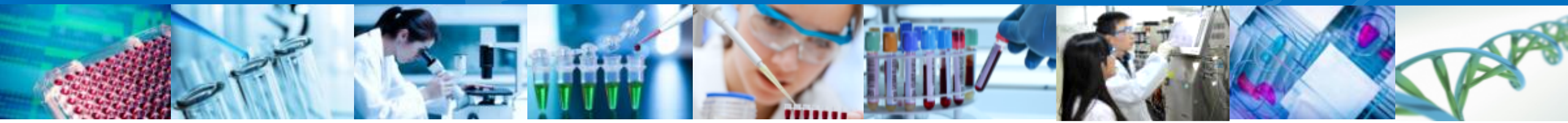
LC-MS Based Large Molecule Bioanalysis in Drug Screening and Preclinical Pharmacokinetics

Zhiyu Li, Ph.D.
Non-GLP BAS, DMPK, WuXi AppTec



药明康德 WuXi AppTec

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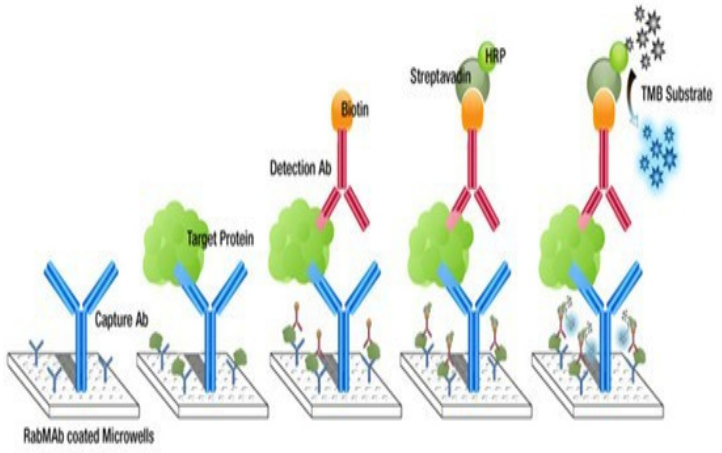
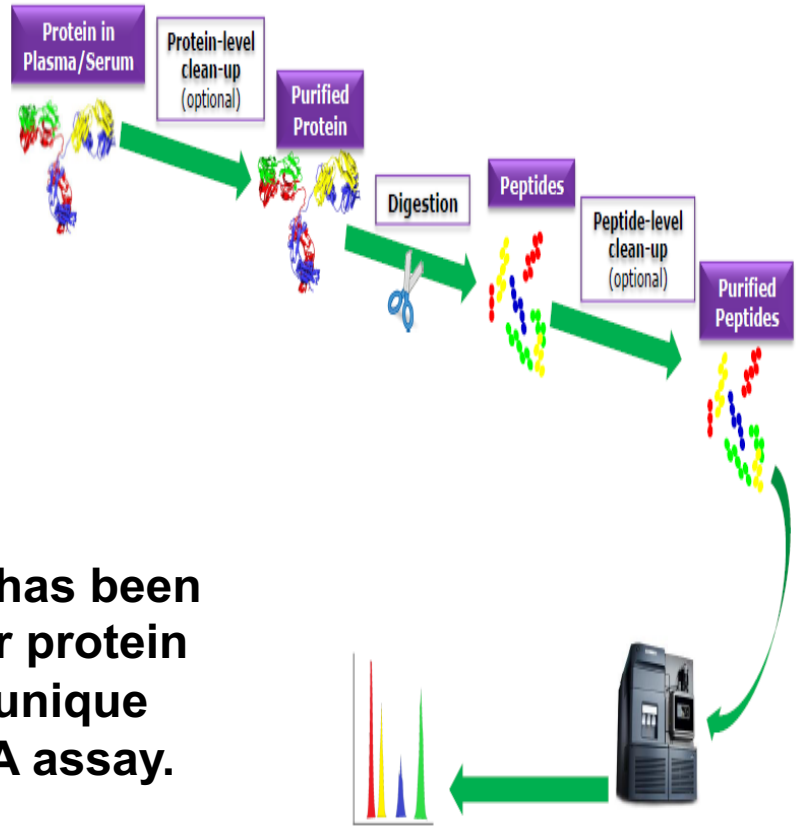
Introduction (1)

Traditional – LBA approach



LC-MS based approach



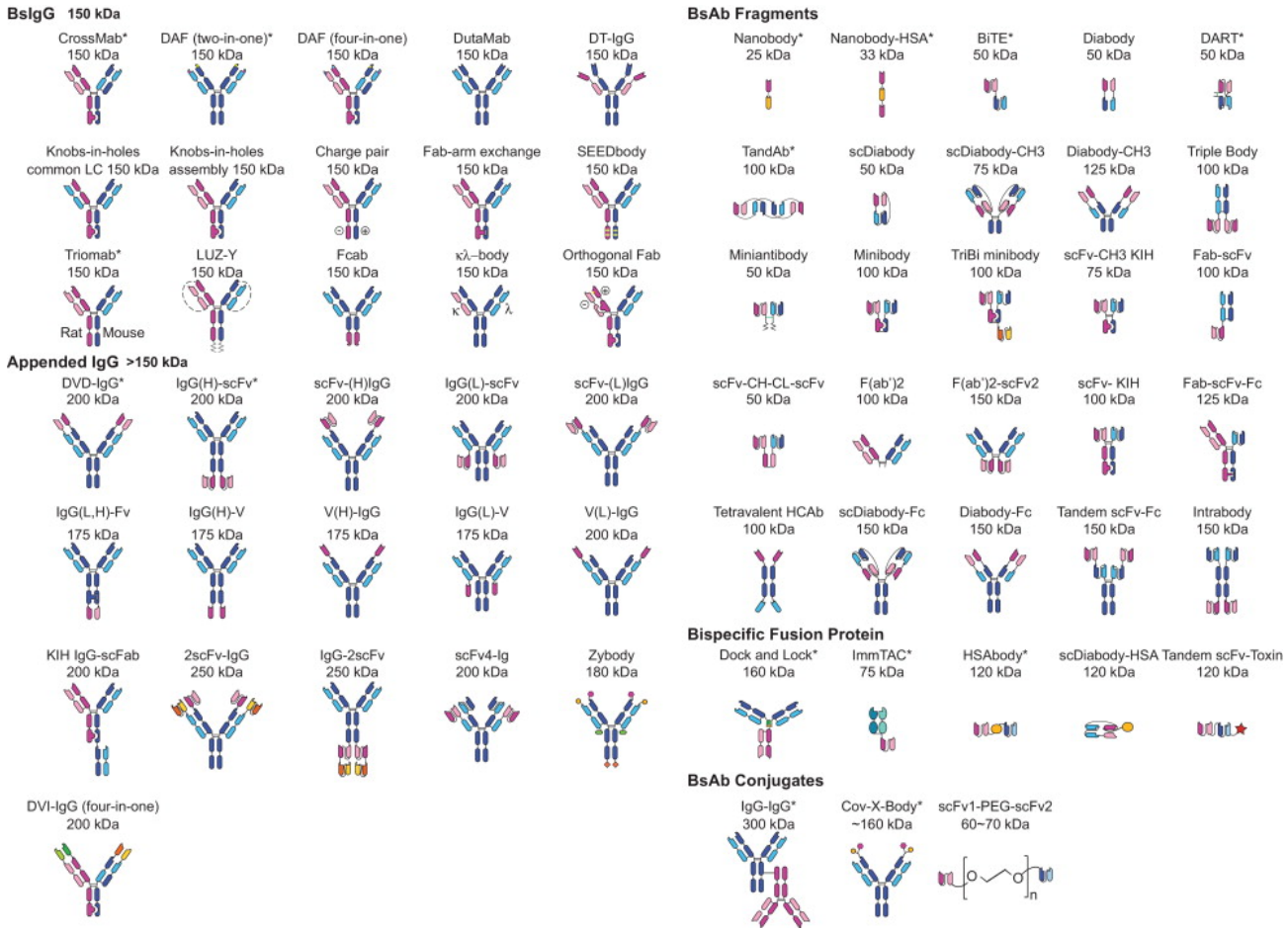
Protein-based therapeutics have shown the fastest growth in recent years.
7 out of top 10 best-selling prescription drugs in 2018 were large molecules
6 were humanized monoclonal antibodies (mAb) or fusion proteins.

LC-MS based assay has been increasingly used for protein bioanalysis provide unique advantages over LBA assay.

https://topforeignstocks.com/2018/09/28/the-top-20-best-selling-drugs-in-2018-chart/, archived June 20th, 2019
Zhiren Yu, WuXi AppTec DMPK Science Day presentation, Nov. 20th, 2016, Shanghai, China
https://www.fda.gov/downloads/drugs/guidances/ucm070107.pdf, archived July 9th, 2018

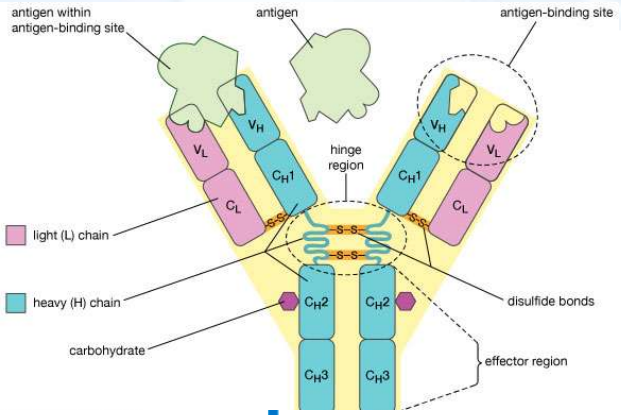
Introduction (2)
-Method of Choice in the Discovery Stage

- No need to develop specific antibodies
  - Development of specific antibodies take 3-6 months
  - Antibody development can be very challenging for small proteins with M.W. ≤ 20 kDa
- Faster method development
- Better specificity
- Cheaper cost
- Good for complicated drug structures



Spiess, Zhai & Carter *Mol. Immunol.* 67(2 Pt A):95-106.

Introduction (3)
-General LC-MS Based Approaches



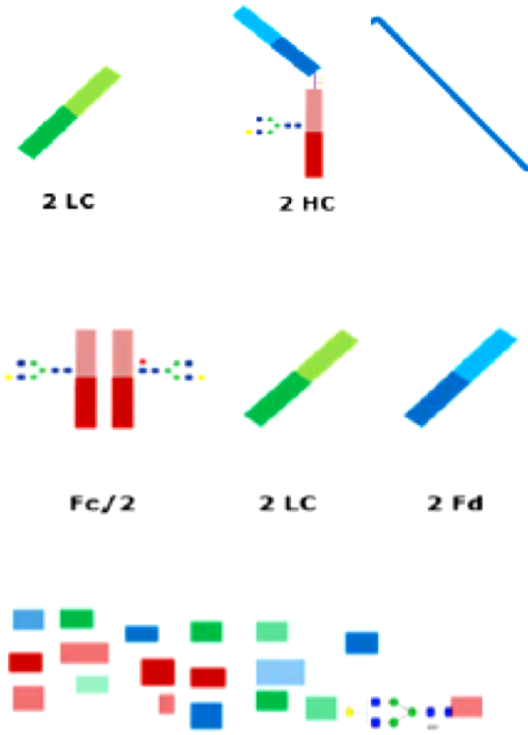
reduction

IdeS

reduction

reduction

digestion



Intact Level analysis

(M.W. ~150 kDa)

High resolution MS

Orbitrap and TOF instruments

Middle-down (subunit)

(M.W. ~20-50 kDa)

Commercial kit for tryptic digestion

Surrogate Peptides

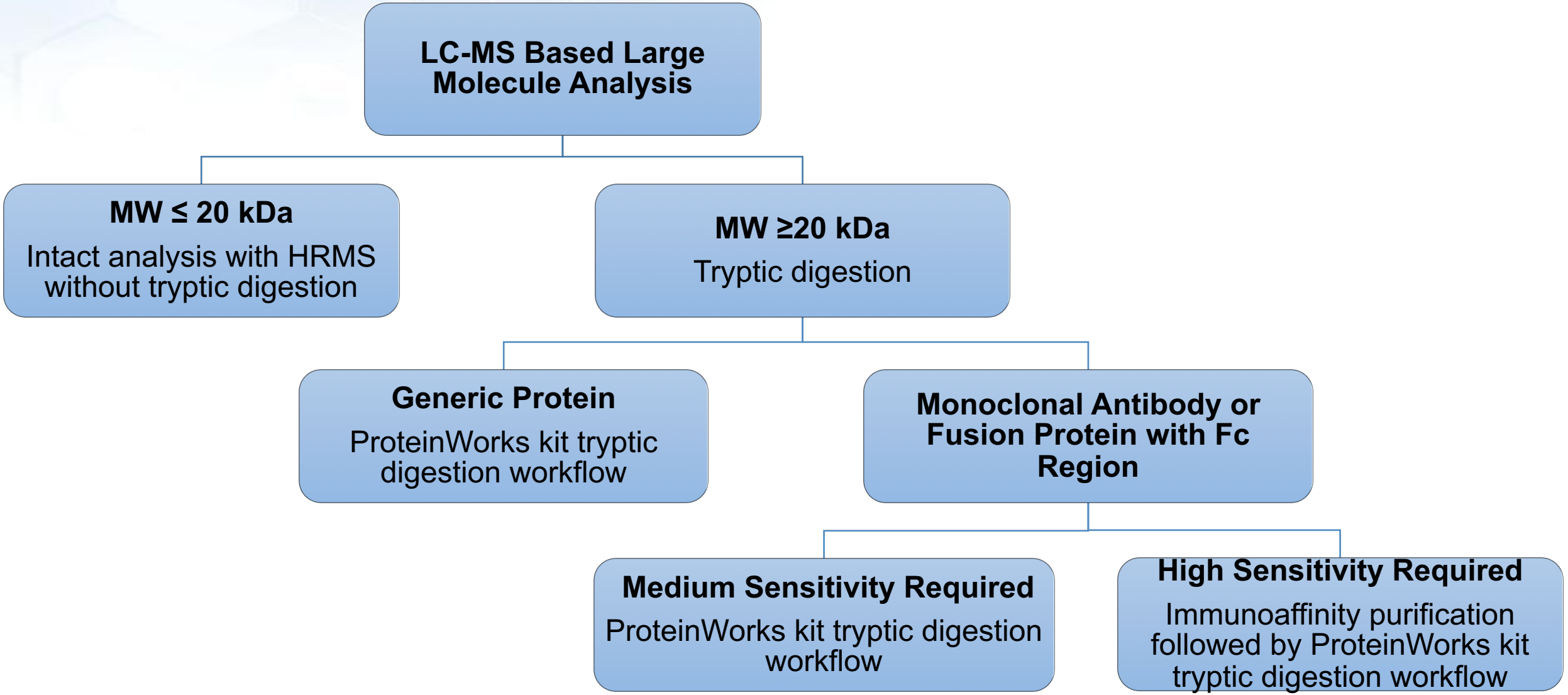
(M.W. ~500-2000 Da)

Low resolution MS

Triple-quad instrument

https://www.britannica.com/science/antibody, achieved July 9th, 2018
Yun Wang Alelyunas, Intact Level Protein Quantification, Waters internal training, June 12th, 2018, Milford, MA, USA

Introduction (4)
- Analysis Strategy



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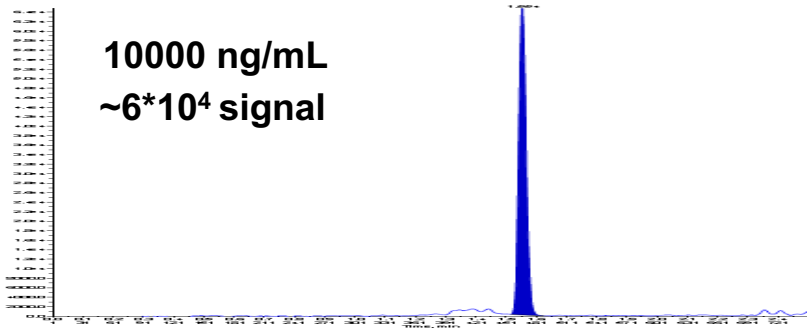
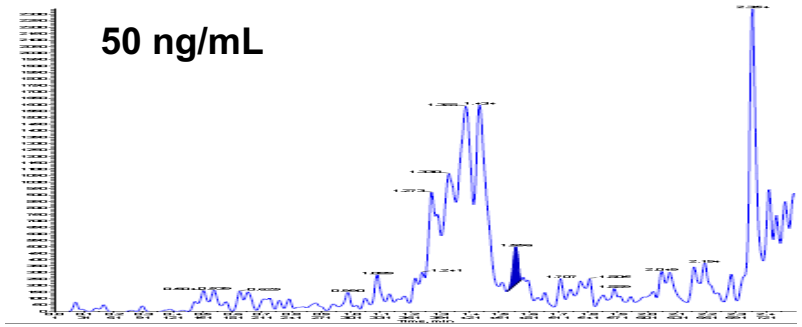
Intact Approach (1)
- Quantitative Analysis for Molecules w/ M.W. ≤ 20 kDa in PK Studies

Small protein w/ M.W. ~11.5 kDa, hard to develop specific antibodies for molecules with M.W. ≤ 20 kDa

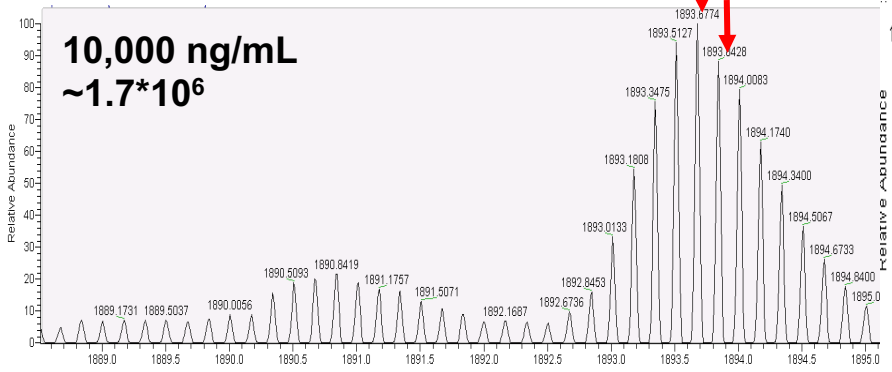
LC-MS/MS with triple-quad

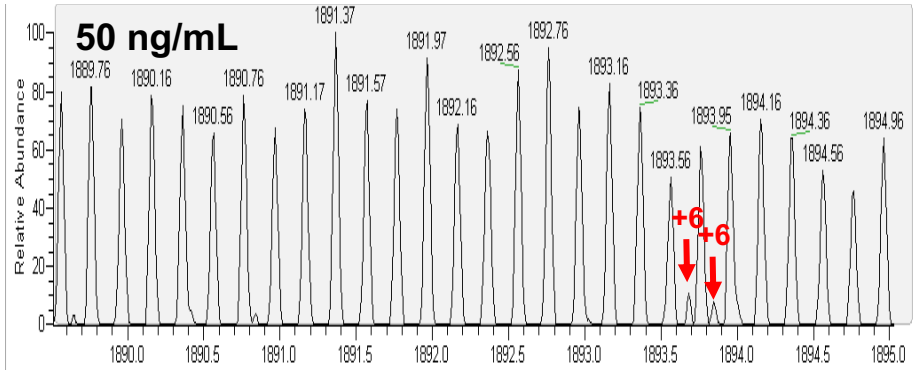




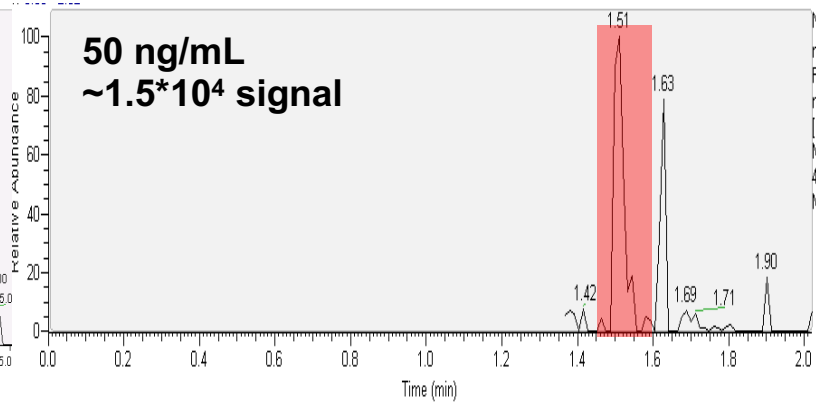
Orbitrap Q-Exactive Plus

140,000 resolution MS spectra

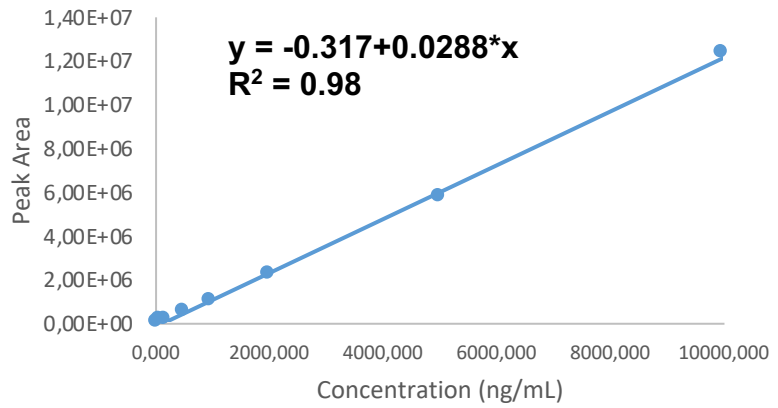




EIC LC-MS Chromatogram



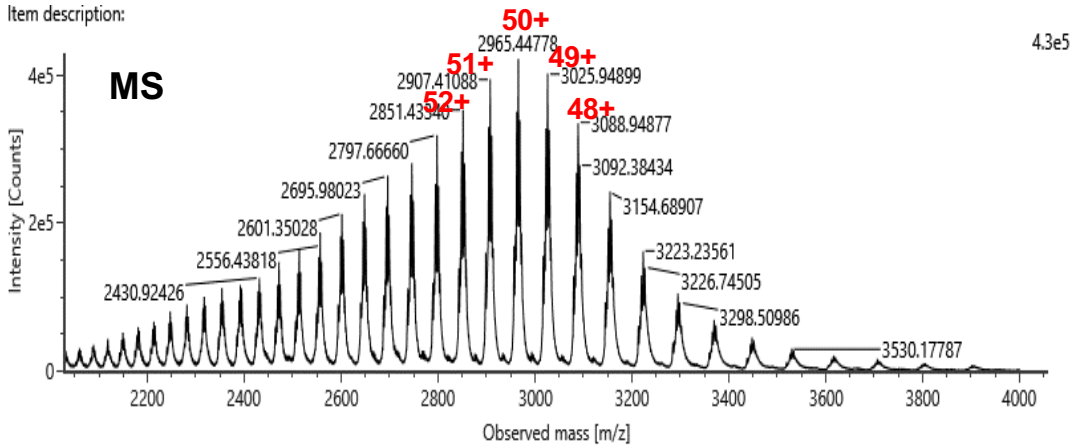
Calibration Curve from LC-Orbitrap QE

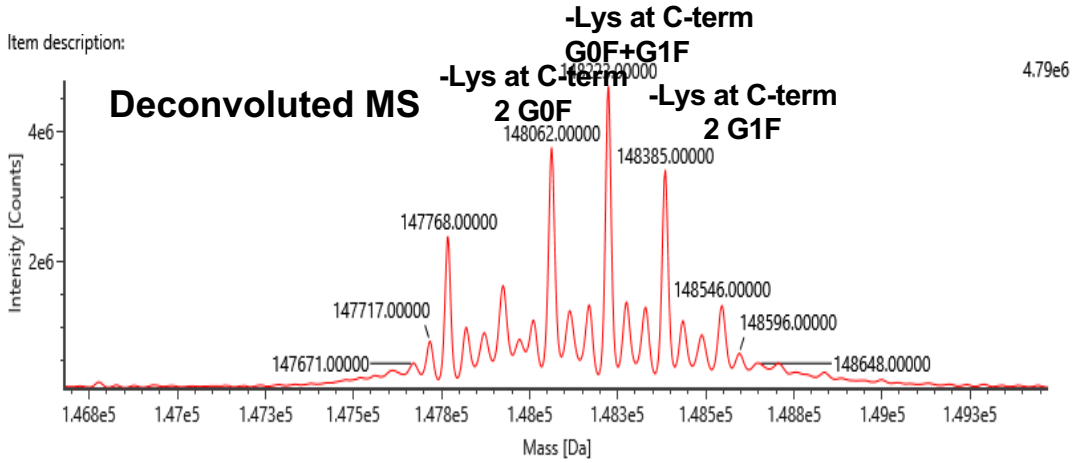


Intact Approach (2)
- Intact Analysis of Larger Protein

- Waters XeVo G2-XS Q-TOF instrument in demo in our lab
- Perform LC-MS analysis of larger molecules, such as protein subunits, and intact protein
- Deconvolution with Unifi

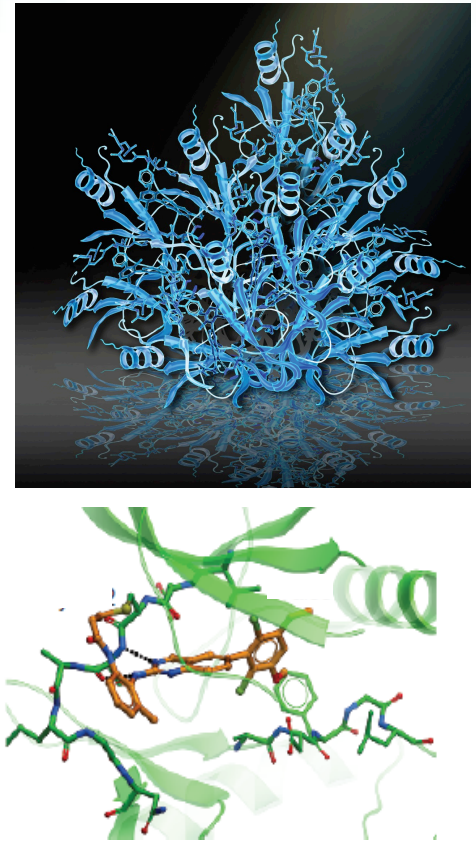
Trastuzumab, neat solution



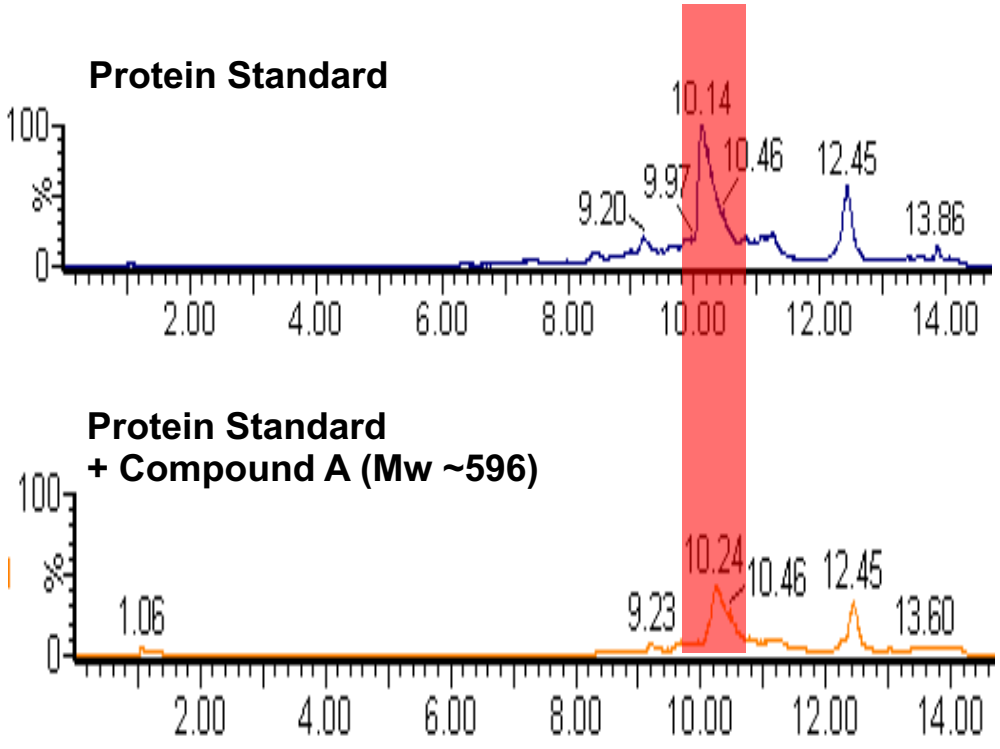


Intact Approach (3)
- Qualitative Analysis for Drug Screening

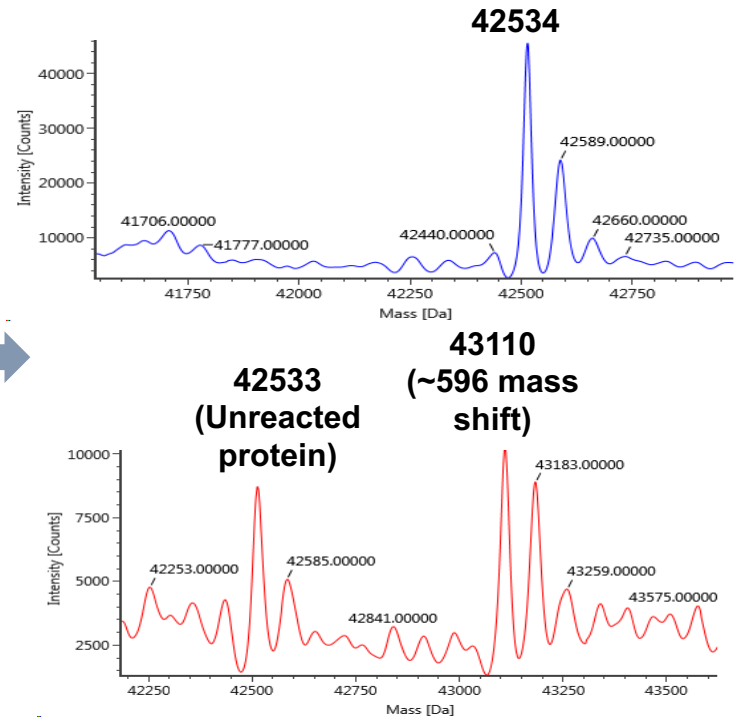
Protein with selective small molecule inhibitor reported in literature



Protein incubated with drug candidates, followed by LC-MS detection on Waters Q-TOF



Peak integration and mass deconvolution with Unifi



M. Hagel, C. Miduturu, M. Sheets, N. Rubin, W. Weng, N. Stransky, N. Bifulco, J.L. Kim, B. Hodous, N. Brooijmans, A. Shutes, C. Winter, C. Lengauer, N.E. Kohl, T. Guzi, Cancer Discov. 5 (2015) 424–437.

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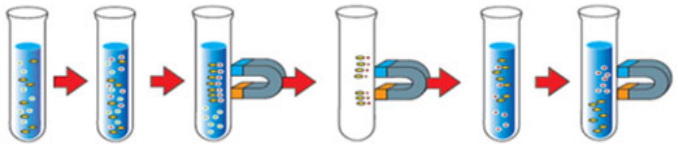
Surrogate Peptide Approach (1)
-Sample Preparation

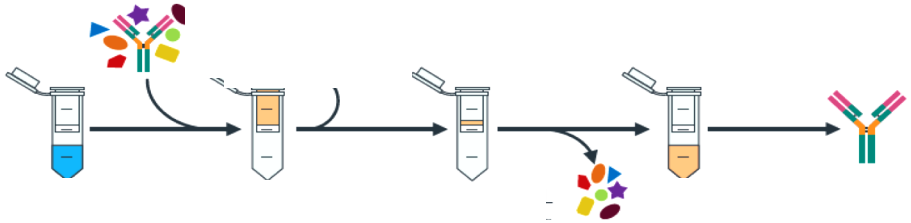
Affinity Binding → Washing → Elution

Denaturation → Reduction → Alkylation → Digestion → Quench → SPE

Magnetic bead immunoaffinity purification and enrichment



Membrane immunoaffinity purification and enrichment

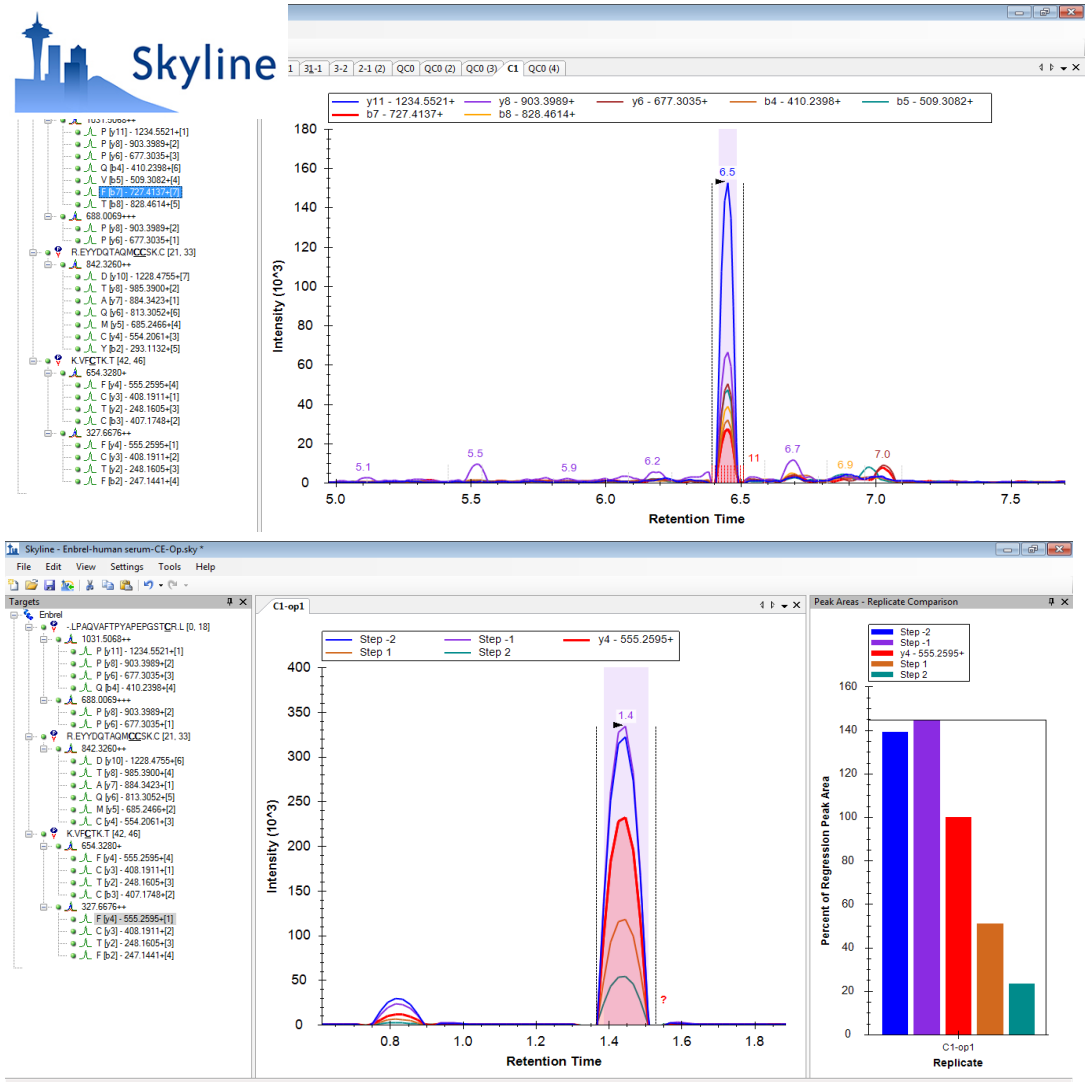


Commercial tryptic digestion kits for proteolysis
Waters ProteinWorks kit

~4-5 hour sample preparation Vs. Overnight sample prep

Surrogate Peptide Approach (2)
-Method Refinement

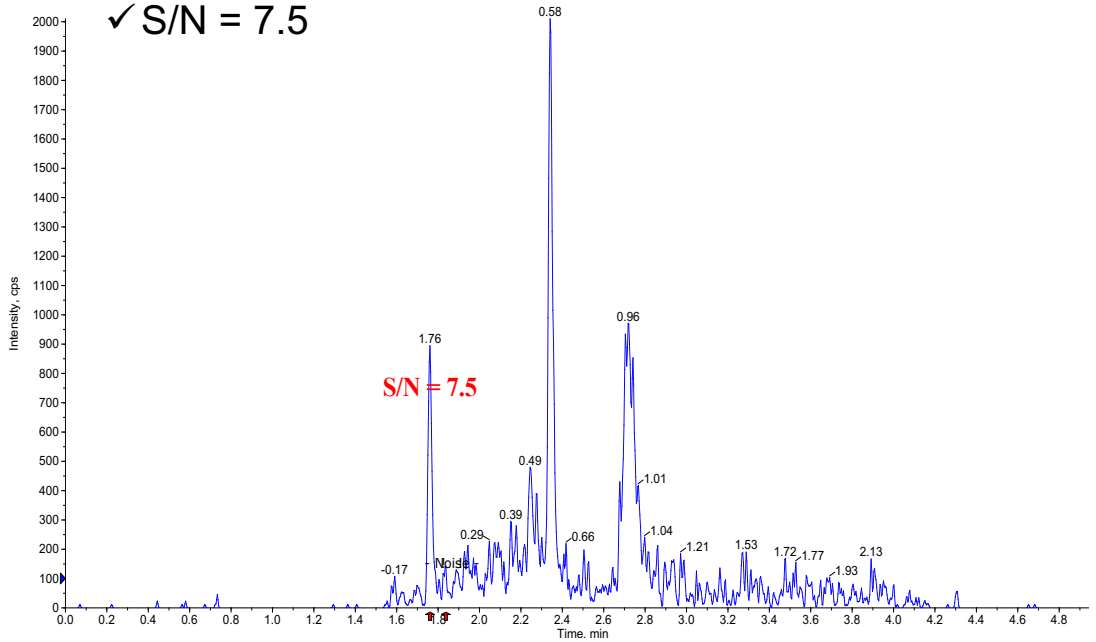
1. Predict potential unique peptides and SRM transitions against background proteome with Skyline
2. Proteolysis of target protein in neat solution
   - MS tuning with chromatographic separation
   - Import data to Skyline, select potential surrogate peptides and SRM transitions, and optimize MS conditions
3. Spike target protein in matrix, conduct proteolysis
   - Optimize LC-MS/MS conditions for surrogate peptides
4. (Optional) Optimize affinity purification and enrichment conditions for target protein
5. LC-MS/MS analysis

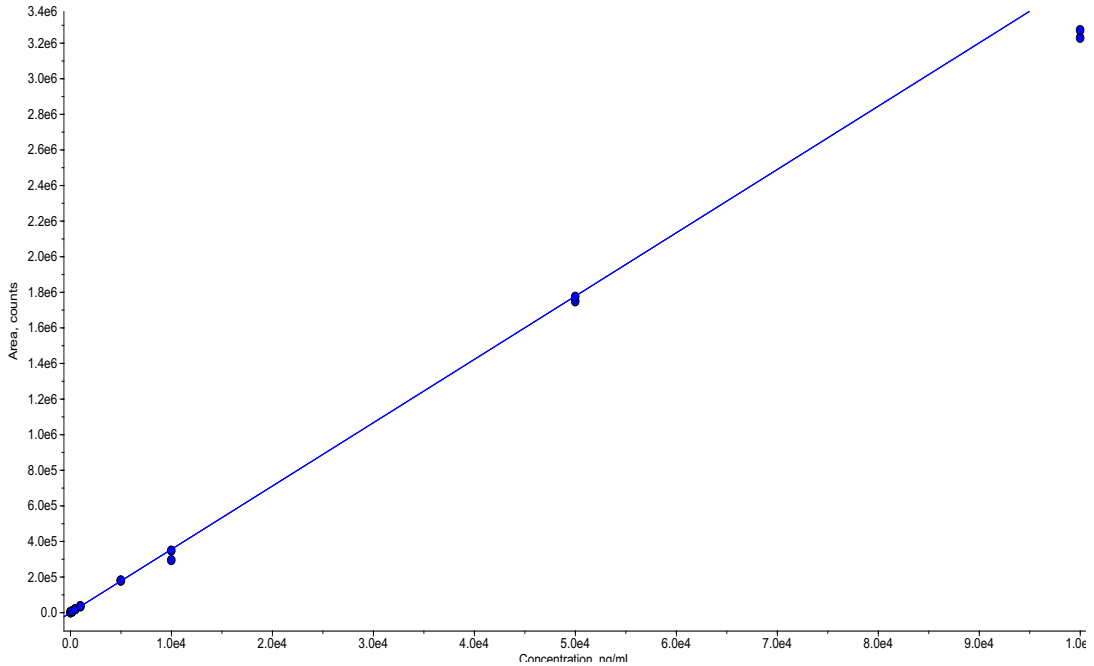


Surrogate Peptide Approach (3) -Generic Protein

Generic protein, M.W. ~32 kDa

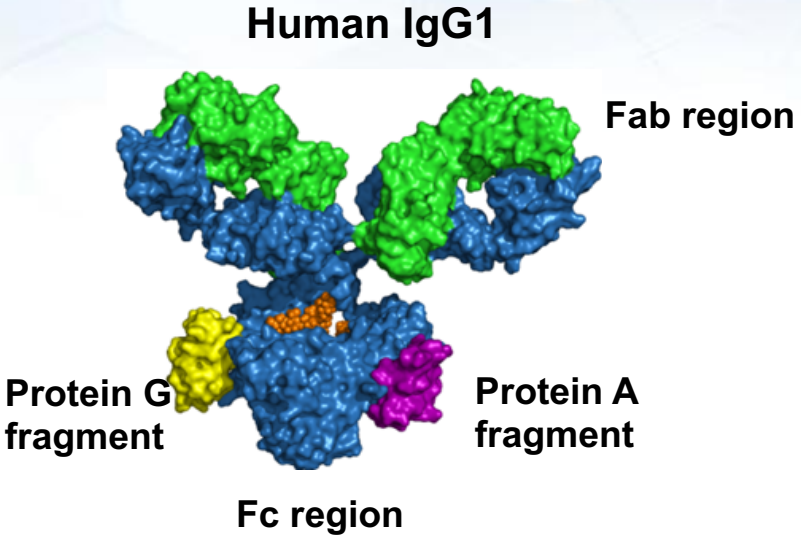
- ✓LLOQ = 0.02 µg/mL
- ✓RT = 1.76 min (Run time = 5.0 min)
- ✓S/N = 7.5

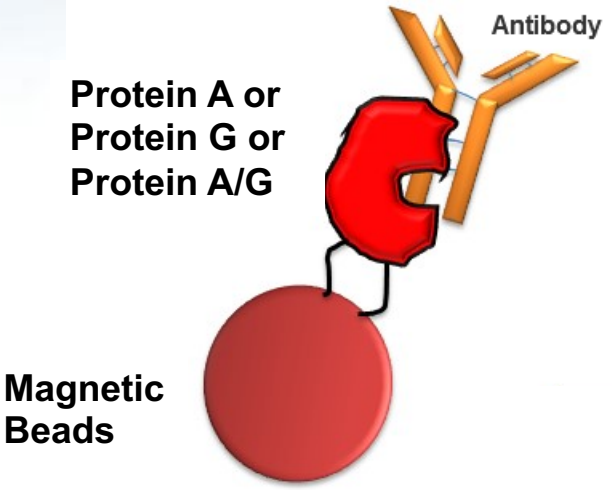


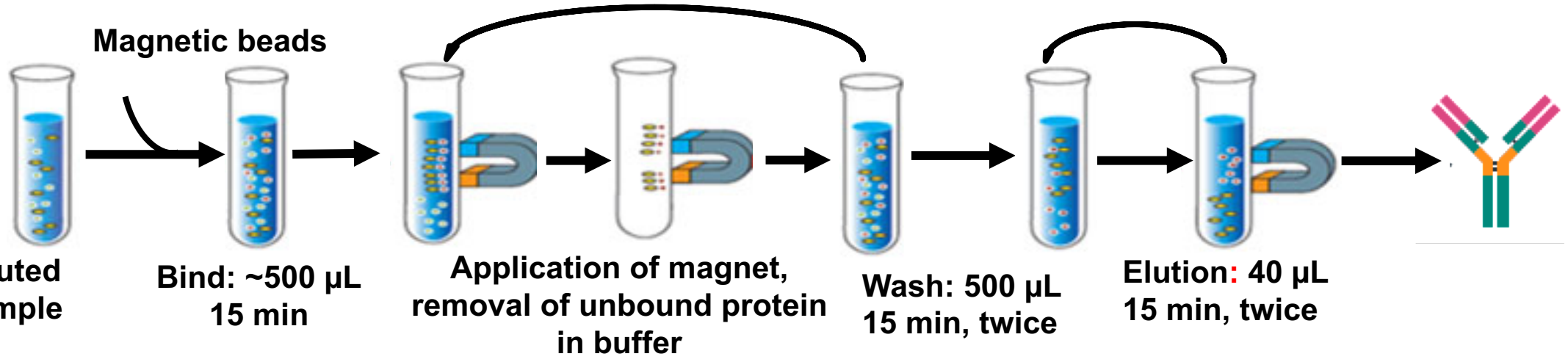


- However, the responses of surrogate peptides, especially those with Cys, were greatly affected by experimental conditions with generic methods.
- Overnight tryptic digestion meant two-day sample prep.
- We started to use Waters ProteinWorks kit for tryptic digestion.

Surrogate Peptide Approach (4)
- Magnetic Beads Immunoaffinity Purification

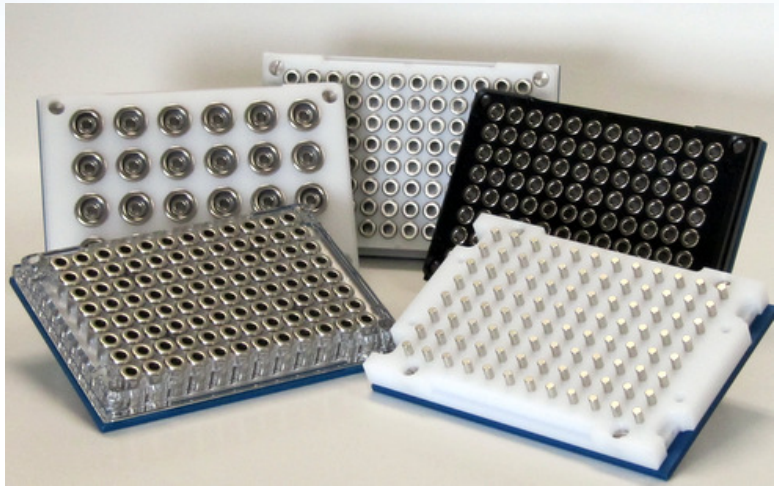






https://absoluteantibody.com/antibody-resources/antibody-overview/other-antibody-interactions/
http://www.sinobiological.com/antibody-purification-immunoprecipitation-ip.html
http://www.emerther.cn/productDetails?id=14&collapseId=1 , achieved July 9th, 2018

Surrogate Peptide Approach (5)
- Practical Difficulties with Magnetic Beads



Various forms of magnetic plates

Insertion of 96-well plate onto magnetic plate

Magnetic beads attached to the side of wells by magnetic forces

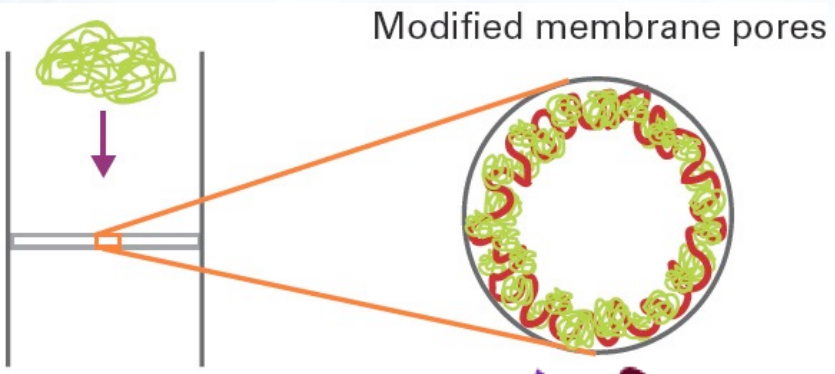
Magnetic beads get into tips

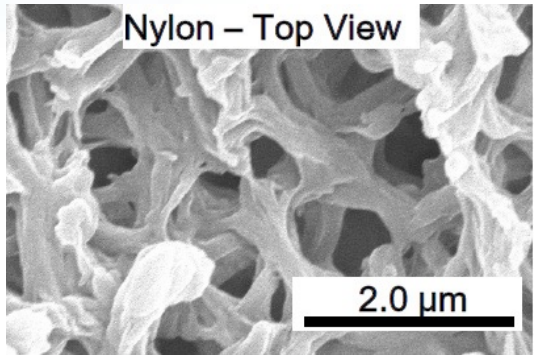
- Difficult for sample preparation
- Low throughput
- Hard to get familiarized with the method
- 3-4 hours for 96-well plate

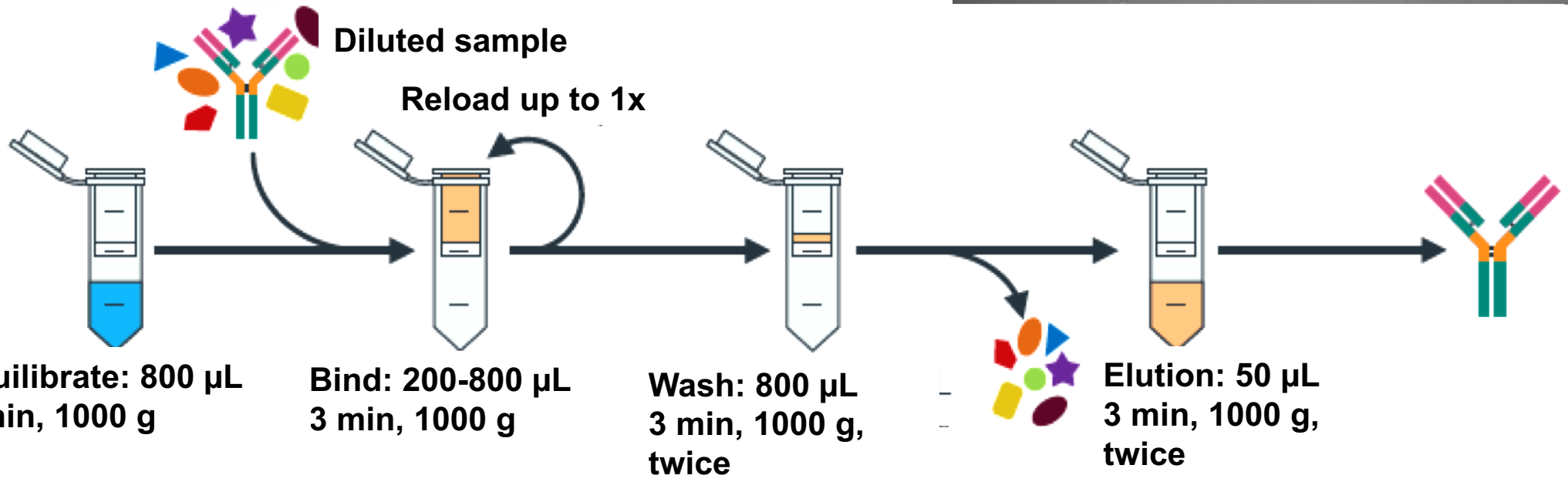
https://www.alpaqua.com/Products/Magnet-Plates/96R-96S-Magnet-Plates
https://www.epigentek.com/catalog/epimag-ht-96-well-magnetic-separator-p-3706.html
http://vp-sci.com/products/magnetic-bead-separation-devices/magnetic-plates-for-microplates/vp-771hh-la.html , achieved Aug. 1st, 2019

Surrogate Peptide Approach (6)
- Membrane IP

TakaraBio Capturem Products



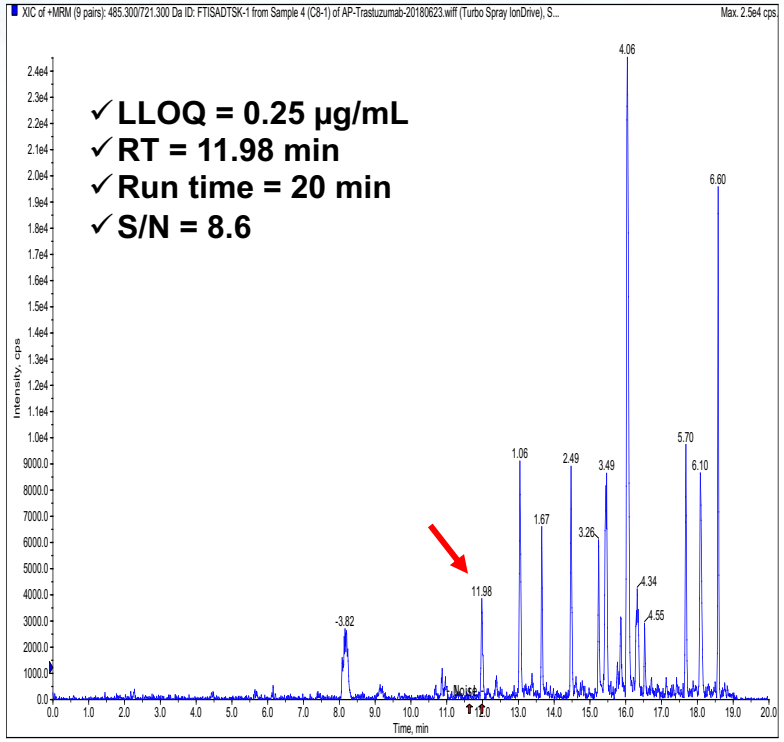




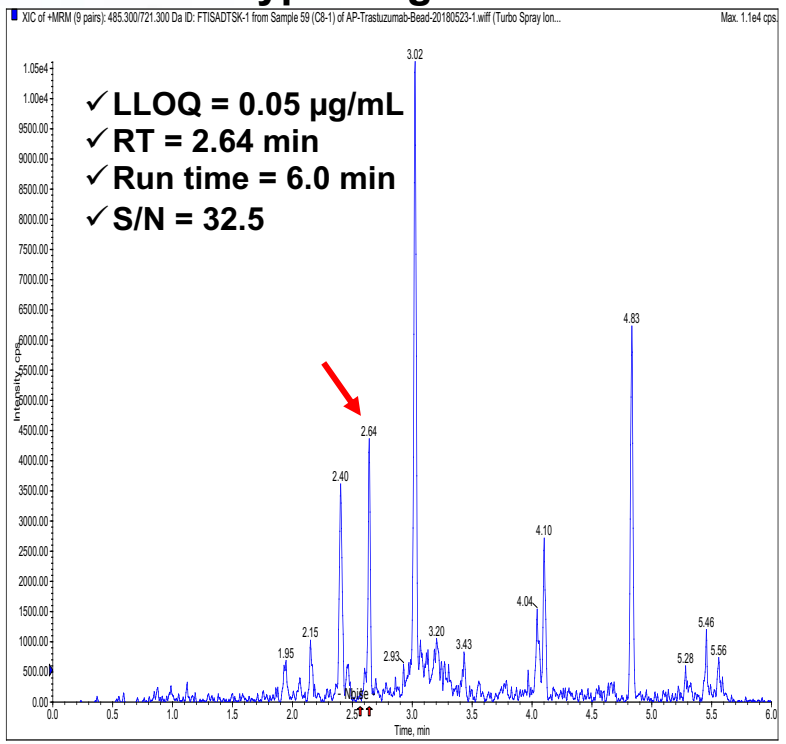
Christian Hoppmann, et. al., WP 055, 66th ASMS, June 3rd – June 7th, 2018, San Diego, CA, USA
Michelle Robinson, et. al., WP 652, 66th ASMS, June 3rd – June 7th, 2018, San Diego, CA, USA
https://www.takarabio.com/products/protein-research/purification-products/antibody-purification/capturem-protein-a, archived Aug 1st, 2019

Surrogate Peptide Approach (7)
- Comparison between Three Methods

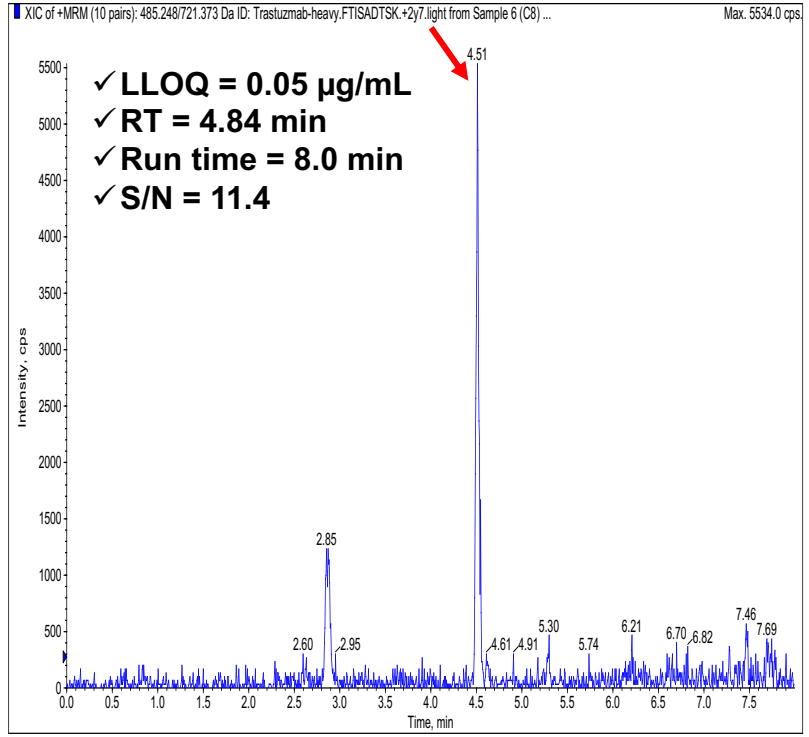
Direct tryptic digestion



Magnetic beads IP + tryptic digestion



Membrane IP + tryptic digestion



	Direct digestion (Rat serum)	Magnetic beads IP + tryptic digestion (Rat serum)	Membrane IP + tryptic digestion (Rat serum)	WuXi ELISA* (Human serum)
LLOQ (µg/mL)	0.25	0.05	0.05	0.03125
Run time (min)	20	6	8	NA

* WuXi BAS protocol, 17BASM185V1

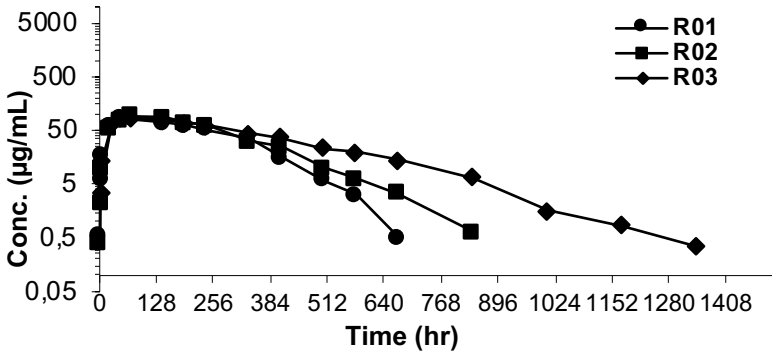
Surrogate Peptide Approach (8)
- Partial Validation Results Comparison

Precision and Accuracy										
Method	Sample ID*	Nominal Conc. (μg/mL)	Intra-day (Within-run)						Inter-day (Between-run)	
			Day 1		Day 2		Day 3			
			Bias%**	CV%**	Bias%**	CV%**	Bias%**	CV%**	Bias%**	CV%**
Direct tryptic digestion	LLOQ	0.25	-0.5	10.7	17.1	4.62	4.7	4.82	7.1	9.65
	Low QC	0.75	0.1	7.52	-5.9	4.01	-4.2	6.33	-3.3	6.41
	Mid QC	15	-1.7	8.53	0.4	5.66	1.6	2.30	0.1	5.80
	High QC	40	-4.3	7.99	-7.7	5.98	2.0	4.11	-3.4	7.19
	Dilute QC	80	4.4	8.10	---	---	---	---	---	---
Magnetic beads IP + tryptic digestion	LLOQ	0.05	-0.4	8.17	-11.4	14.7	11.4	19.7	-0.1	17.4
	Low QC	0.15	-3.1	1.78	-10.0	9.17	10.7	10.5	-0.8	11.9
	Mid QC	20	-8.5	5.44	0.7	12.1	12.5	12.4	1.6	13.4
	High QC	40	-4.4	3.24	1.1	7.18	7.0	8.06	1.2	7.85
	Dilute QC	80	-12	4.63	---	---	---	---	---	---
Membrane IP + tryptic digestion	LLOQ	0.05	4.0	9.4	-6.4	4.1	-4.0	10.6	-2.2	9.3
	Low QC	0.15	-2.7	11.9	-0.7	4.9	-1.3	10.8	-2.0	9.1
	Mid QC	20	1.5	8.3	8.5	9.1	-2.5	4.6	2.5	8.5
	High QC	40	2.8	2.6	-1.8	4.8	-4.0	8.1	-1.0	5.9
	Dilute QC	80	11.5	9.5	---	---	---	---	---	---

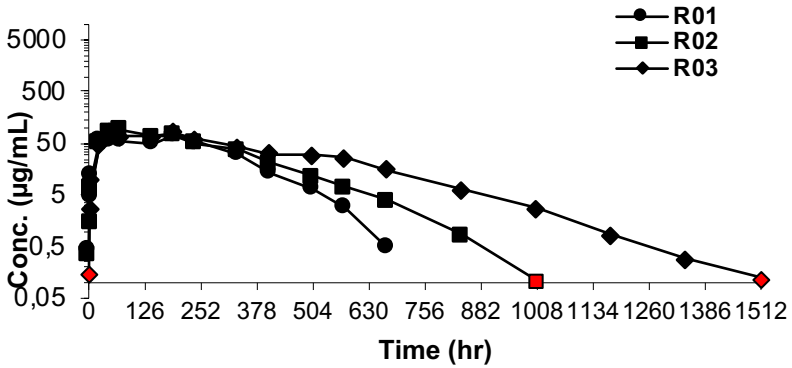
* Six sets of QCs were tested on each level in each run.
**Within-run and between-run %Bias must be less than or equal to 15.0%, except for LLOQ, which was 20.0%; Within-run and between-run %CV must be within ±15.0%, except for LLOQ, which was ±20.0%.

Surrogate Peptide Approach (9)
- Pharmacokinetics Parameters Comparison

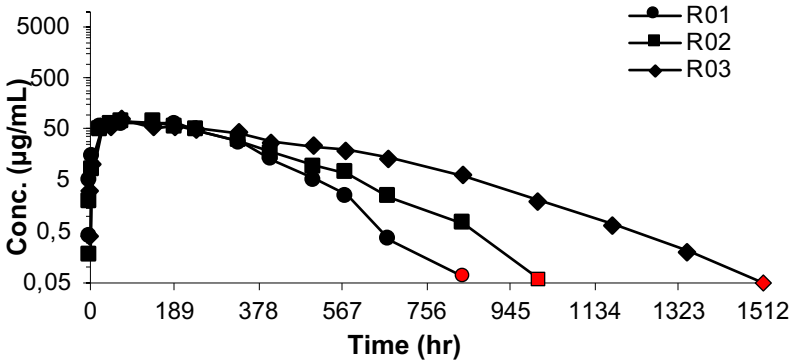
Direct tryptic digestion



Magnetic beads IP + tryptic digestion



Membrane IP + tryptic digestion



Method	Direct Tryptic Digestion			Magnetic Beads IP + Tryptic Digestion			Membrane IP + Digestion		
PK Parameters	Mean	SD	CV (%)	Mean	SD	CV (%)	Mean	SD	CV (%)
C_{max} (µg/mL)	86.1	5.65	6.56	81.3	8.44	10.4	71.9	5.06	7.03
T_{max} (h)	64.0	13.9	21.7	104.0	77.1	74.2	96.0	41.6	43.3
$T_{1/2}$ (h)	89.8	34.9	38.9	76.3	38.7	50.7	104	43.7	42.2
$AUC_{0\text{-last}}$ (µg·h/mL)	27612	5172	18.7	26135	6361	24.3	23881	4604	19.3

Surrogate Peptide Approach (10)
- Proficiency Testing Held by NIFDC

- The National Institute for Food and Drug Control (NIFDC, 中国食品药品检定研究院) held the first proficiency testing for large molecule quantitation in biological samples in June, 2019
- DMPK Department of WuXi AppTec submitted results from both direct tryptic digestion and membrane IP followed by tryptic digestion
- Both our results passed NIFDC criteria
- No systematic deviation between LC-MS and LBA for large molecule quantitation

证 书

CERTIFICATE

兹证明：上海药明康德新药开发有限公司药性评价部 参加了2019年度由中国食品药品检定研究院举办的能力验证项目。

计划名称：生物样本检测-测定猴血清中贝伐珠单抗的浓度

计划编号：NIFDC-PT-195

评价结果合格，特发此证。

中国食品药品检定研究院

二〇一九年七月

NIFDC

CERTIFICATE

This is to certify that the DMPK Department of WuXi AppTec (Shanghai) Co., Ltd. participated in the Proficiency Testing program administered by the National Institutes for Food and Drug Control in 2019.

Program name: **Biological sample analysis – Measurement of monoclonal antibody bevacizumab concentration in monkey serum**

Program number: NIFDC-PT-195

The evaluation result is **SATISFACTORY**, and this certificate is hereby issued.

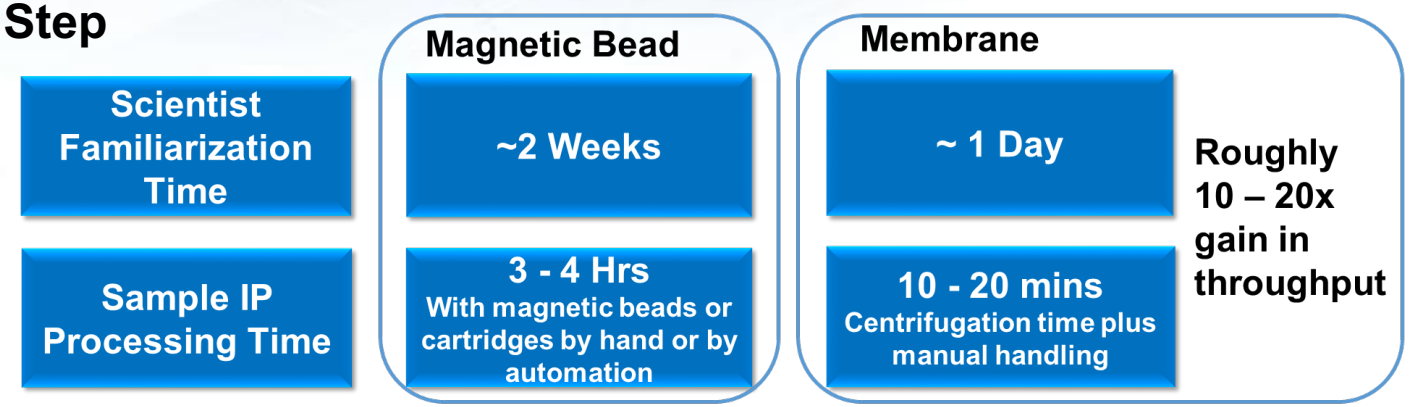
National Institutes for Food and Drug Control

(Special Seal for Proficiency Testing of National Institutes for Food and Drug Control)

July, 2019

Surrogate Peptide Approach (11)
- Efficiency Comparison

➢ IP Step



➢ Total time for a typical monoclonal antibody

Time	Direct digestion w/ generic tryptic digestion method	Direct digestion w/ ProteinWorks kit	Magnetic beads IP + tryptic digestion w/ ProteinWorks kit	Membrane IP + tryptic digestion w/ ProteinWorks kit
Immunoaffinty Purification (hrs)	NA	NA	4	0.3
Tryptic digestion (hrs)	22	5	5	5
Total sample prep (hrs)	22	5	9	5.3
LC-MS for 100 injections (hrs)	34	34	10.5	13.7
Total time (hrs)	56	39	19.5	19
Number of Days	3	2	1	1

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Summary

- LC-MS based large molecule bioanalysis platform for drug screening and preclinical pharmacokinetics was established in the DMPK department of WuXi AppTec
  - Better specificity, no need for specific antibody
  - More than 100 proteins analyzed
- Intact Approach
  - Quantitative analysis for small proteins (M.W. ≤ 20 kDa): better specificity
  - Qualitative/semi-quantitative analysis for small molecule drug screening assay
- Surrogate Peptide Approach
  - Quantitative analysis for larger proteins (M.W. ≥ 20 kDa, with various IP options)
  - Validated method that passed NIFDC proficiency testing
  - No systematic differences between the LC-MS approach and LBA approach
  - Combination of immunoaffinity purification and Waters ProteinWorks kit greatly improved efficiency

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Thank you

DMPK Department

Lab Testing Division