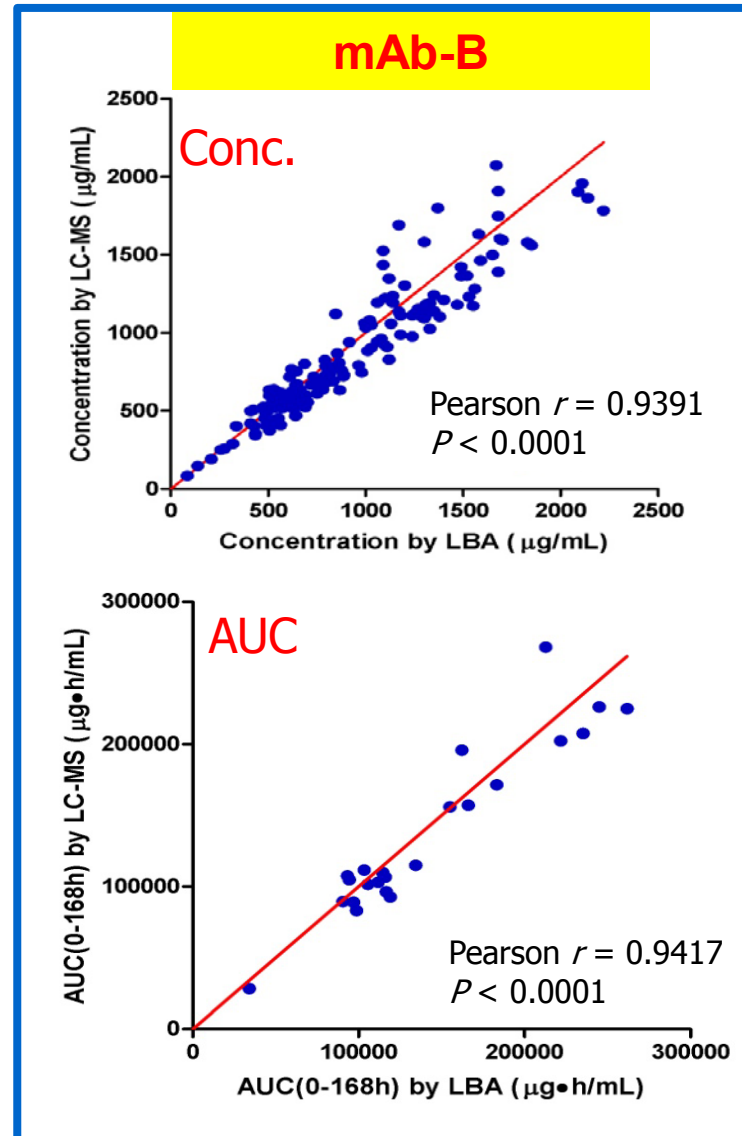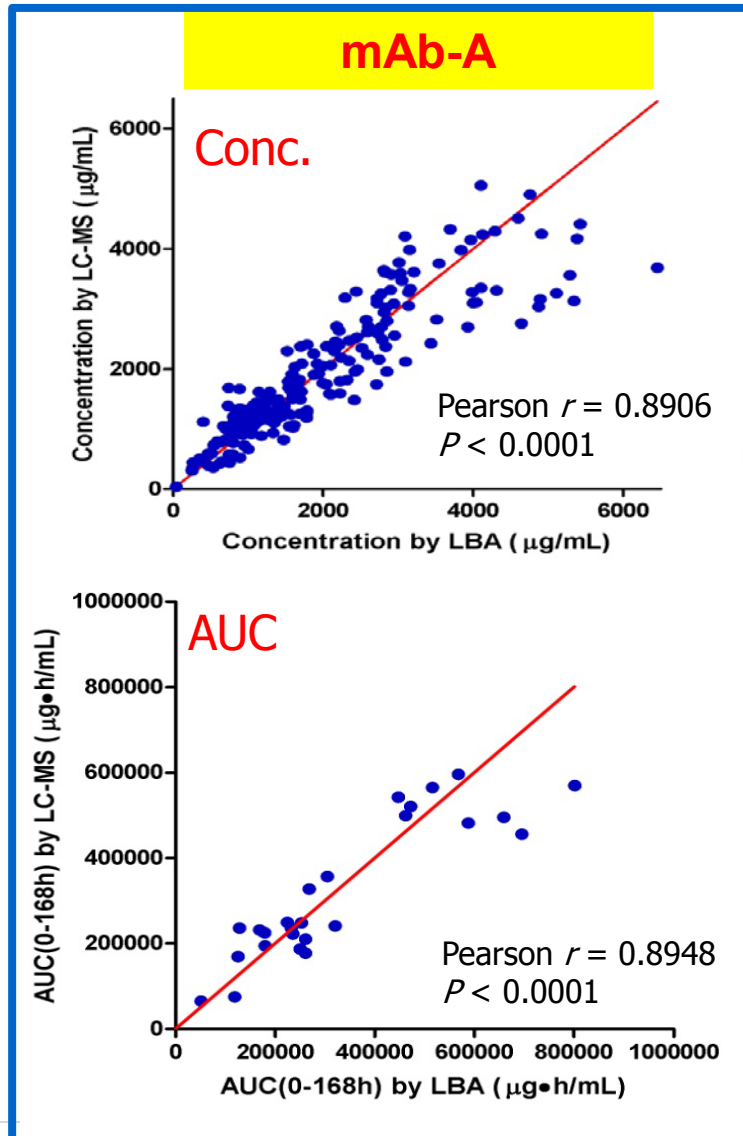
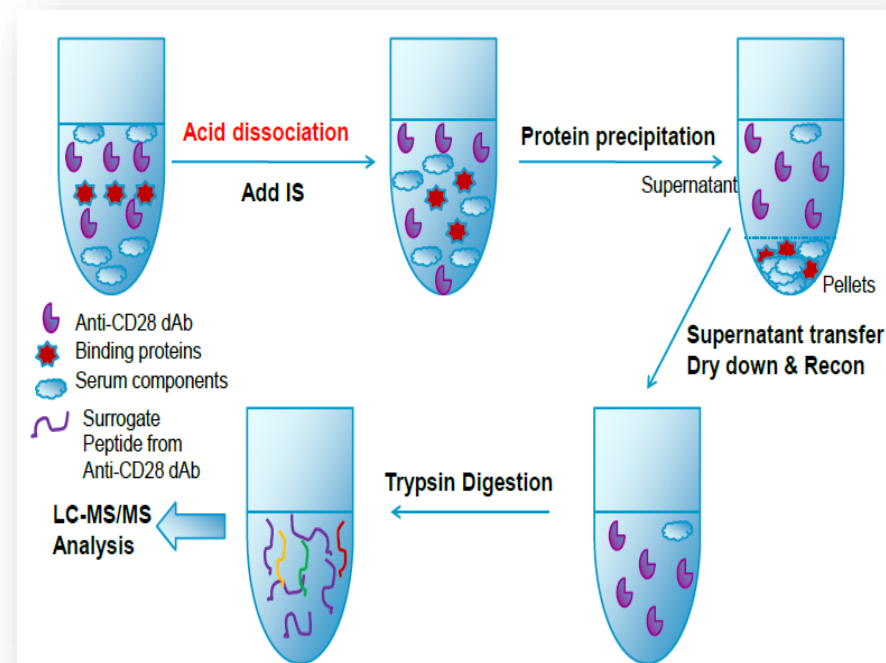# Case Study #2: PEG-dAb in clinical pharmacokinetic (PK) study

- 12 kDa domain Ab with 40 kDa PEG
- Needed a more sensitive assay than the LBA to cover PK profile at the lowest dose
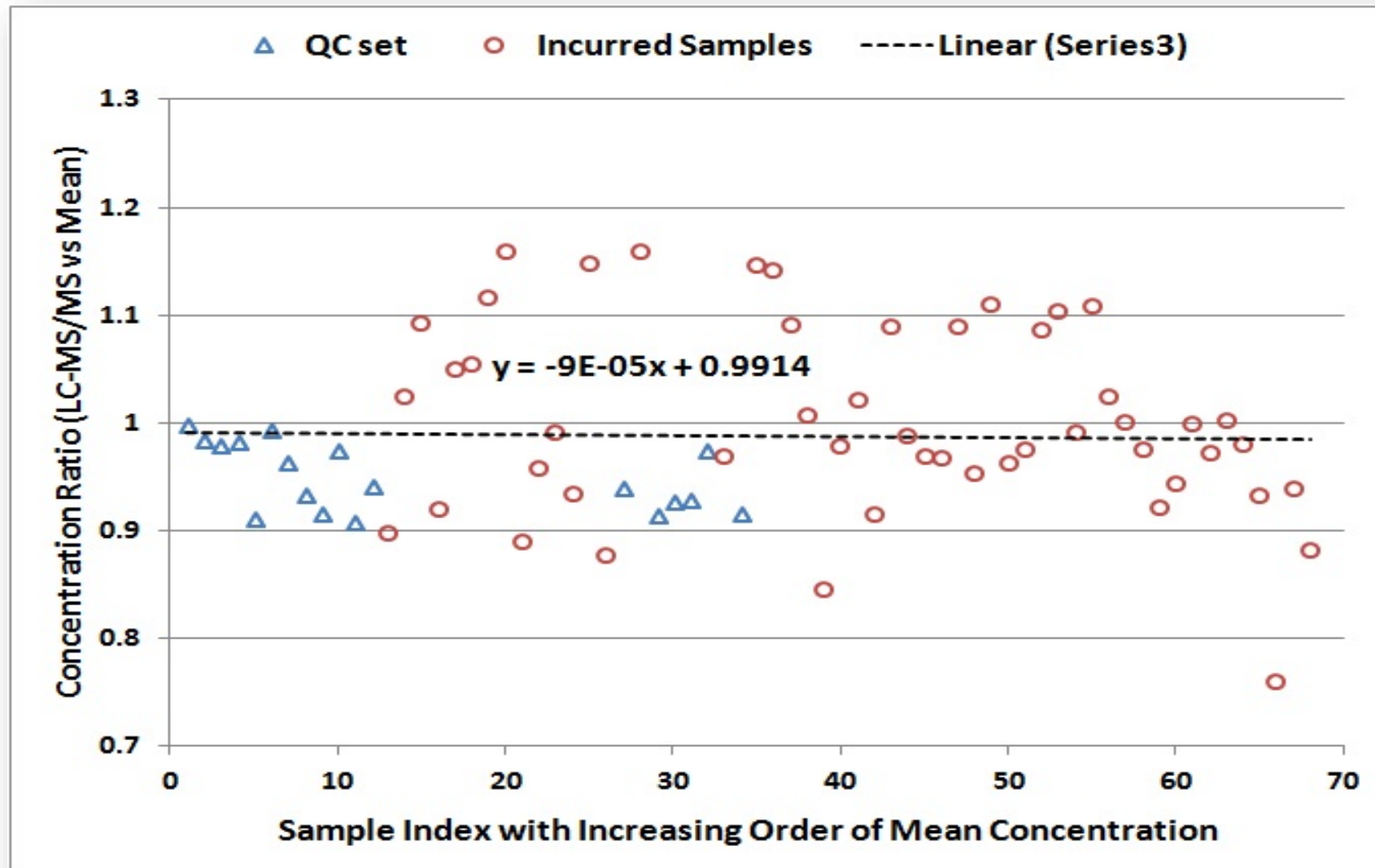
LLOQ 80 ng/mL

LBA to measure "free" drug

LLOQ 10 ng/mL

LC-MS to measure "total" drug

Gong C, Bioanalysis. 2014, 6:2371

Comparable Data of LC-MS vs. LBA

Case Study #3: PEG-Protein In-Vivo Biotransformation (BTX)

AdPKE-Linker-	PIPDSSPLL	QFGGQVRQRY	LYTDDAQQTE
AHLEIREDGT	VGGAADQSPE	SLLQLKALKP	GVIQILGVKT
SRFLCQRPDG	ALYGSLHFDP	EACSFRELLL	EDGYNVYQSE
AHGLPLHLPG	NKSPHRDPAP	RGPARFLPLP	GLPPALPEPP
GILAPQPPDV	GSSDPLSMVG	PSQGRSPSYA	S

D/P cleavage by formic acid hydrolysis

In-vivo C10-clipping (inactive)

Fung E, et al, Bioanalysis, 2014, 6: 2985
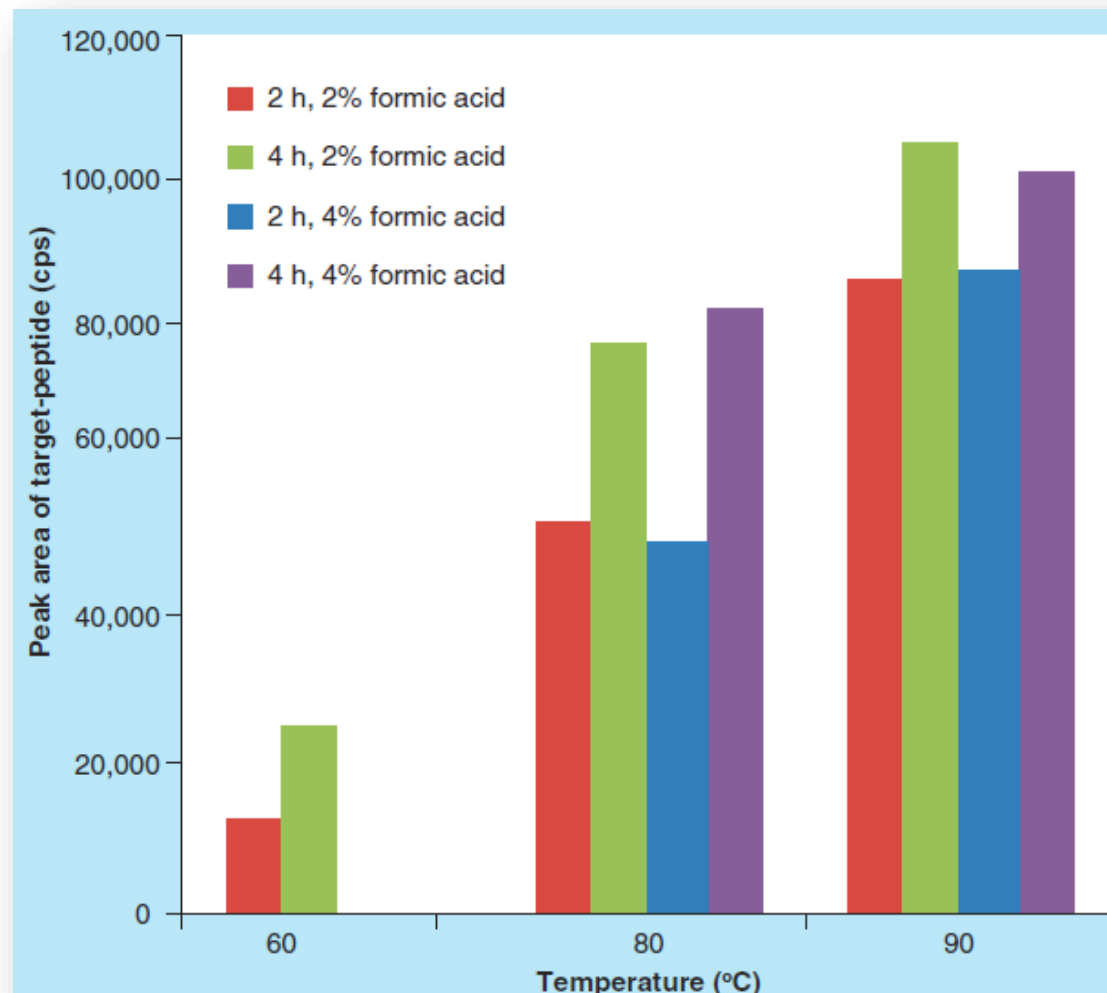
Biotransformation Assay

C10 Clipping metabolite
PLSMVG**P**$^+$

Parent drug
PLSMVG**P**SQGRSPSYAS^{2+}

Fung E, et al, Bioanalysis, 2014, 6: 2985

Bristol-Myers Squibb 40
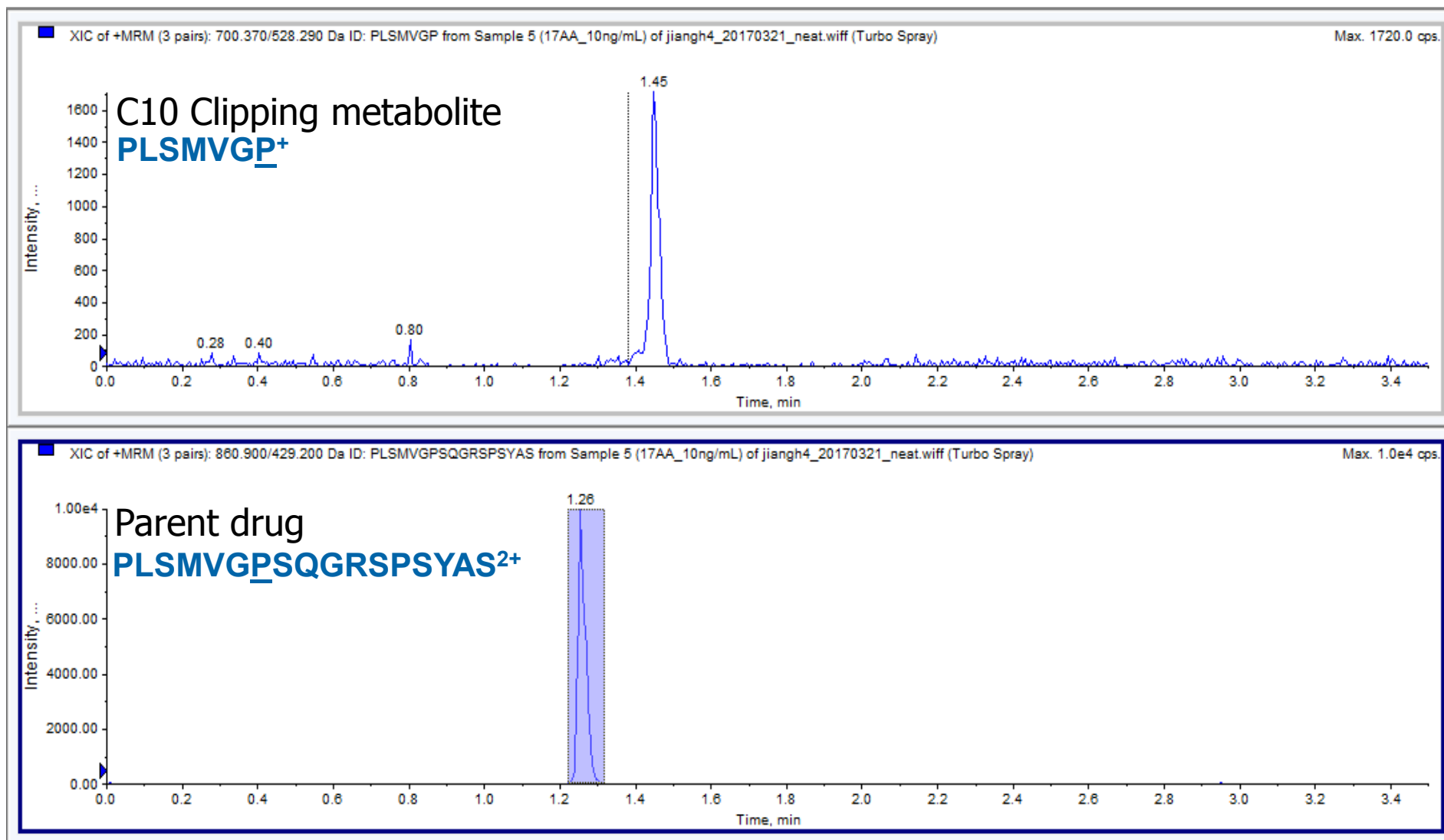
Case Study #4: Neutralizing Antibody (NAb) Assay Development
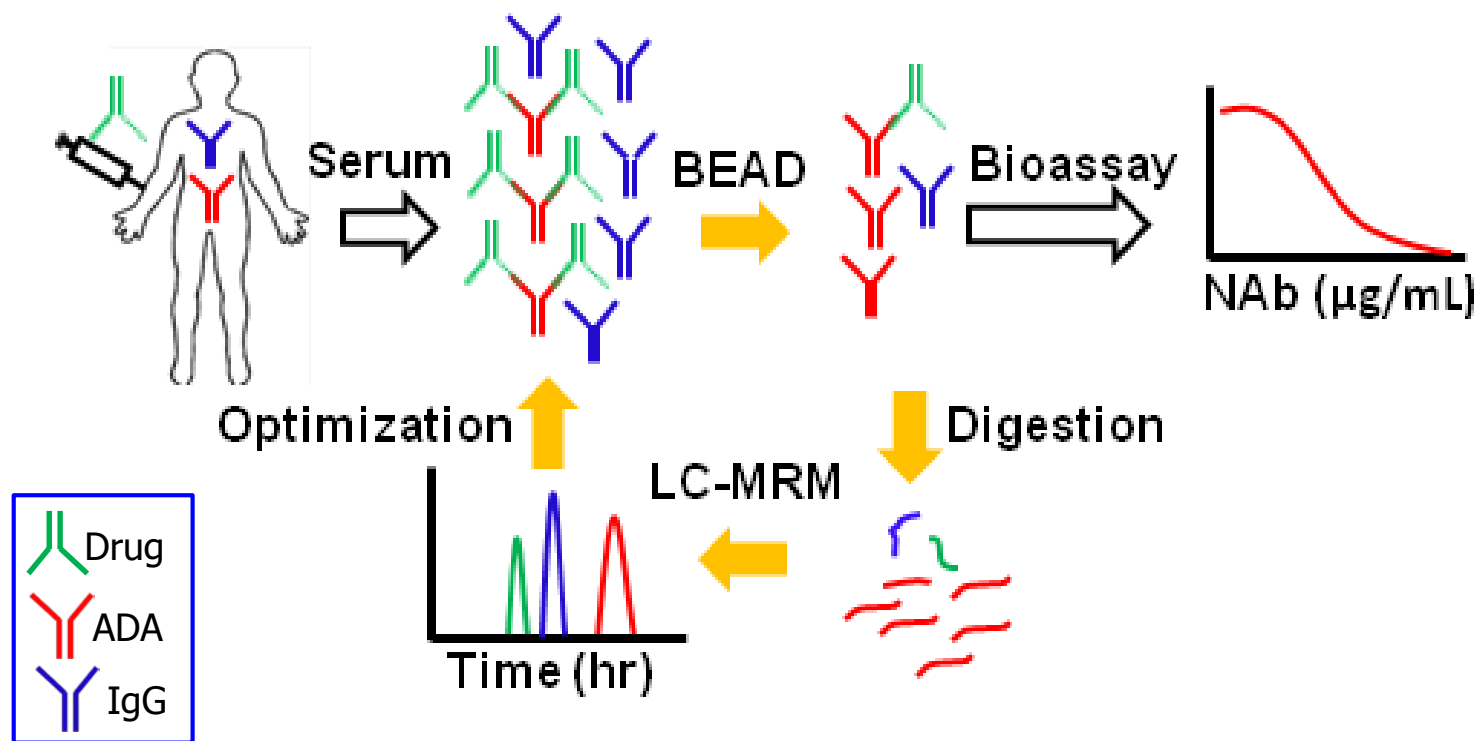

- Cell-based neutralizing antibody (NAb) assay is to detect ADAs that can neutralize drug activity.
- The assay is interfered by the drug and endogenous serum factors.
- Bead extraction with acid dissociation (BEAD) refers to the extraction of ADA from human serum by the beads coated with biotin-drug, after the ADA is dissociated from drug-ADA complex with acid.
- LC-MS assay to evaluate if the drug and serum factors have been removed prior to the downstream cell-based assay

LC-MS Analytes in the BEAD Elutes

Jiang H, Anal. Chem. 2014, 86: 2673
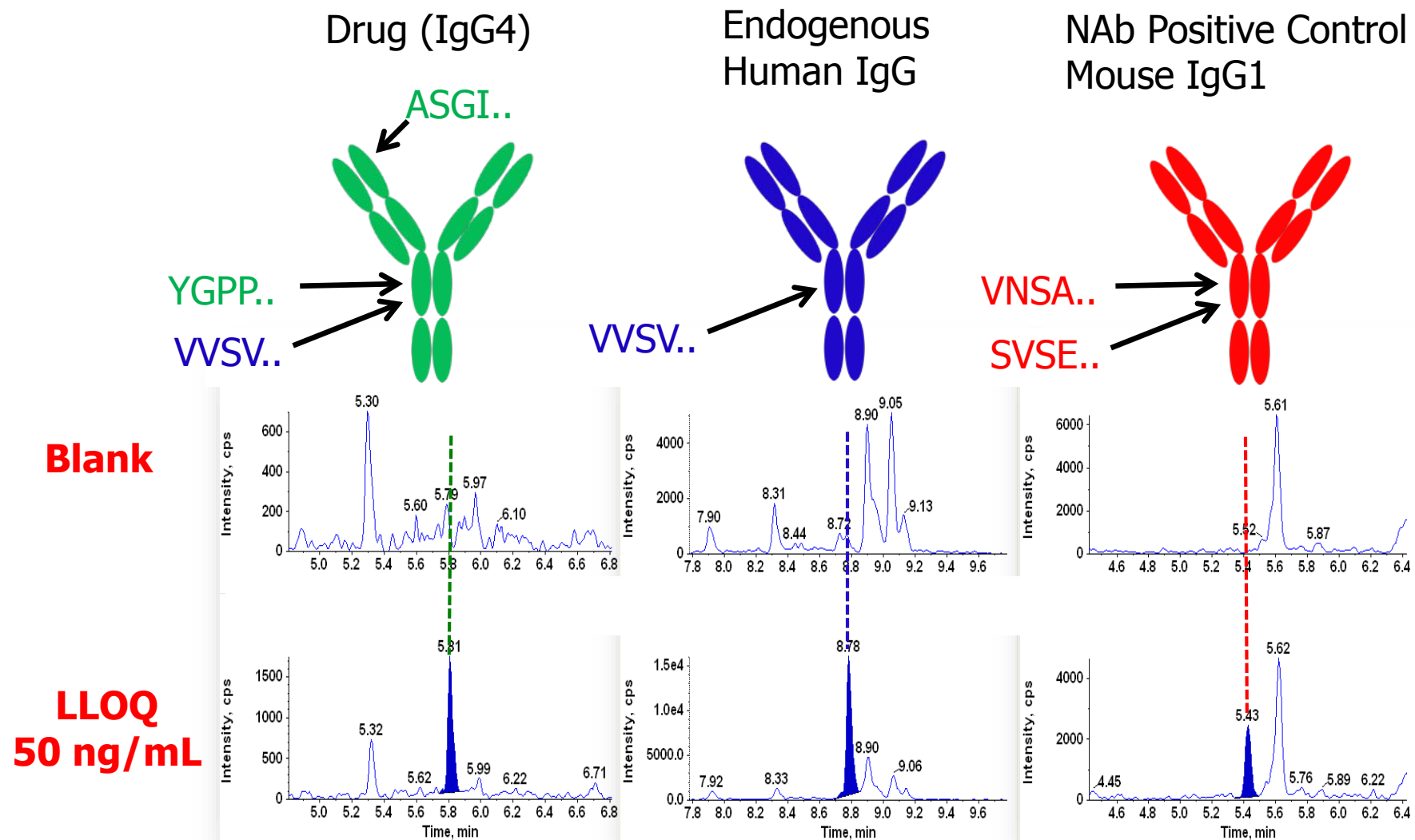
Summary

- LC-MS is a powerful bioanalytical tool for biologics development due to its good specificity, multiplexing capacity, and flexibility.

- The LC-MS assay sensitivity is continuously being improved by advancement of LC and MS technologies and innovative applications in hybrid LC-MS.

- Most importantly, it can be employed to overcome the technical issues observed in the LBA.

  - High LBA detection background (case study #1)

  - Poor LBA assay sensitivity (case study #2)

  - LBA not able to differentiate parent drug and its metabolite (case study #3)

  - LBA not able to detect residual drug (case study #4)

3. Strategy and applications of LC-MS in immunogenicity assessment

液质联用在免疫原性评估中的应用

Hao Jiang, PhD

Bristol-Myers Squibb, Princeton, NJ, USA

Immunogenicity Testing

- Based on FDA guidance[1], immunogenicity tests should be designed to detect ADA that could mediate unwanted biological or physiological consequences.

  - ➤ Screening assays are used to detect all antibodies that bind to the therapeutic protein product.

  - ➤ The specificity is established using confirmatory assays.

  - ➤ Further characterized using tittering and neutralization assays.

- The sponsor should implement preliminary validated assays early, before and during phase 1, and obtain data in real time.

- Information on immune responses observed during clinical trials is crucial and should be included in the prescribing information.

- Therefore, the development of valid, sensitive, specific, and selective assays to measure ADA responses is a key aspect of therapeutic protein product development.
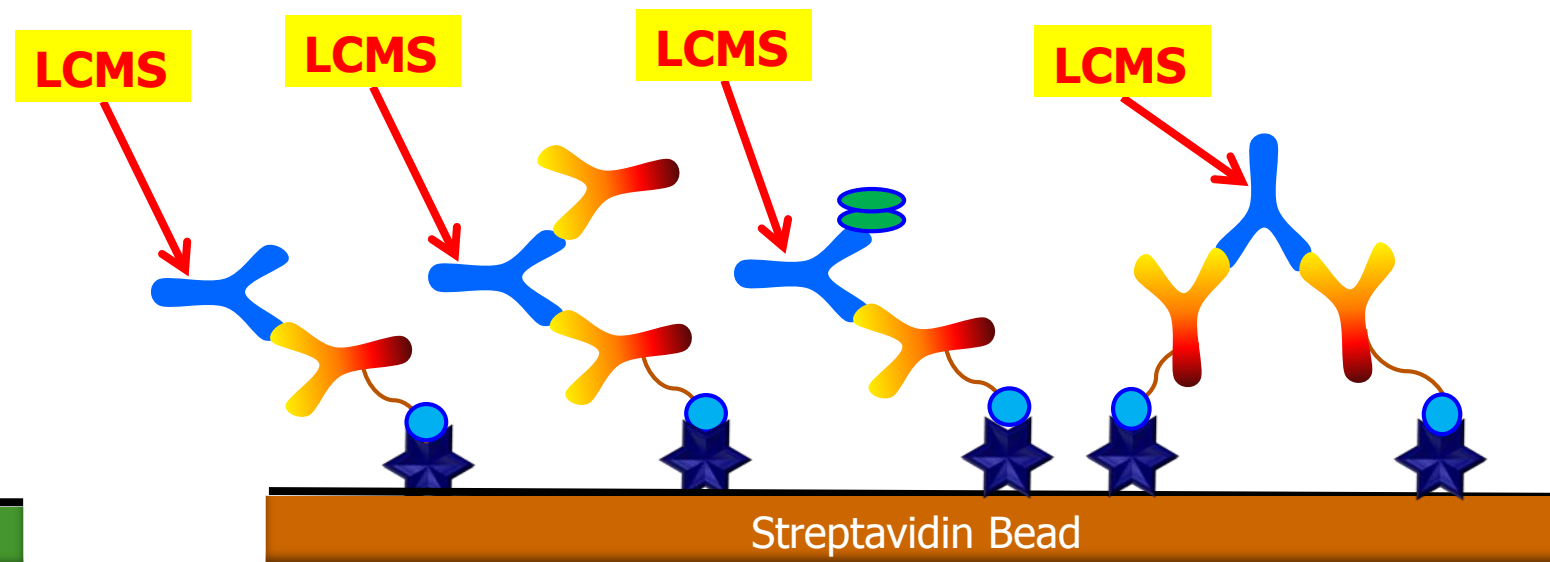
[1]Assay Development and Validation for Immunogenicity Testing of Therapeutic Protein Products, Guidance for Industry (draft guidance)", by US FDA/CDER, CBER,CDRH, April 2016
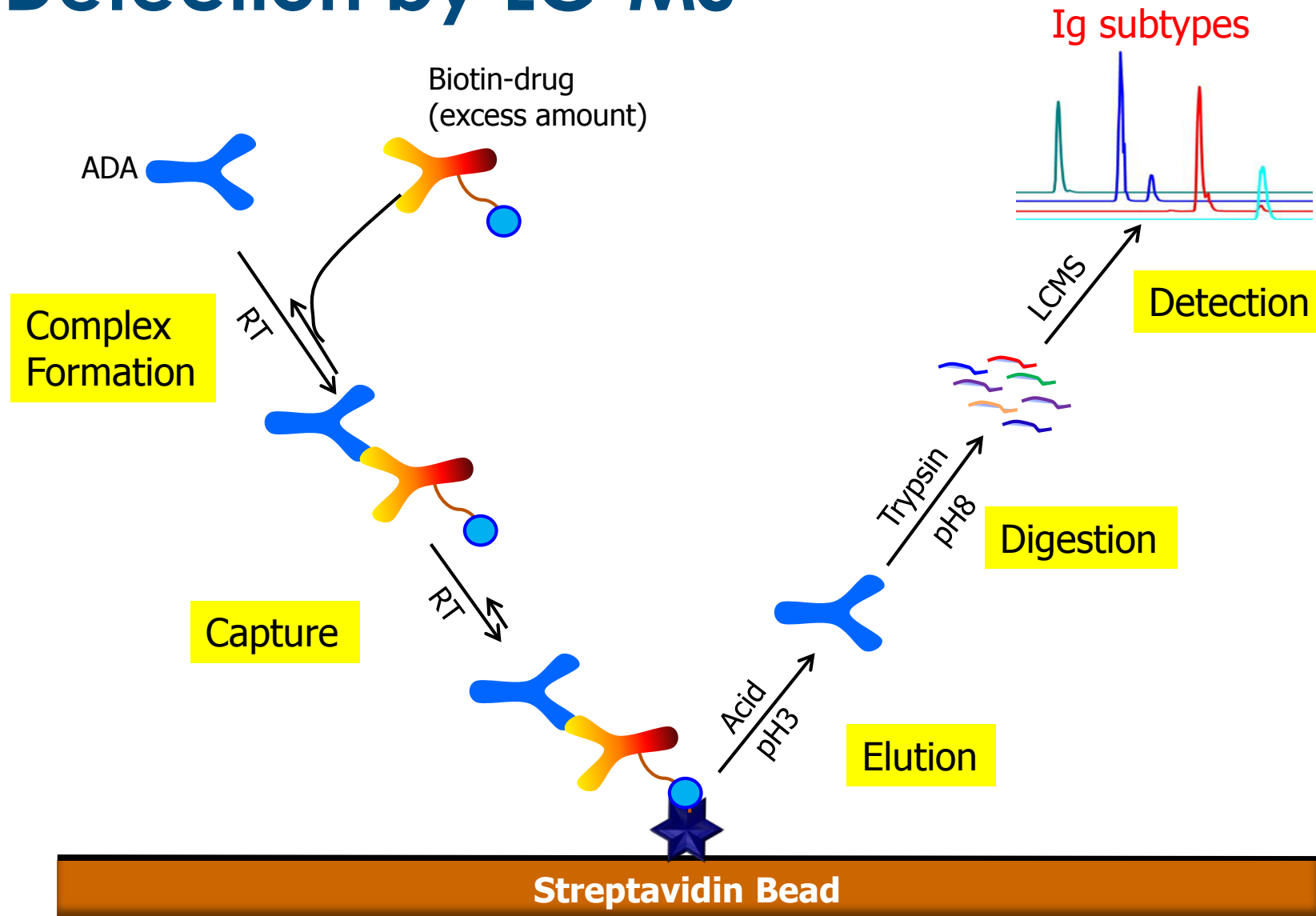
Bridging LBA and LC-MS Assay Formats

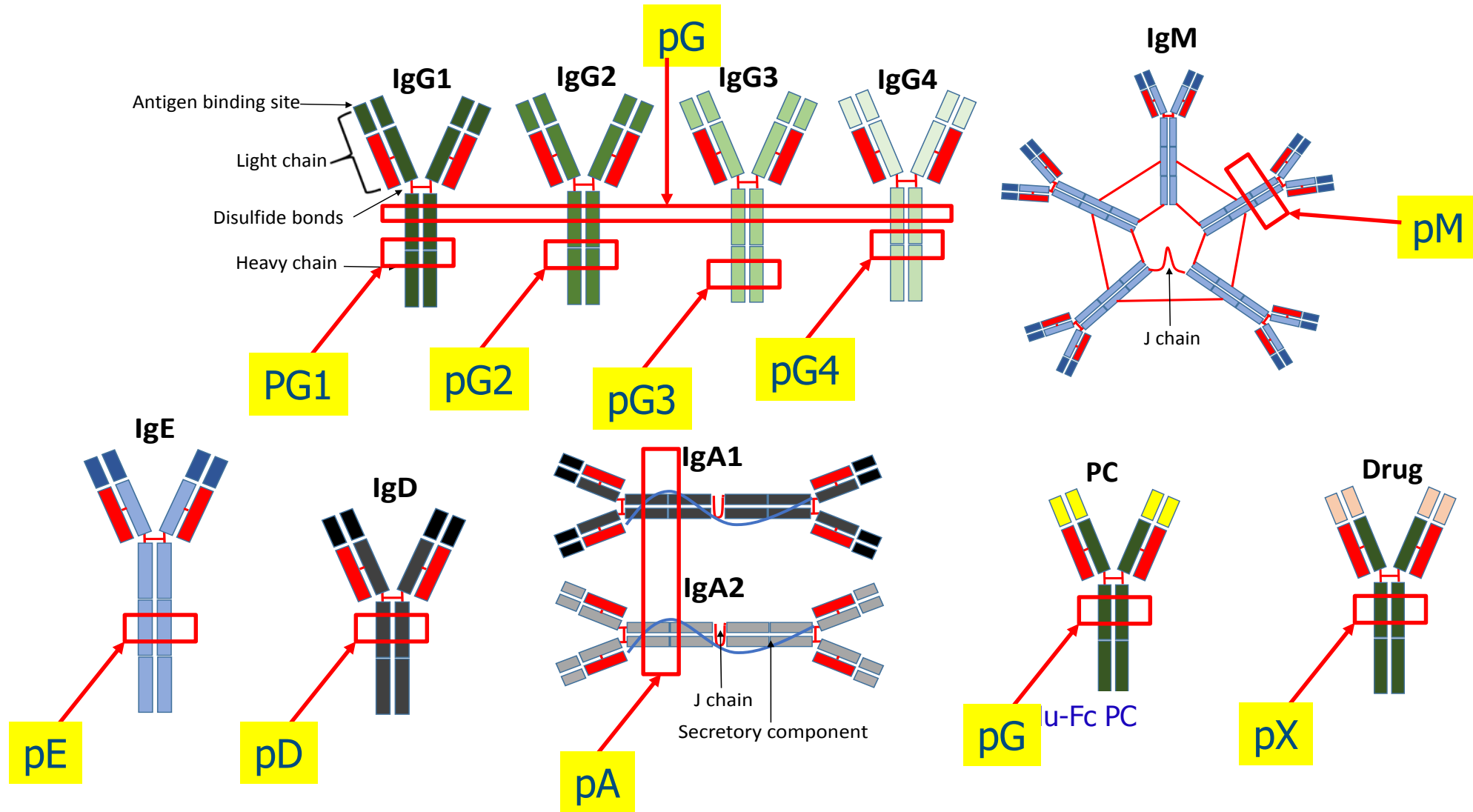Bridging LBA
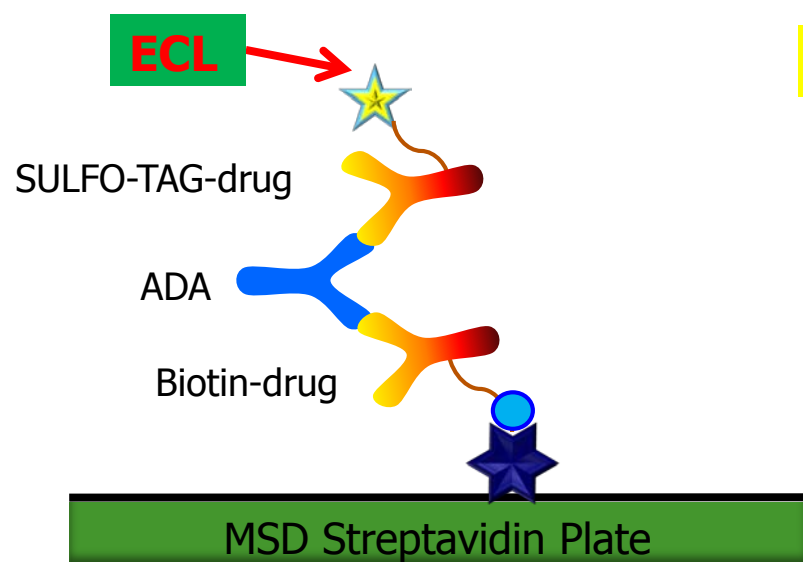
Immunocapture LCMS (direct format)

- Jiang H, et al. Anal Chem, 2014, 86, 2673–2680
- Xu W, et al. J Immunol Methods, 2015, 416, 94-104
- Chen L, et al. J Immunol Res, 2016, Article ID 7682472

ADA Detection by LC-MS

Jiang H, et al. Bioanalysis. 2018, to be published

Bristol-Myers Squibb 47

Multiplex ADA Isotyping by LC-MS

Jiang H, et al. Bioanalysis. 2018, to be published

Specific Surrogate Peptides for ADA Isotype/Subclass

Human Ig Isotype/subclass	Composition%*	Conc. (mg/mL)*	Surrogate peptide sequence	Peptide ID
IgG	80%	7.23 – 16.85	NQVSLTCLVK DTLMISR	pG
(IgG1)	65% of IgG	4.5 – 9.0	GPSVFPLAPSSK FNWYVDGVEVHNAK	pG1
(IgG2)	25% of IgG	1.8 – 5.3	VVSVLTVVHQDWLNGK GLPAPIEK	pG2
(IgG3)	5% of IgG	0.13 – 0.80	SCDTPPPCPR WYVDGVEVHNAK	pG3
(IgG4)	5% of IgG	0.08 – 1.0	YGPPCPSCPAPEFLGGPSVFLFPPKPK GLPSSIEK	pG4
IgM	8%	0.48 – 2.7	FTCTVTHTDLPSPLK GQPLSPEK VSVFVPPR	pM
IgE	<0.03%	$< 4.3 \times 10^{-7}$	AEWEQK LEVTR	pE
IgA	12%	0.8 – 4.6	YLTWASR VAAEDWK	pA
IgD	<1%	≤ 0.14	SLWNAGTSVTCTLNHPSLPPQR	pD

LC-MS Procedure

LC Separation

1) C18 LC column 2.1 x 100 mm, 3 μm

2) Mobile phases: 0.1% formic acid/water (A); 0.1% formic acid/acetonitrile (B)

3) LC gradient: 5% - 50% for 4 min

4) Flow-rate: 0.4 mL/min

5) Injection volume: 10 μL

6) Run time/sample: 6 min

MS Detection

1) Triple Quadrupole (TQ) instrument (SCIEX 5500/6500)

2) Multiple Reaction Monitoring (MRM)

3) Monitoring isotype/subclass specific surrogate peptides

4) Peptide specific parameters: Declustering Potential (DP), Collision Energy (CE)

This generic LC-MS conditions are NOT project-specific and can be used for different projects

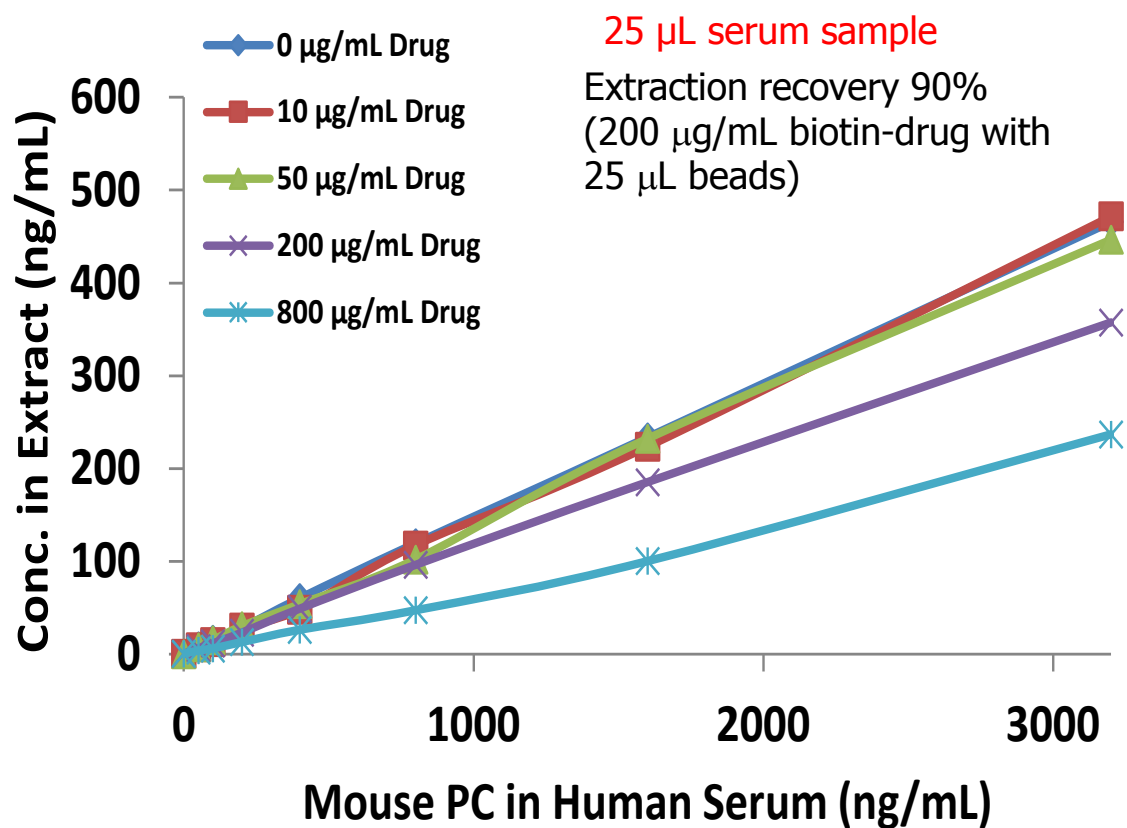Bristol-Myers Squibb

Representative Chromatograms of Surrogate Peptides

Human ADA controls at 1 μg/mL in elution buffer
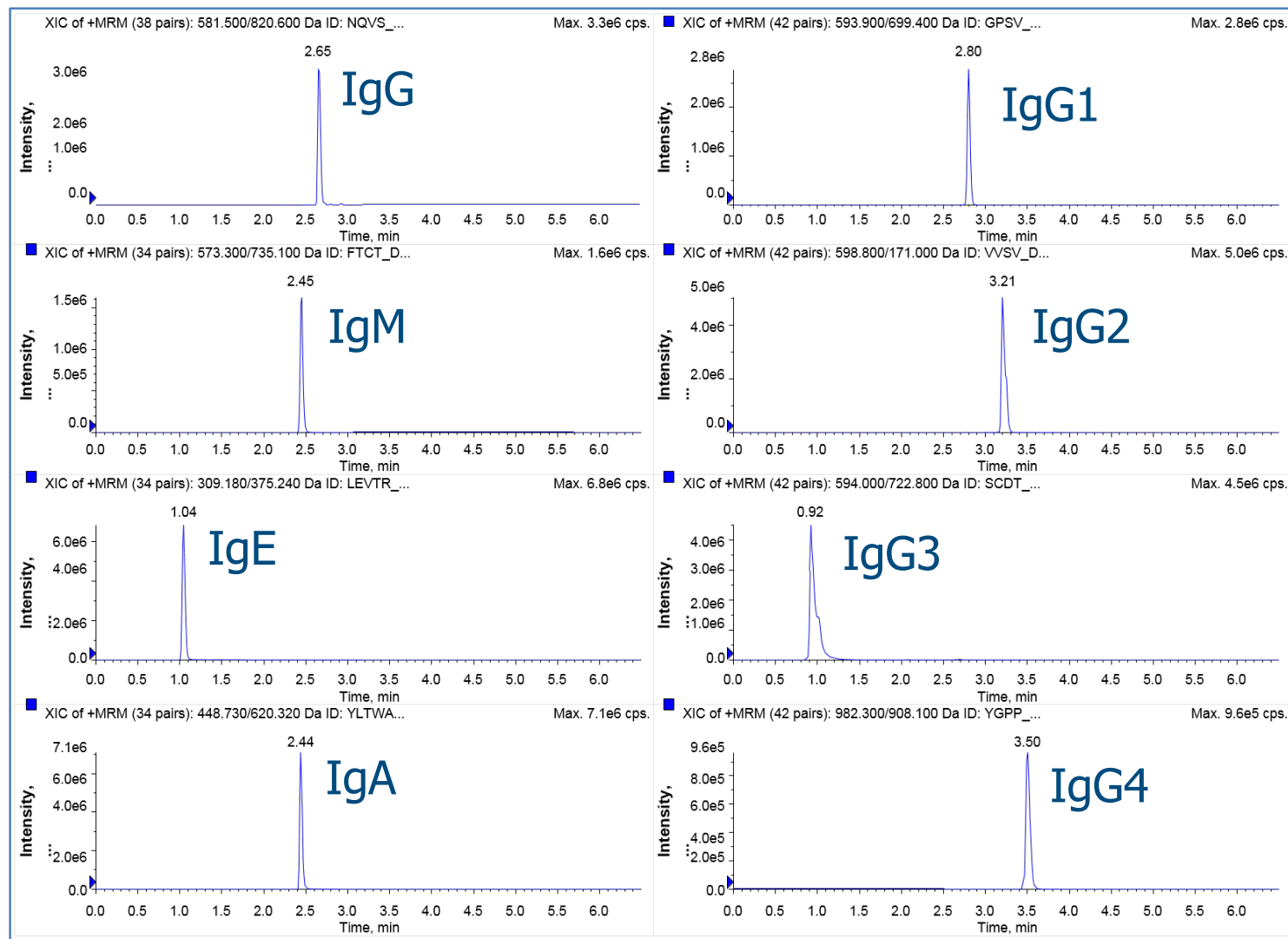
- Assay sensitivity at 50 ng/mL PC in serum (5 ng/mL for processed samples)

- Each surrogate peptide was monitored at a specific MRM ion transition

- The positive control and the residual drug in the extract can also be simultaneously measured

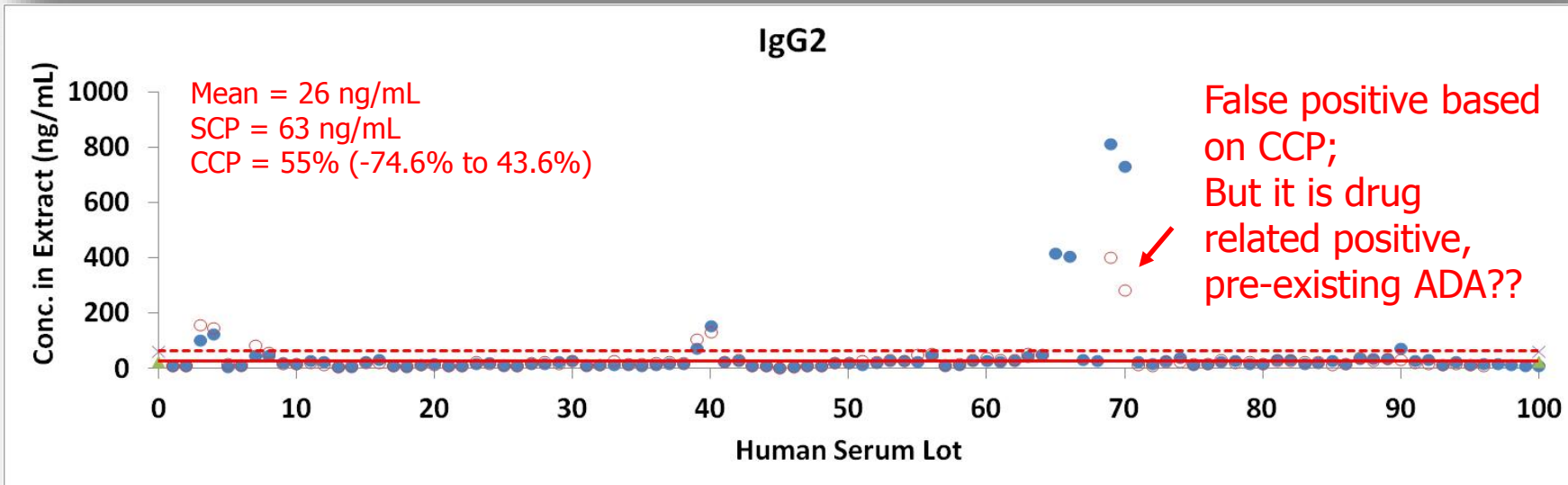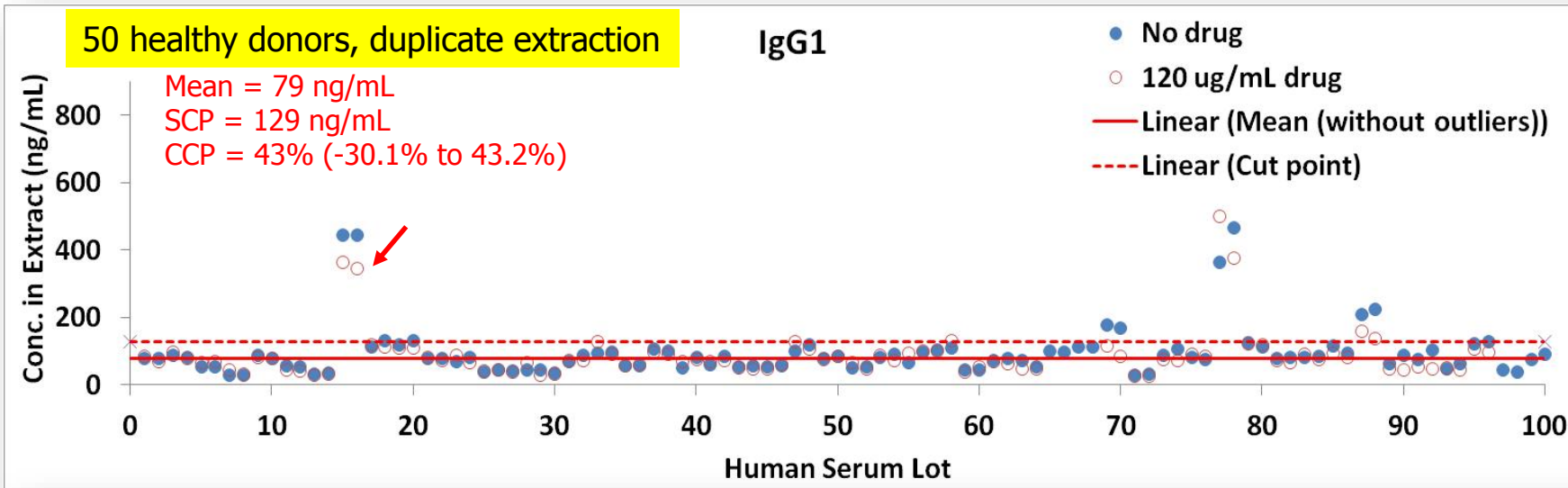- The MS parameters for every surrogate are generic and suitable for different projects

Jiang H, et al. Bioanalysis. 2018, to be published

Drug Interferences

Drug Sensitivity and Tolerance

25 µL serum sample

Extraction recovery 90%
(200 µg/mL biotin-drug with
25 µL beads)

PC (ng/mL)	Drug (µg/mL)				
	0 µg/mL Drug	10 µg/mL Drug	50 µg/mL Drug	200 µg/mL Drug	800 µg/mL Drug
0	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
50	8	7	7	6	<LLOQ
100	16	13	15	12	6
200	28	29	30	23	13
400	60	48	54	49	26
800	119	117	102	96	47
1600	234	223	232	185	100
3200	466	472	447	357	237

Jiang H, et al. Bioanalysis. 2018, to be published

Screening and Confirmatory Cut Points (SCP, CCP)

50 healthy donors, duplicate extraction

IgG1

- ● No drug
- ○ 120 ug/mL drug
- — Linear (Mean (without outliers))
- ---- Linear (Cut point)

Mean = 79 ng/mL
SCP = 129 ng/mL
CCP = 43% (-30.1% to 43.2%)

IgG2

Mean = 26 ng/mL
SCP = 63 ng/mL
CCP = 55% (-74.6% to 43.6%)

False positive based on CCP;
But it is drug related positive, pre-existing ADA??

IgG3 and IgG4 were not detectable by LCMS

SCP = Mean-Response + 1.645*SD (95% CI)

CCP = Mean-%Inhibition + 2.33*SD (99% CI)

Titer CP = Mean-Response + 3.09*SD (99.9% CI)

Jiang H, et al. Bioanalysis. 2018, to be published

LC-MS Reduces Drug Interference (or False Negative)
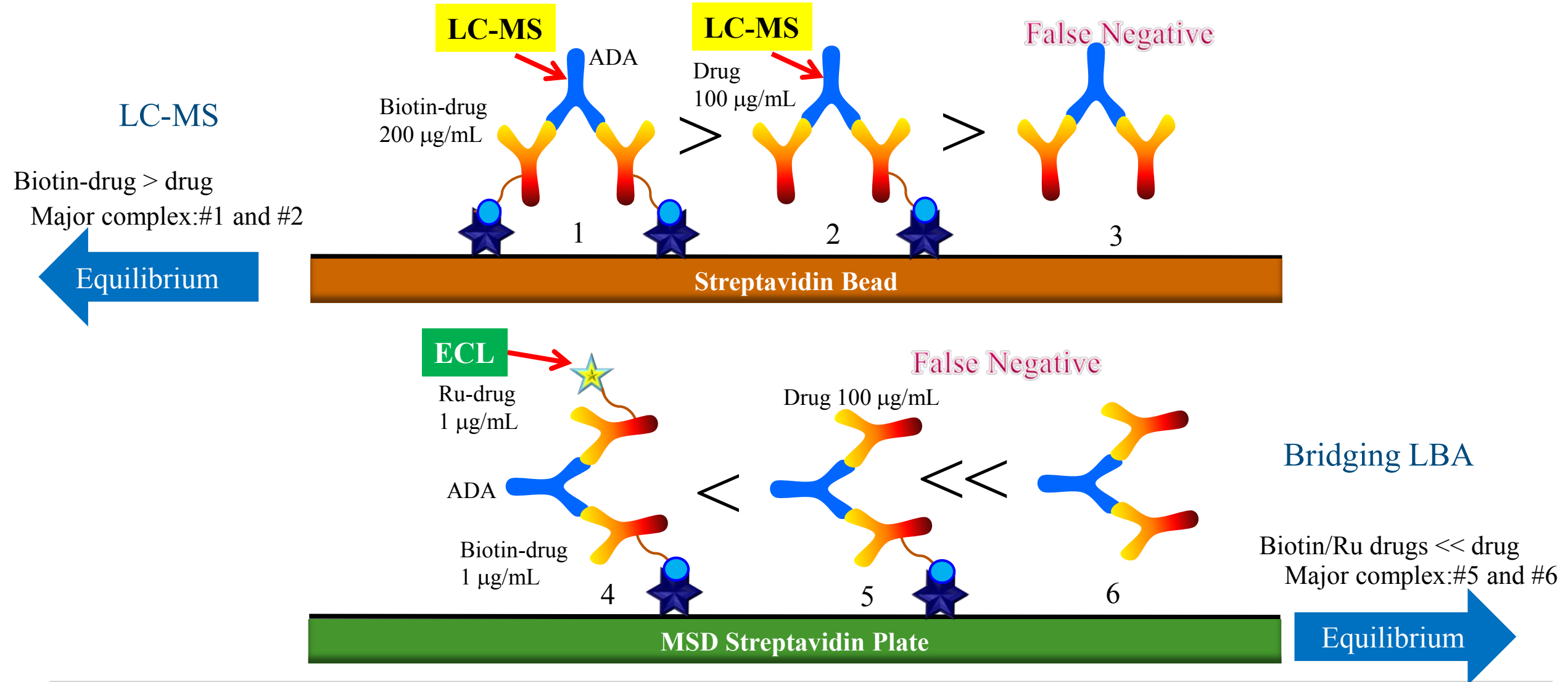
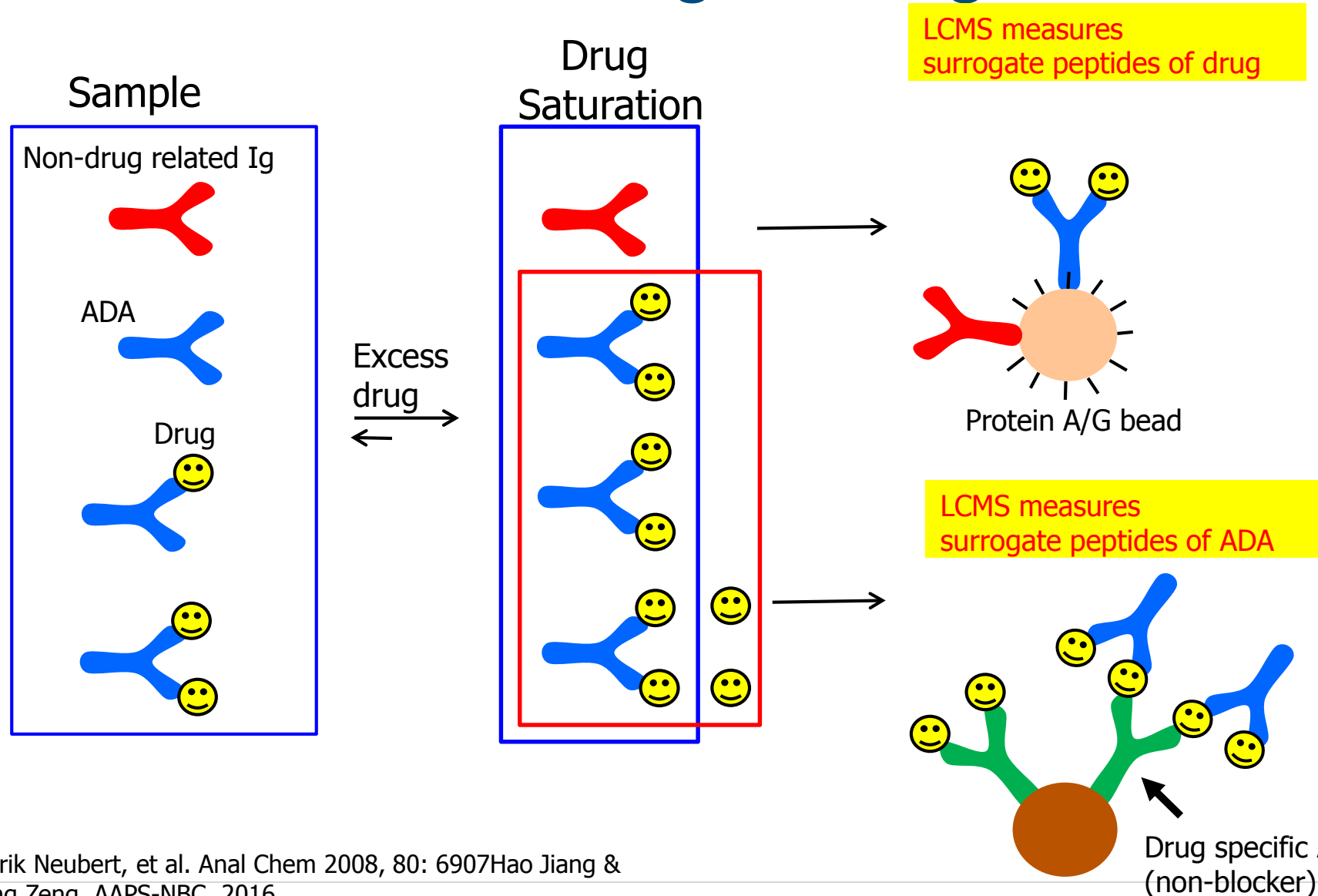# LC-MS Reduce False Positives
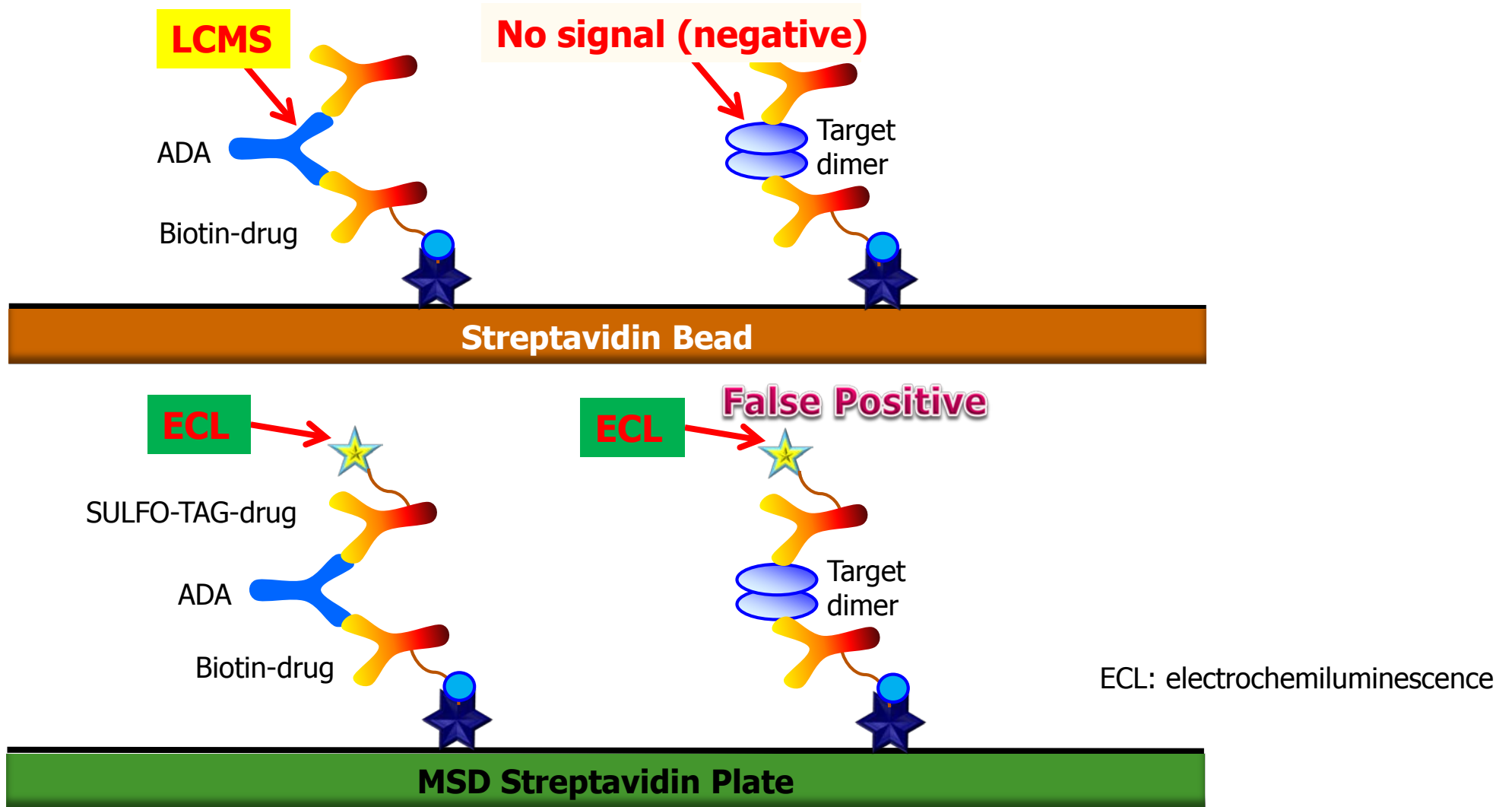
Pros and Cons

Pros

- Reduced drug interference
- Reduced false positive
- Generic assay
- Multiplex (ADA isotypes/drug)
- Direct ADA measurement
- Relative conc. of ADA
- Single labeled drug

Cons

- Less assay sensitivity
- Need PC with species-specific Fc
- Higher cost (beads, trypsin)

Other Formats To Mitigate Drug Interference

Sample

Non-drug related Ig

ADA

Drug

Drug
Saturation

Excess
drug

LCMS measures
surrogate peptides of drug

Protein A/G bead

Pros:
• No drug interference

Cons:
• Indirect ADA measurement
• Drug without Fc
• Maybe poor assay sensitivity (high background)

LCMS measures
surrogate peptides of ADA

Drug specific Ab
(non-blocker)

Pros:
• No drug interference
• Direct ADA measurement
Cons:
• Drug without Fc or with Fc from different species
• Poor assay sensitivity (epitope masking)

• Hendrik Neubert, et al. Anal Chem 2008, 80: 6907Hao Jiang & Jianing Zeng. AAPS-NBC, 2016
• Lin-zhi Chen, et al. J Immunol Res, 2016, Article ID 7682472

Summary

- In this presentation, we discussed "why" and "how" to apply LC-MS in ADA measurement which is a novel application of LC-MS technology.

- LC-MS approach has unique features such as simple assay format, improved specificity/drug tolerance, and its capability of isotyping and multiplexing.

- The established experimental parameters are NOT project-dependent and need minimal method development work.

- Less sensitivity and higher cost (on supplies and instruments) are the major concerns.

- Right now, LC-MS approach is suitable for discovery studies. Its application in regulated clinical studies would be very challenging and needs more applications in the industries to obtain solid data and the feedback from regulatory agencies.

Plenty of Online Resources

Paper Reviews
- Wei C, et al, Current Pharmacology Reports, 2018: https://doi.org/10.1007/s40495-017-0118-x (Newest review for antibody and ADC bioanalysis)
- An B, et al, Drug Metab Dispos, 2014, 42:1858 (antibody and ADC bioanalysis)
- van den Broeka I, et al, Bioanalysis, 2015, 7: 1943 (intact protein bioanalysis)
- van den Broeka I, et al, Journal of Chromatography B, 2013, 929: 161 (peptide-based bioanalysis)

Intact protein ("top-down")
- Lanshoeft C, et al, Anal. Chem. 2017, 89: 2628
- Zhao Y, et al, Anal. Chem. 2017, 89: 5144
- Paula M, et al, J. Am. Soc. Mass Spectrom., 2016, DOI: 10.1007/s13361-016-1566-y
- David F. Keren, et al, Clin Chem Lab Med, 2016, 54: 947
- https://www.chromacademy.com/_downloads/LCGC0817_ChromAcademy_Agilent%20eBook_16[3].pdf

Peptide-based ("bottom-up")
- Furlong M, et al, Biomed. Chromatogr., 2012, 26: 1024
- Li H, et al, Anal. Chem. 2012, 84: 1267
- Jiang H, Anal. Chem. 2013, 85: 9859
- Palandra et al, Anal Chem, 2013, 85:5522
- Neubert H, et al, Anal. Chem. 2013, 85, 1719
- Gong C, Bioanalysis. 2014, 6:2371
- Jiang H, et al. Anal Chem, 2014, 86:2673
- Xu K, et al, Bioanalysis, 2014, 6: 1781
- John T Mehl, et al, Bioanalysis, 2016, 8: 1611
- Lin-zhi Chen, et al. J Immunol Res, 2016, Article ID 7682472
- Hendrik Neubert, et al. Anal Chem 2008, 80, 6907–6914
- Jiang H, et al. Bioanalysis. 2018 (to be published)

Webinars
- Agilent Webinars, "Improve Your Monoclonal Antibody Separations by Leveraging the Advantages of Superficially Porous Particle Columns" https://event.on24.com/wcc/r/1665069/BC2D61CD7E1AAB523C85370A88AB9CD8
- LCGC Webcast, http://www.chromatographyonline.com/lcgc/webcasts
- LCGC's CHROMacademy, https://www.chromacademy.com/index.html

Need to register for free and then get notice for webinar broadcast

Acknowledgements

Backup Slides

Quantification of the IgG2/4 kappa Monoclonal Therapeutic Eculizumab from Serum Using Isotype Specific Affinity Purification and Microflow LC-ESI-Q-TOF MS
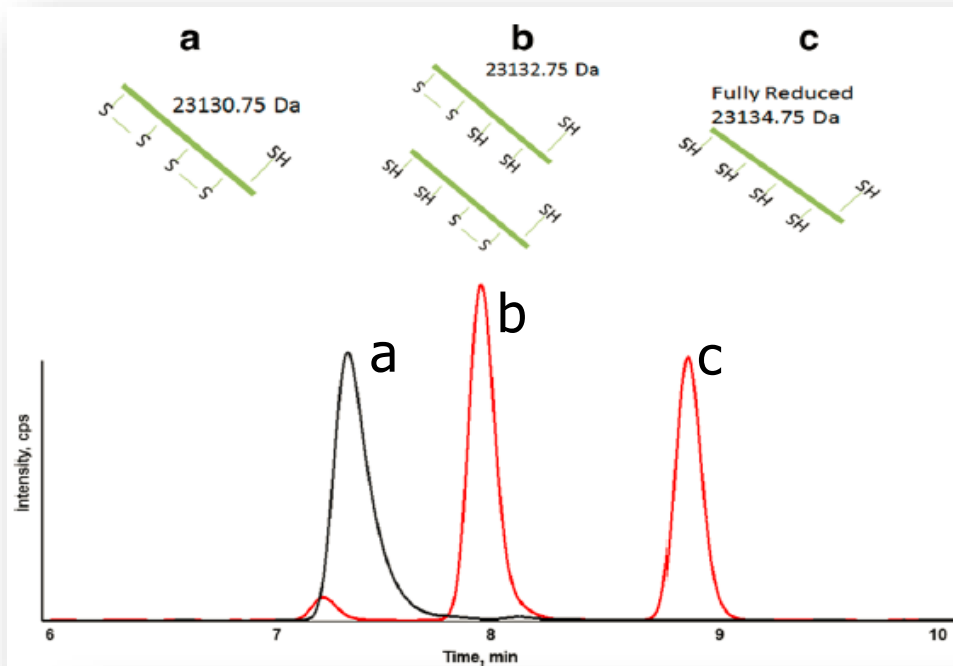

- Dithiothreitol (DTT) only reduces disulfide bond between light and heavy chains
- Tris(2-carboxyethyl)phosphine (TCEP), a stronger reducing agent, also reduces light chain disulfide bond inconsistently
- Eksigent Ekspert 200 microLC
- MP-A: 0.1% FA in water; MP-B: 0.1% FA in 90% ACN/10% IPA
- Poroshell 300SB-C3 column (1.0 × 75 mm), 60 °C
- LC gradient: 20-47%B for 16.5 min

Table 2. IgG4 Affinity Matrix Intact Light chain Imprecision (all in mcg/mL)

|	Repeatability				Within-laboratory				
	1.0	1.5	15	75	1.5	15	50	75	150
N	19	19	19	20	16	15	15	15	17
Mean	1.1	1.2	18.3	88.2	1.6	16.0	57.0	79.3	153
SD	0.12	0.13	1.03	4.81	0.24	1.28	8.66	7.37	14.1
%CV	10.4%	10.8%	5.6%	5.5%	14.9%	8.0%	15.2%	9.3%	9.2%
Recovery	113%	81%	122%	118%	107%	107%	114%	106%	102%

Sequential Protein and Peptide Immunoaffinity Capture for Mass Spectrometry-Based Quantification of Total Human β-Nerve Growth Factor
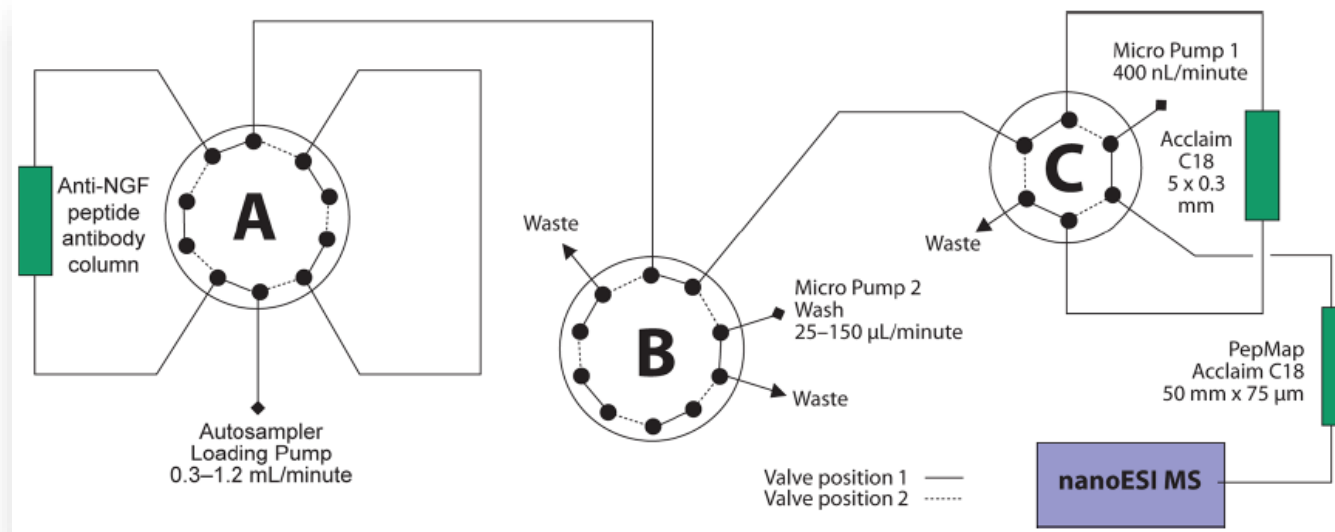
- Protein analyte immunocaptured (IC) with capture Ab on beads followed by trypsin digestion
- Tryptic peptide then IC by the online IC column followed by LC-MS analysis
- Peptide IC column: beads immobilized with the anti-peptide antibody which generated by peptide-(KLH); keyhole limpet hemocyanin (KLH), 390kDa
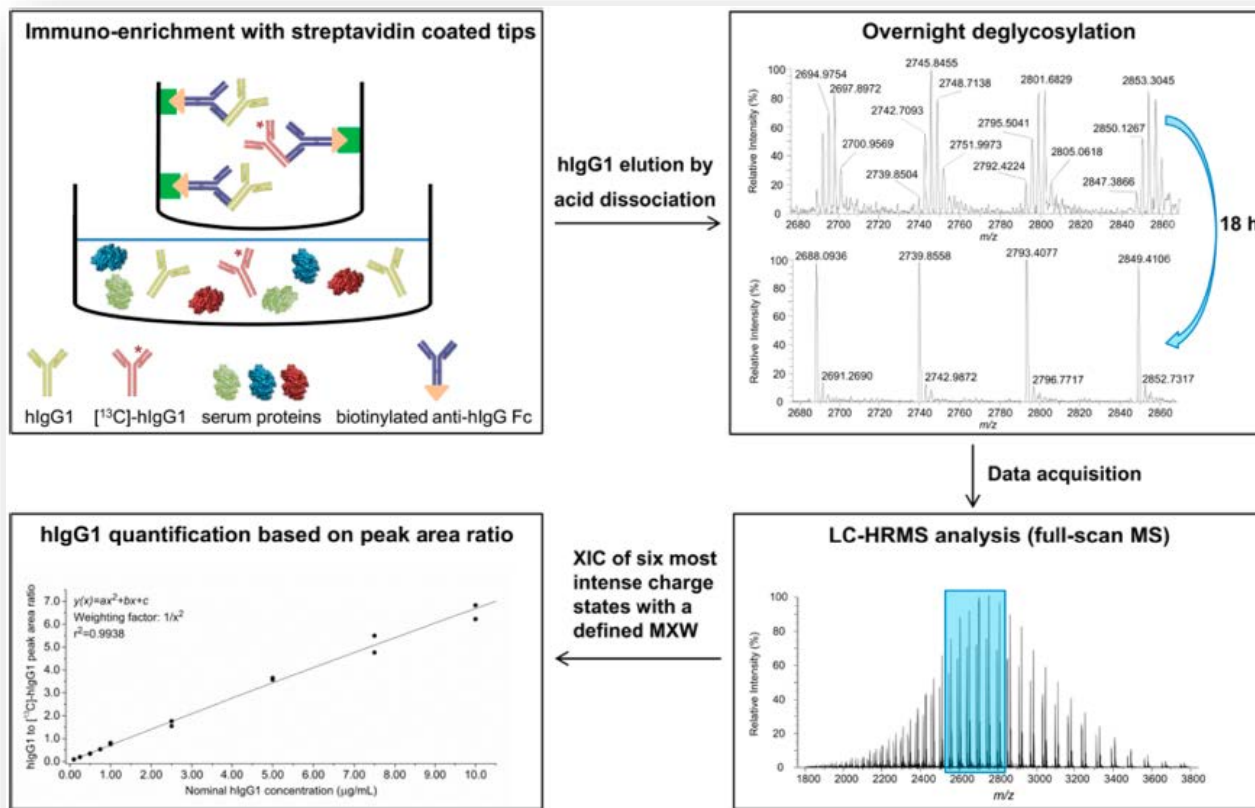
Neubert H, et al, Anal. Chem. 2013, 85, 1719
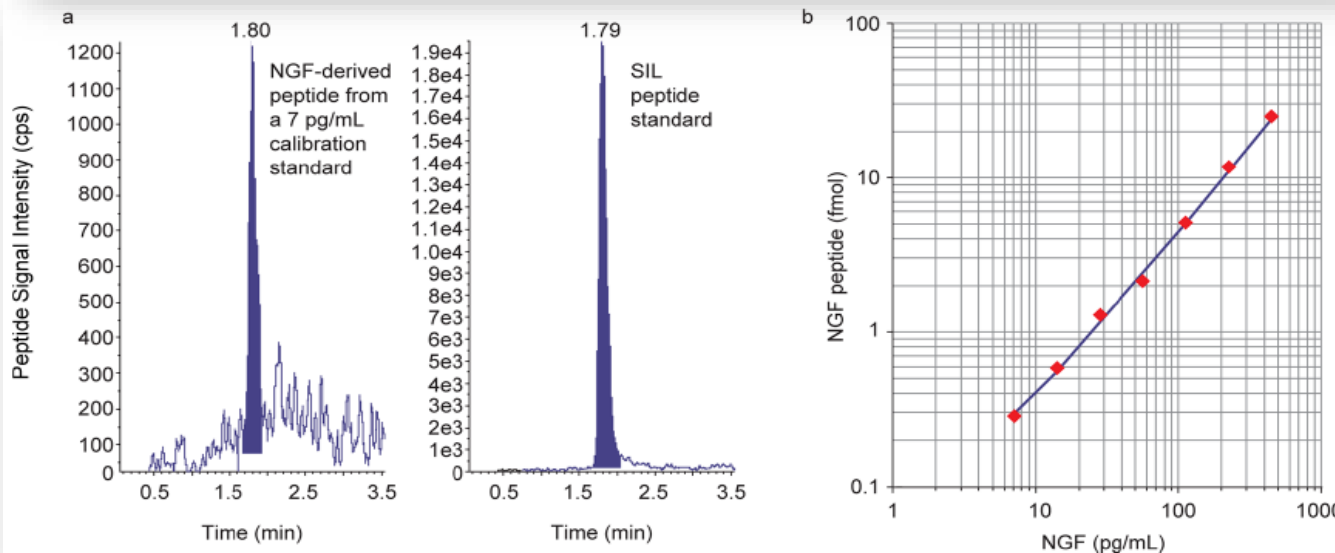
Quantification of *in vivo* Site-specific Asp Isomerization and Asn Deamidation of mAbs in Animal Serum Using IP-LC–MS
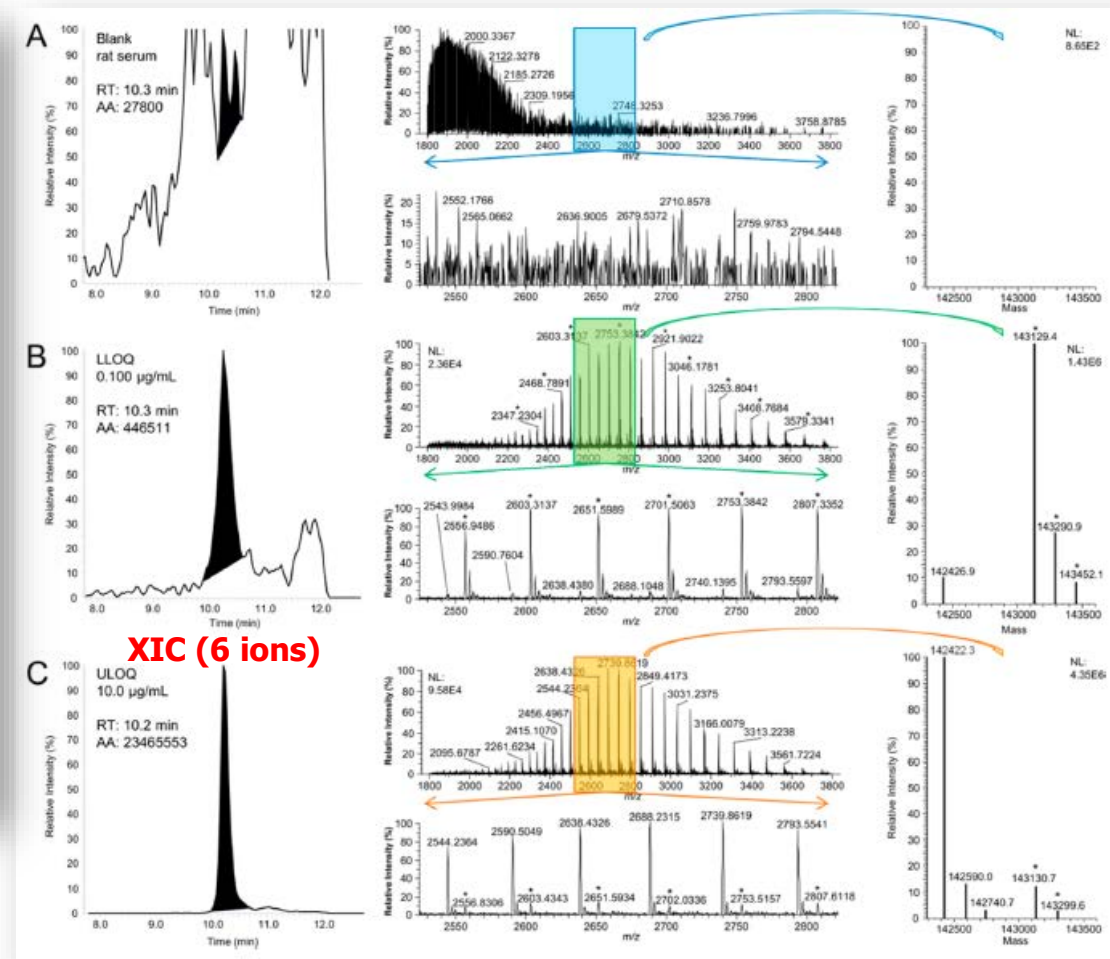

Mehl JT, et al, Bioanalysis, 2016, 8: 1611
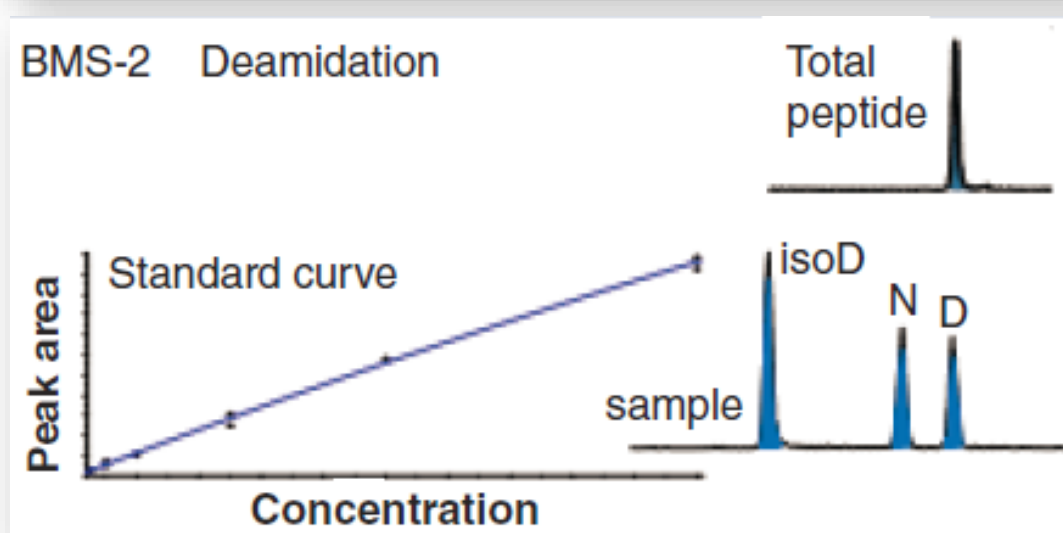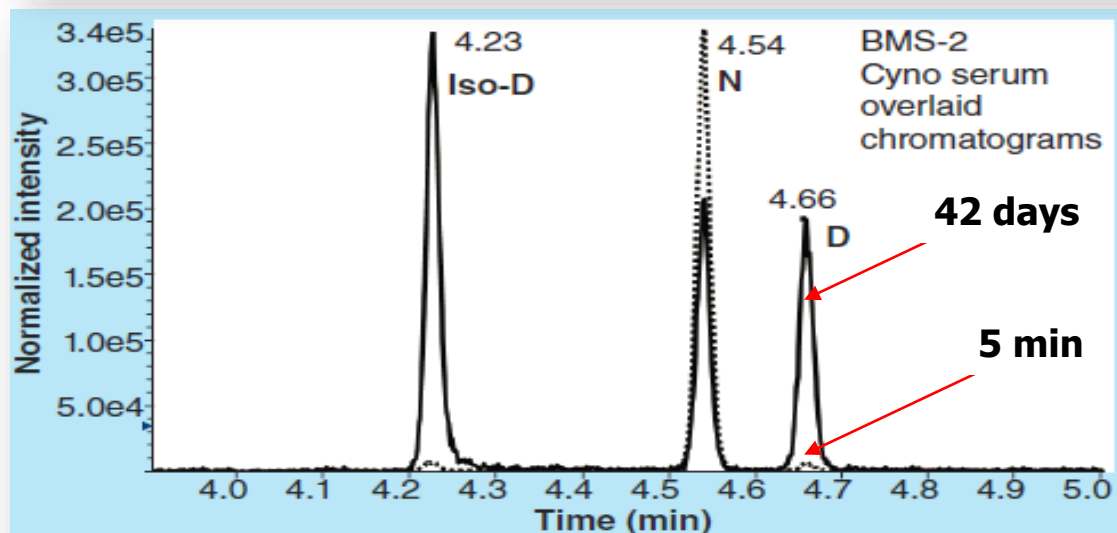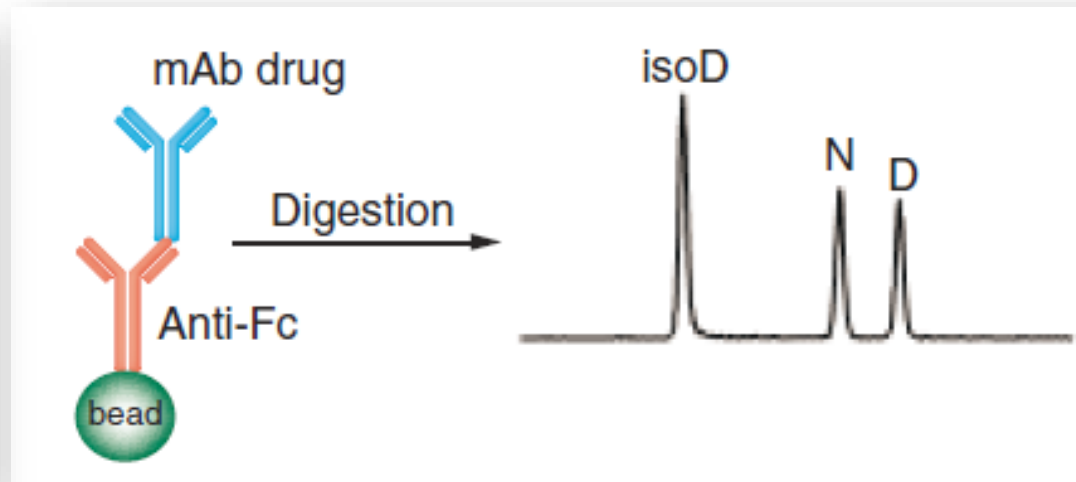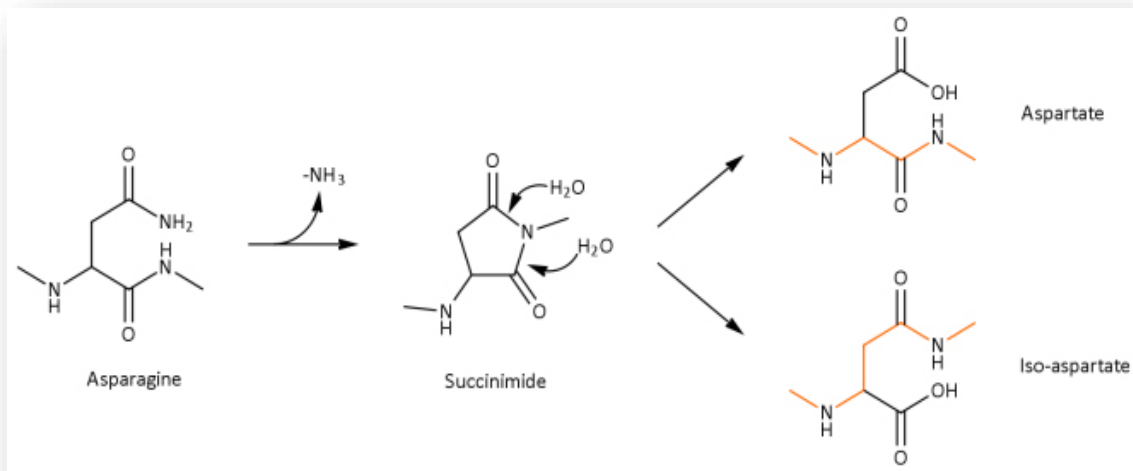
Generic Hybrid LC-HRMS-Based Workflow for Multiplexed Human IgG1 Quantification at Intact Protein Level: Application to Preclinical Pharmacokinetic Studies

- ProSwift RP-4H polymer column at 70°C, 0.2 mL/min
- Mobile phases of 0.1% FA in water and ACN; gradient 20-60% within 12 min

Measuring Monoclonal Proteins in Serum from Multiple Myeloma Patients

Keren DF, et al, Clin Chem Lab Med, 2016, 54: 947
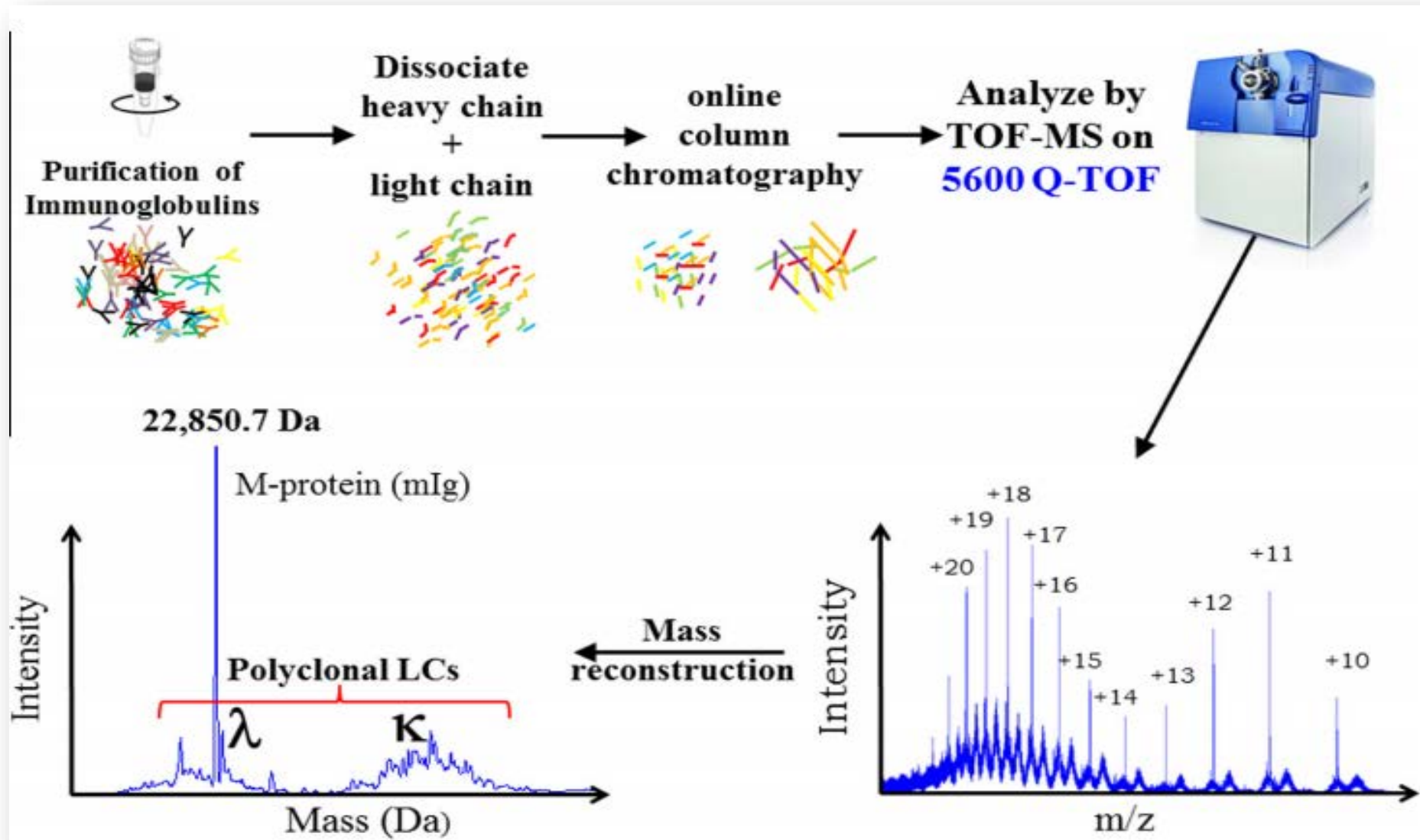
A Multiplexed Immunocapture LC-MS/MS Assay for Simultaneous Measurement of Myostatin and GDF-11 in Rat Serum Using an Automated Sample Preparation Platform
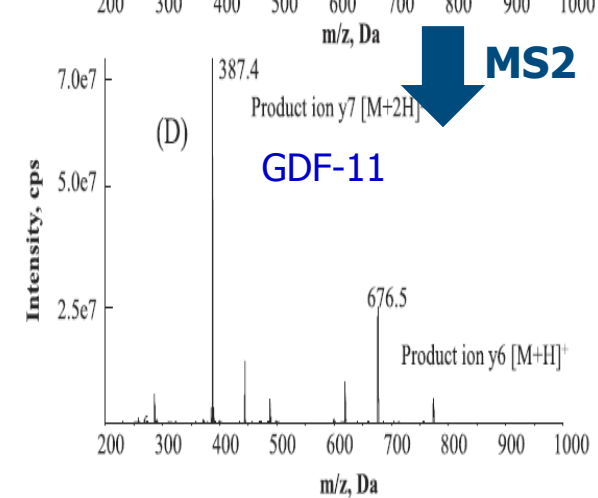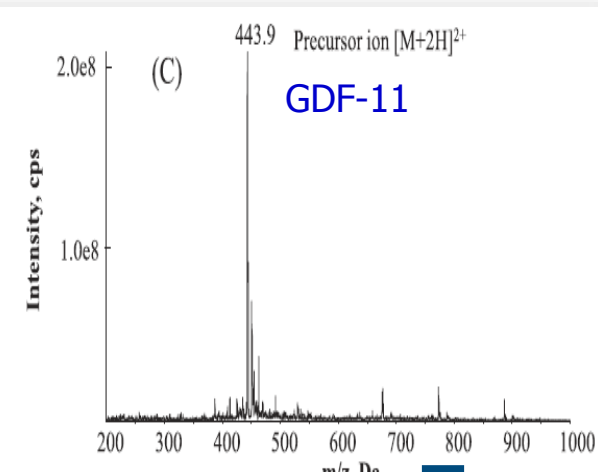
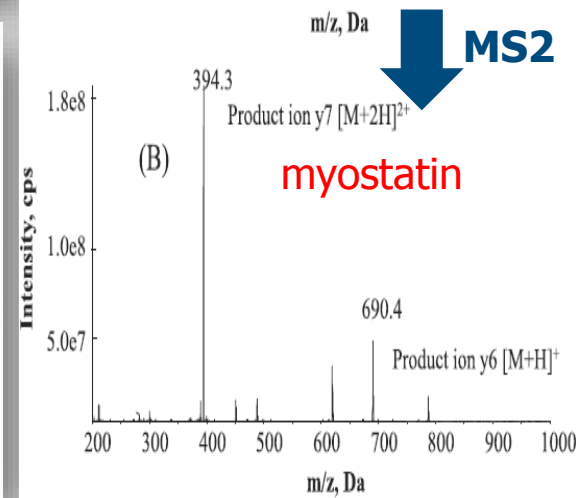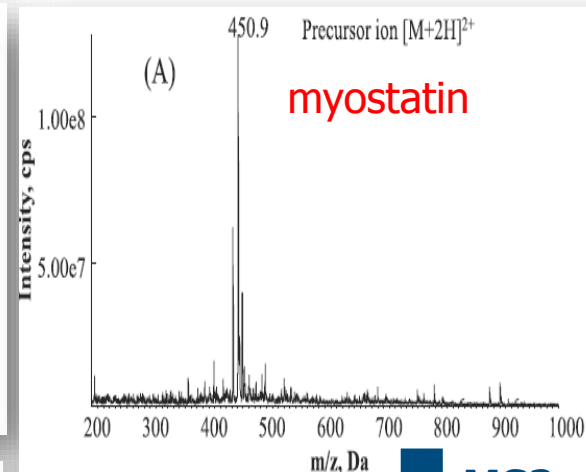Zhao Y, et al, Analytica Chimica Acta, 2017, 979: 36e44

Strategy for the Quantitation of a Protein Conjugate via Hybrid Immunocapture-Liquid Chromatography with Sequential HRMS and SRM-Based LC-MS/MS Analyses

Intact protein — HRMS

Peptides from digestion — MS/MS

HRMS

(A)
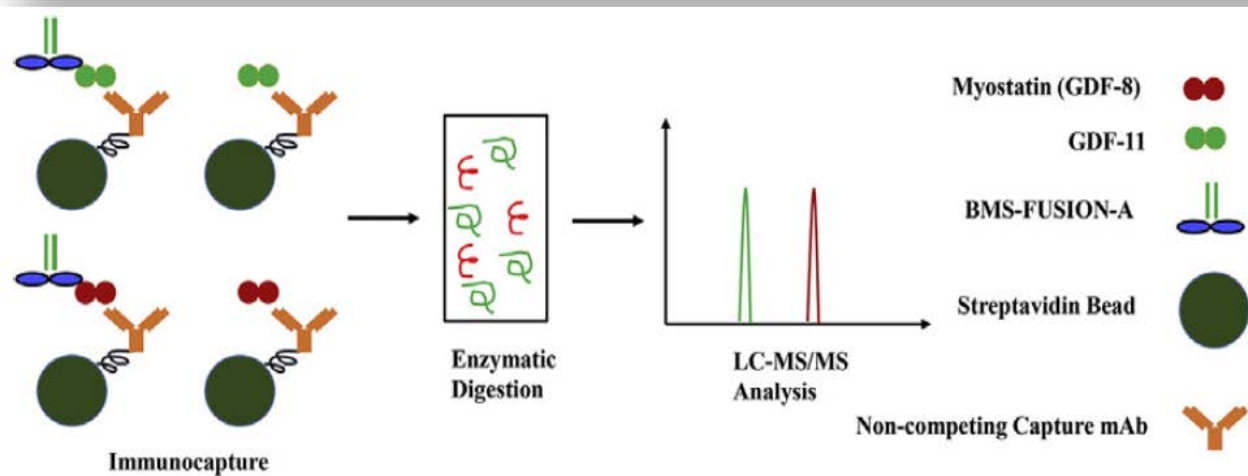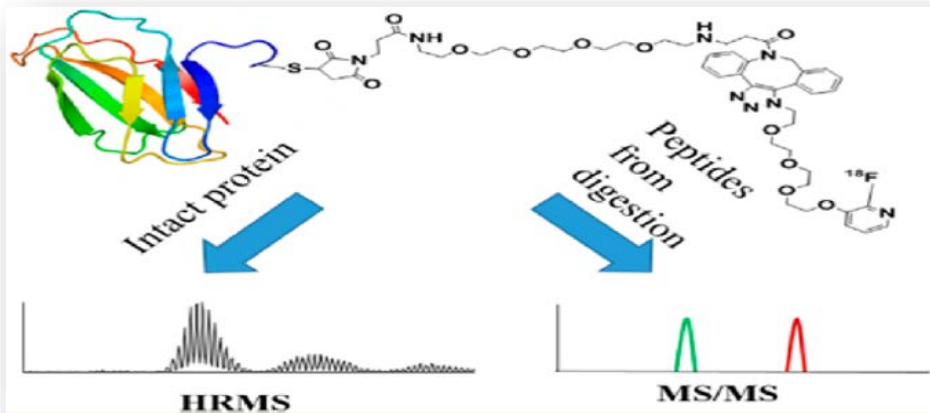
nominal concn (ng/mL)	low (60.00)	GM (200.00)	medium (500.00)	high (800.00)
mean observed concn	58.24	207.05	531.43	754.46
%Dev	−2.9	3.5	6.3	−5.7
between run precision (%CV)	2.1	5.3	2.0	6.1
within run precision (%CV)	6.3	6.8	2.6	6.5
total variation (%CV)	5.3	6.5	2.8	6.8
n	8	8	8	8
number of runs	2	2	2	2

MS/MS

(B)

nominal concn (ng/mL)	low (3.00)	GM (30.00)	medium (500.00)	high (800.00)
mean observed concn	2.89	30.01	475.14	725.16
%Dev	−3.7	0.0	−5.0	−9.4
between run precision (%CV)	9.5	1.5	0.0	3.8
within run precision (%CV)	4.7	2.7	3.3	6.6
total variation (%CV)	10.6	3.1	2.9	7.6
n	8	8	8	8
number of runs	2	2	2	2

(A) Total Ion Chromatogram (TIC)

(B) HRMS Spectra
2358.9714 z=5
1965.8114 z=6
1685.1247 z=7
1474.7359 z=8
2948.1959 z=4

(C)
2358.9745
2358.7754
2358.5765
2359.1735
2358.3732
2359.3800
2359.5784
2358.1718
2359.7783
2357.9690
2359.9787
2360.1743
11782.76
11800.75

(D)
(Z5 + z6)

Zhao Y, et al, Anal. Chem. 2017, 89, 5144−5151

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