Quantitation of Proteins and Monoclonal Antibodies In Serum by LC-MS/MS Using Full-Length Stable Isotope Labeled Internal Standards

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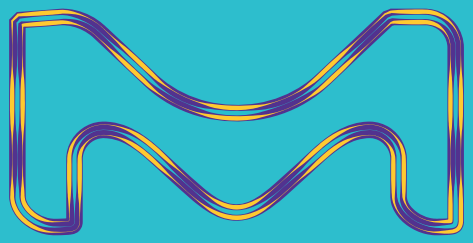
MilliporeSigma

MilliporeSigma is a business of Merck KGaA, Darmstadt, Germany



MERCK

Outline

- Why Quantitative MS vs ELISA
- Quantitative MS workflow
- Why SIL protein as an internal standards
- Expression and characterization of SIL proteins and antibodies
- Quantitative MS assays using SIL proteins and antibodies

Serum Protein Measurement Methods

ELISA / LBA

Pros:

- High sensitivity
- High throughput
- Traditional methodology
- Minimal sample prep

Cons:

- Assay specific reagents
  - Long lead times
  - Poor standardization
- Specificity concerns
- Difficult to multiplex

LC-MS/MS

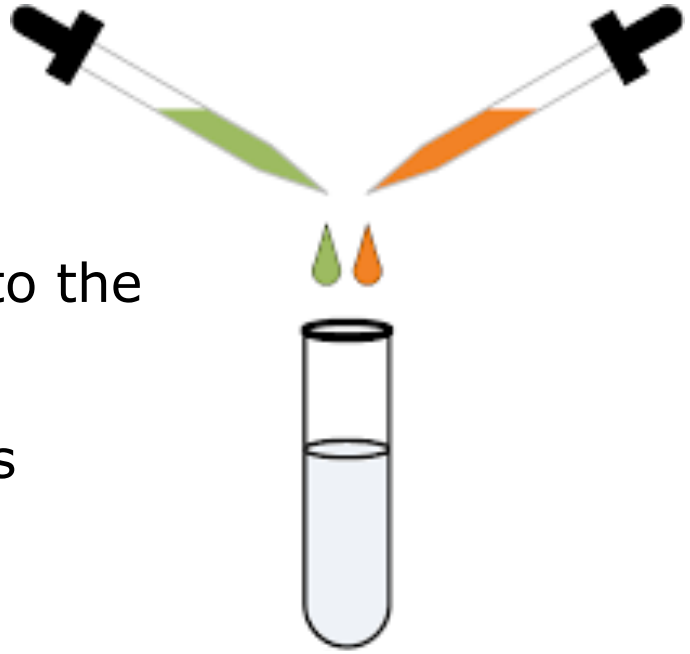
Pros:

- Highly selective
- Faster assay development
- Ability to multiplex
- Can be combined with immunoaffinity enrichment

Cons:

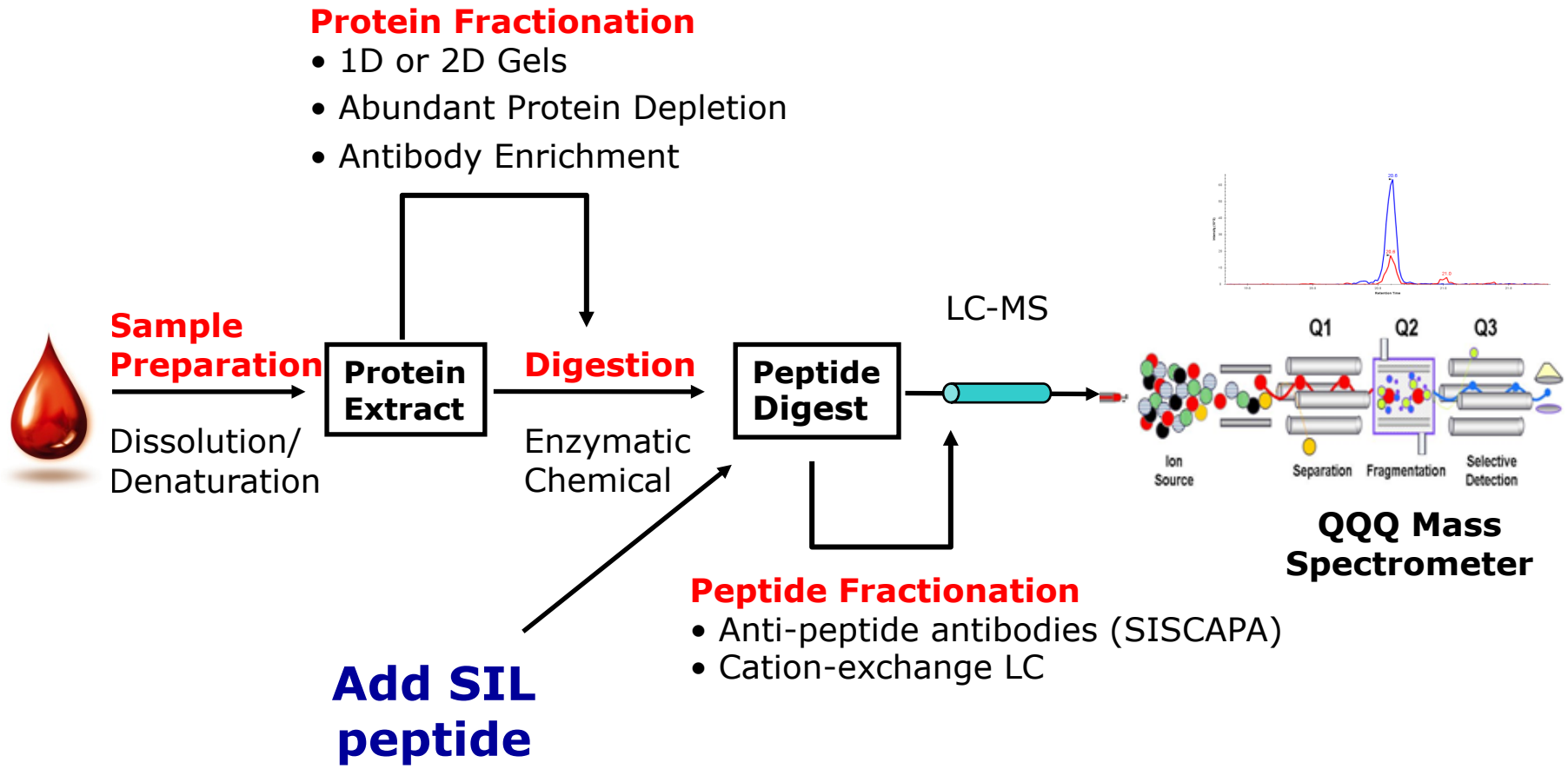
- Expensive instrumentation
- Extensive sample prep
- Requires an internal standard

General Design of an Internal Standard



- Not be present in any of the samples
- Similar in physiochemical properties to the target analyte
- Added as early on in the procedure as possible
  - recovery during transfer and clean-up
  - variability in extraction efficiency
  - injection volume variability
  - matrix suppression
- for LC-MS , preferably an isotopically labeled version of the analyte (SIL)

Typical Quantitative MS Workflow



SIL peptides are typically added late in the workflow

Protein Internal Standard Workflow

Protein Fractionation
- 1D or 2D Gels
- Abundant Protein Depletion
- Antibody Enrichment

Sample Preparation

Dissolution/
Denaturation

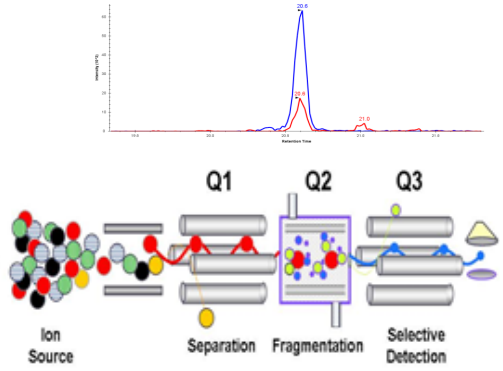
Protein Extract

Digestion

Enzymatic
Chemical

Peptide Digest

LC-MS



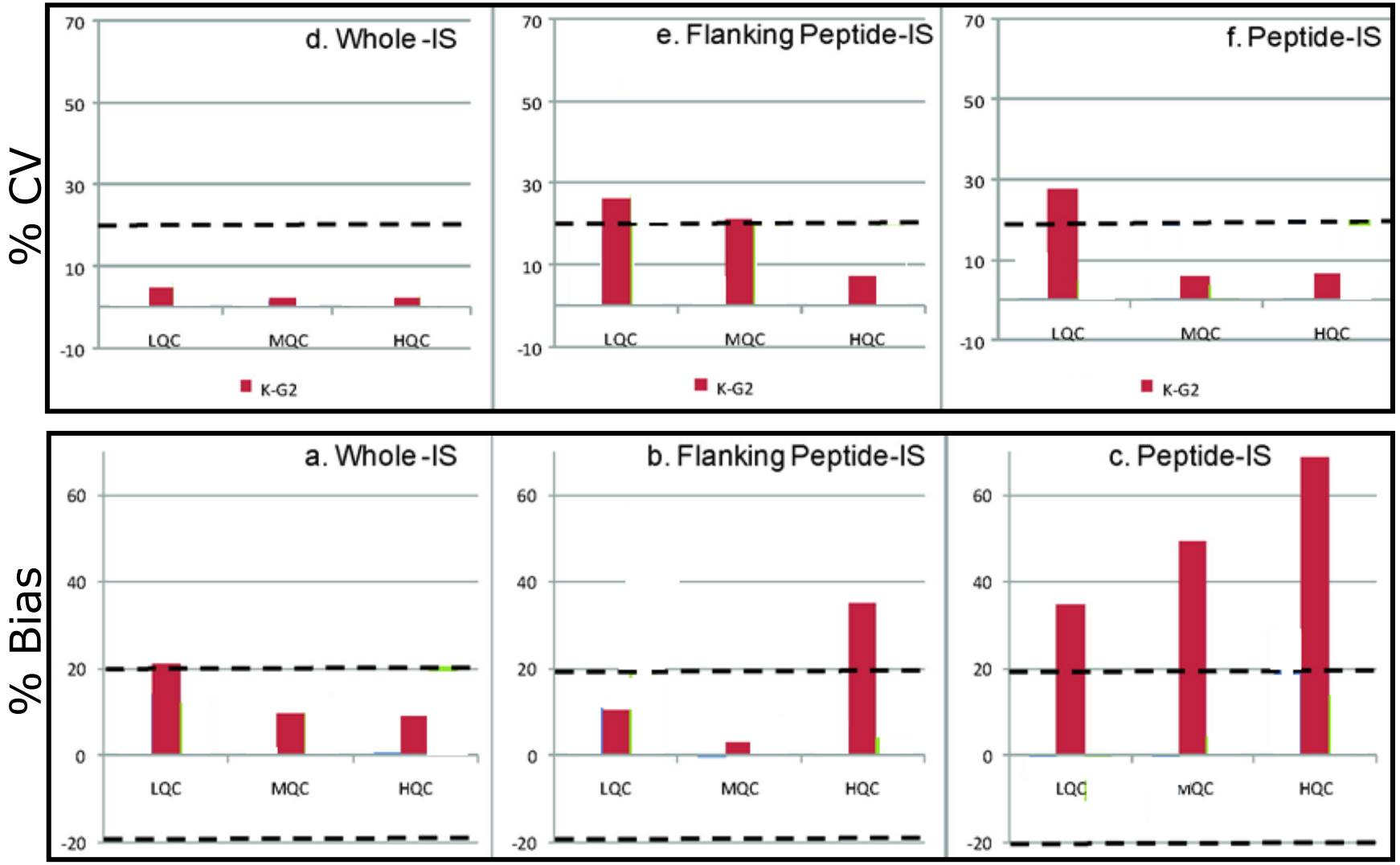
QQQ Mass Spectrometer

Peptide Fractionation
- Anti-peptide antibodies (SISCAPA)
- Cation-exchange LC

Add SILuMab or SIL Protein

SIL protein is added early in the workflow

Accuracy and Precision with Three SIL-IS's



Hongyan Li et al; *Anal. Chem.* **2012,** 84, 1267-1273.

Desired SIL-Protein Properties

- High protein purity
- Matches native protein sequence
- High incorporation of stable isotopes
- Similar PTM to native protein (ie, glycosylation)
- Digestion kinetics same as native protein
- Similar enrichment or fractionation as native protein

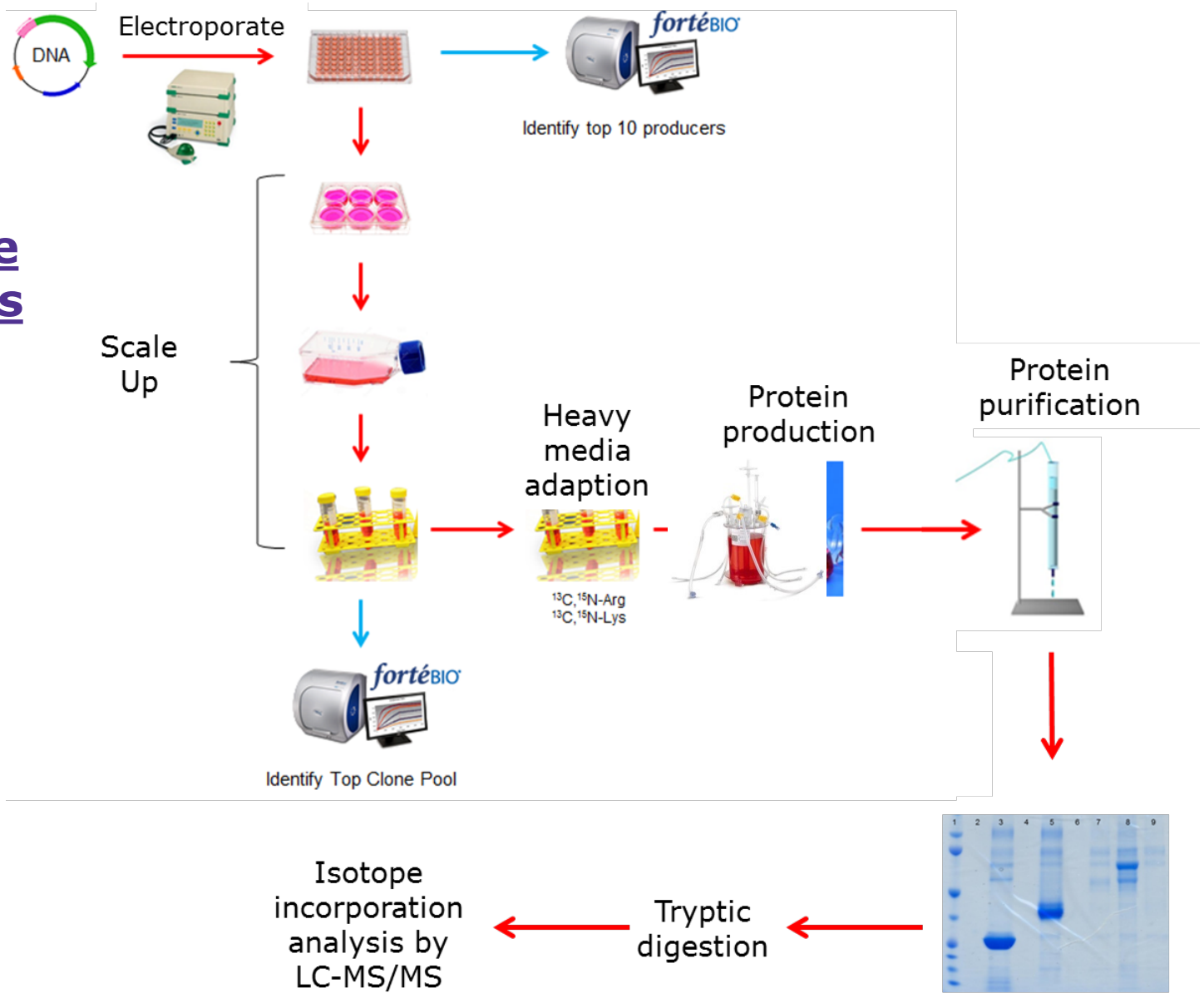
SIL Protein and SILuMab Development

Available Cell Lines

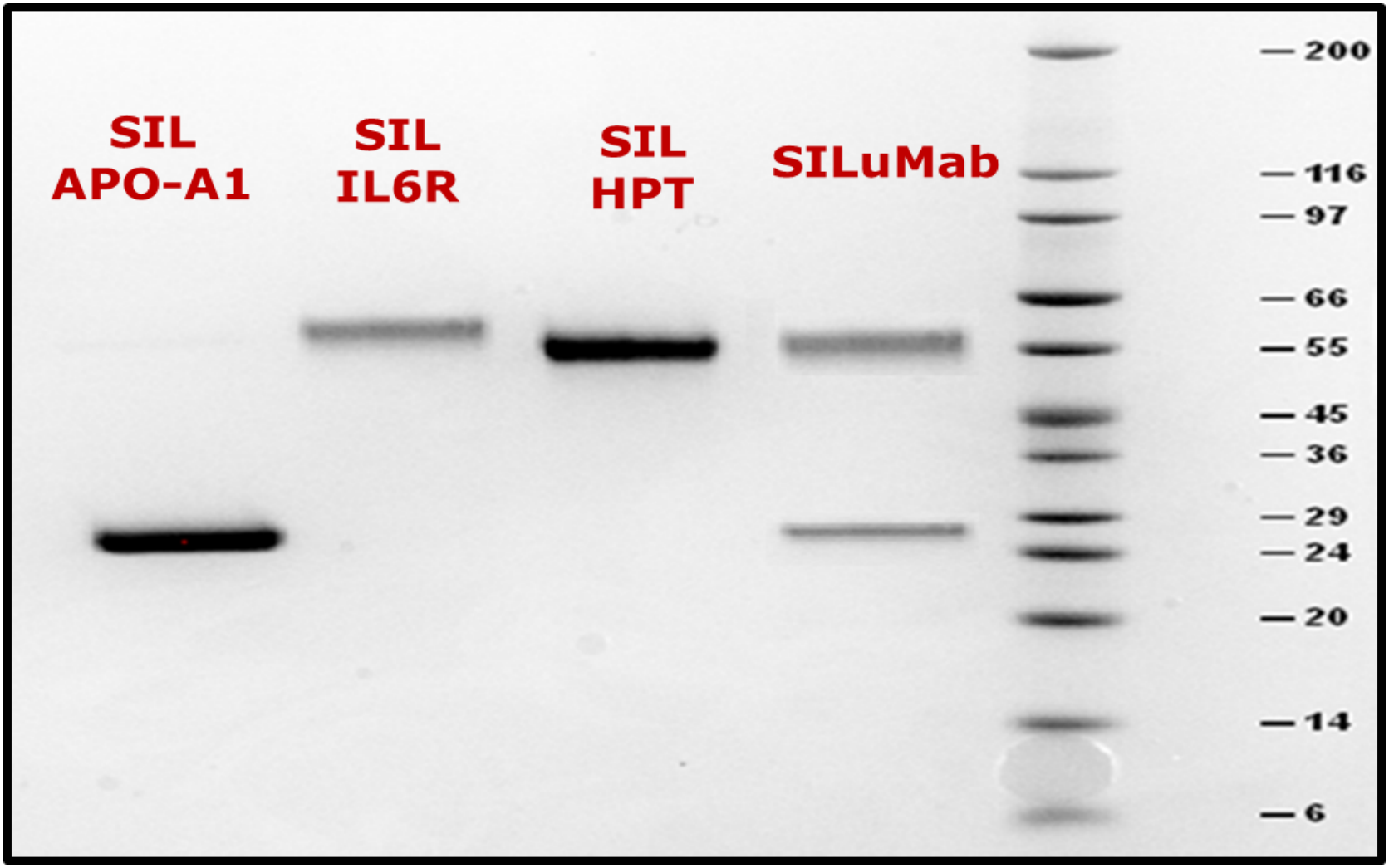
CHO

HEK

E. coli



SIL-Protein Purity by SDS-PAGE

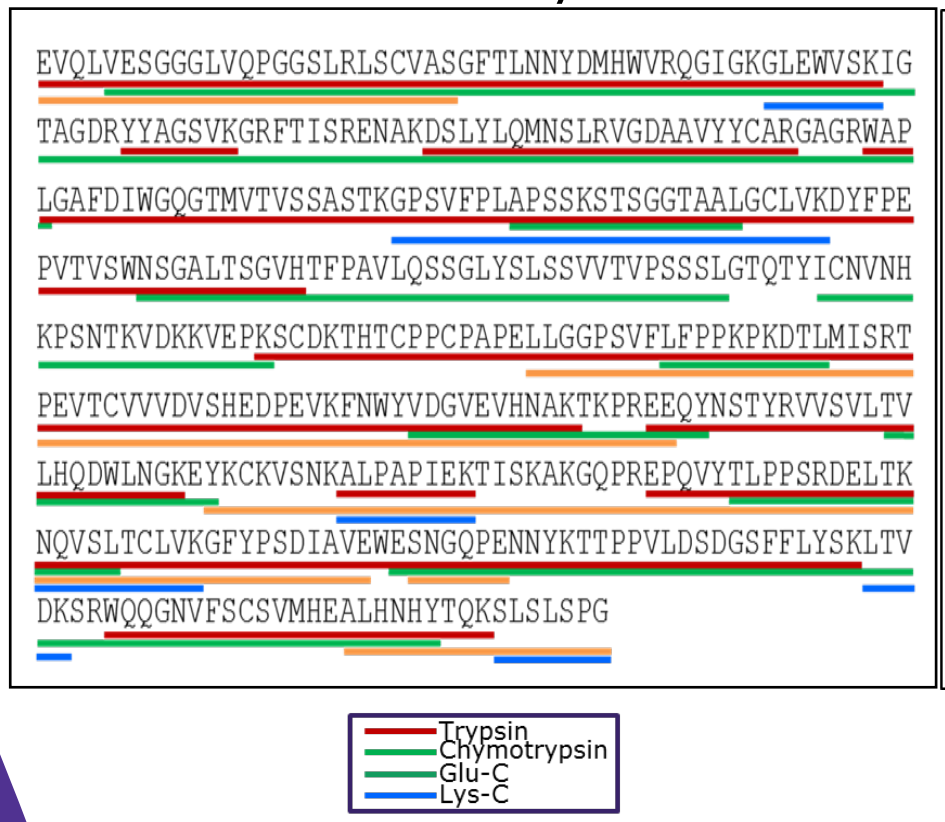


Purity greater than 95% achieved

Characterization of SILUMab

Sequence confirmation by peptide mapping

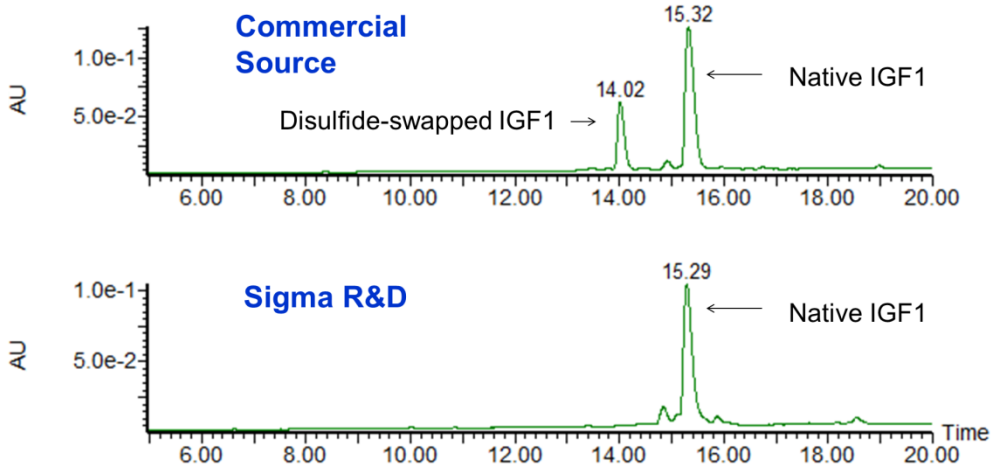
SILuMab Heavy Chain

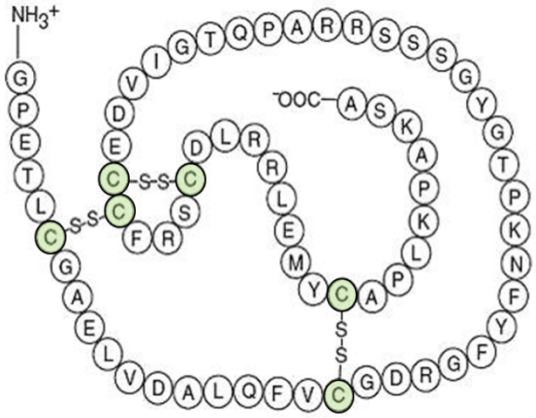


Apolipoprotein A1 (APOA1)

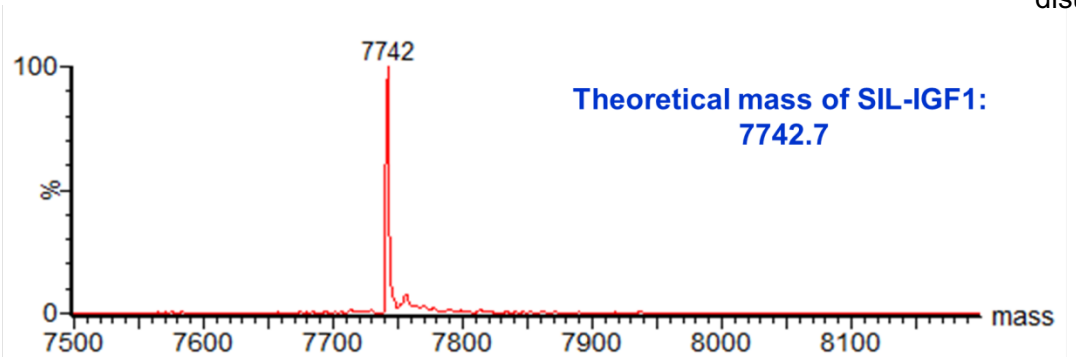
High sequence coverage obtained

RP-LC-MS Analysis of Intact SIL-IGF1





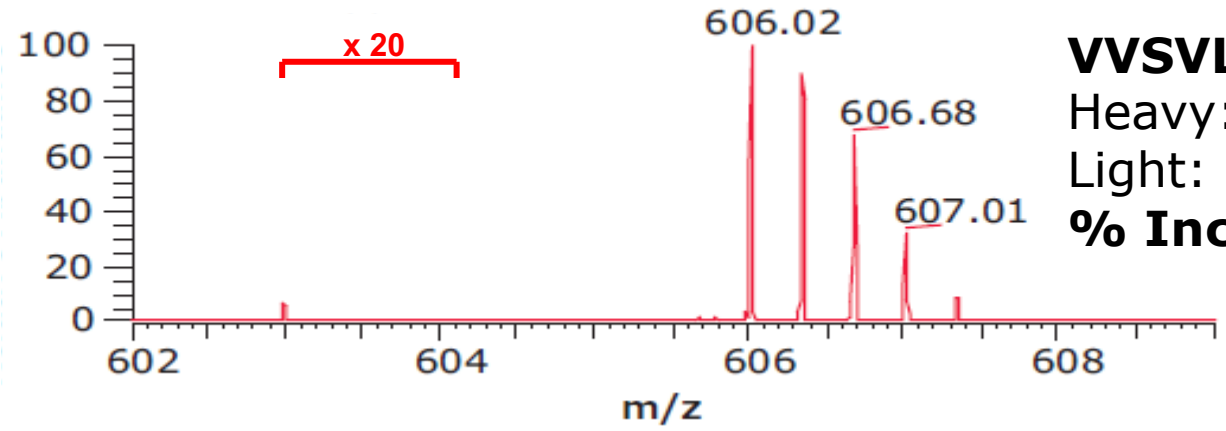
Sequence of IGF-1 consisting of 70 amino acids in a single chain and three intramolecular disulfide bonds



Sequence and structure verified by RP-LC-UV-MS

Isotope Incorporation

$^{13}C_6{}^{15}N_2$ Lys in SILuMab and $^{13}C_6{}^{15}N_4$ Arg in SIL-Thyroglobulin

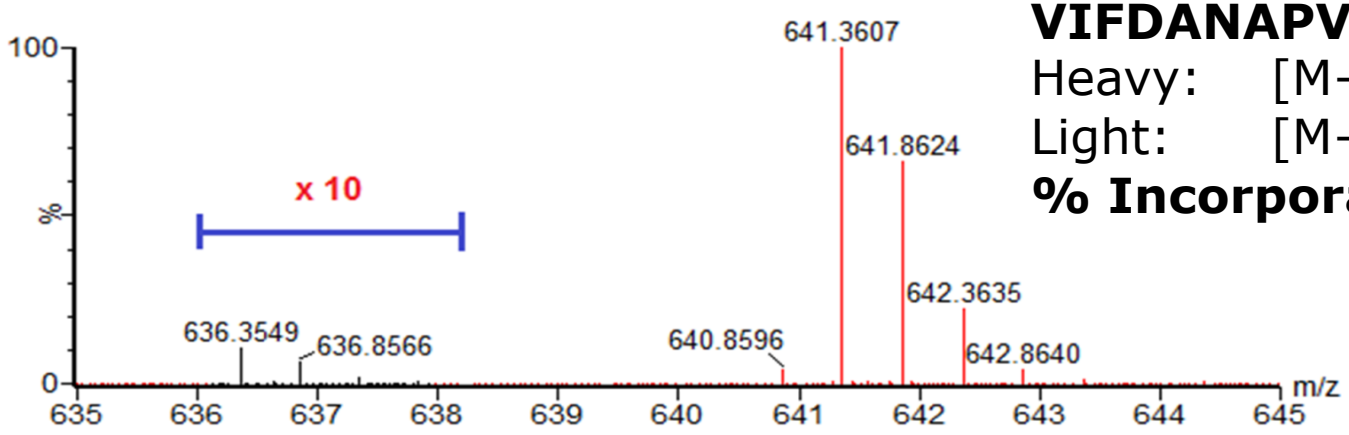


VVSVLTVLHQDQLNG<u>K</u>

Heavy: $[M+3H]^{+3}$ 606.0117

Light: $[M+3H]^{+3}$ 636.3403

% Incorporation > 99%



VIFDANAPVAV<u>R</u>

Heavy: $[M+2H]^{+2}$ 641.3631

Light: $[M+2H]^{+2}$ 636.3590

% Incorporation = 98.2%

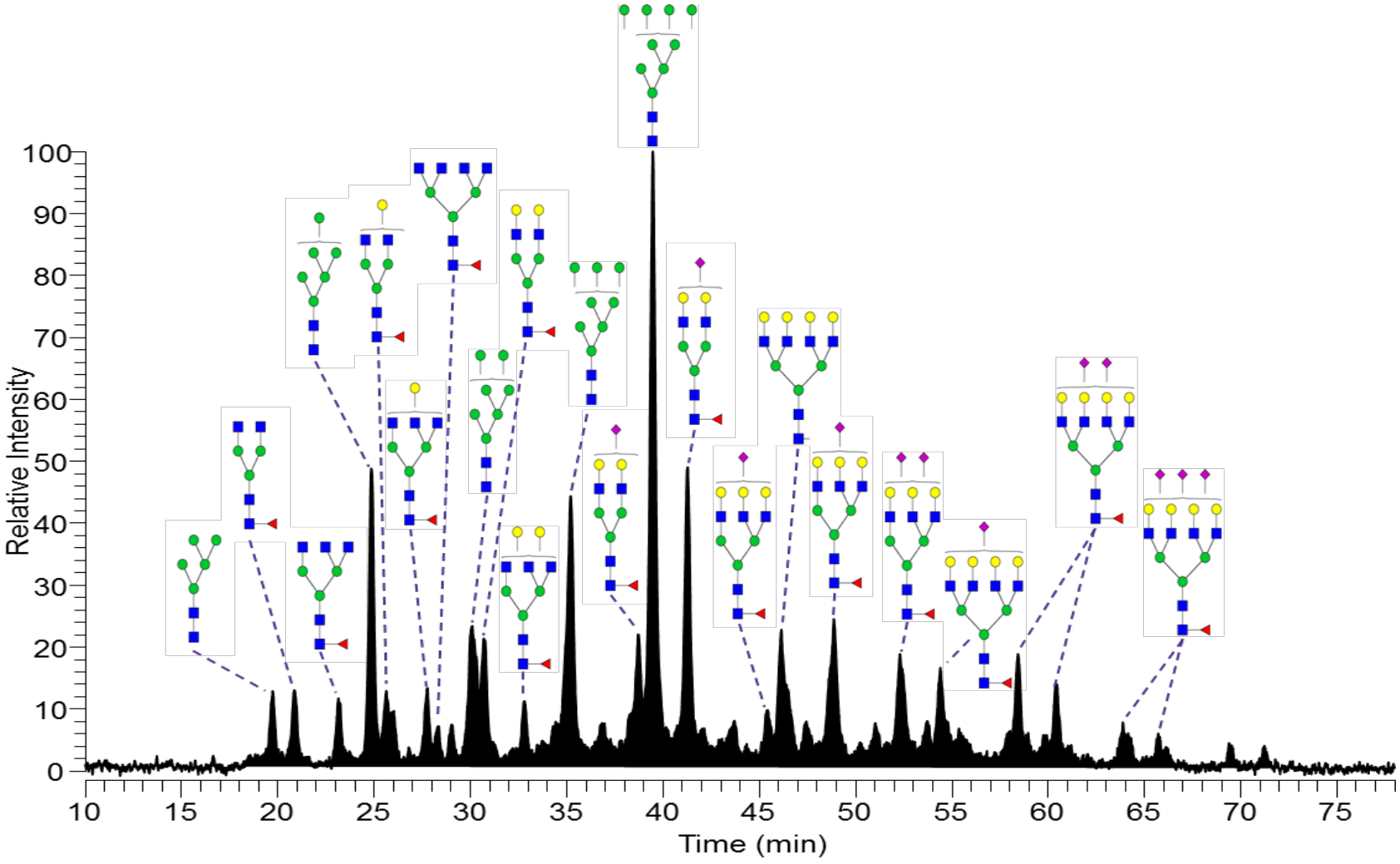
Incorporation > 98%

Desired SIL-Protein Properties

- ☑ High protein purity
- ☑ Matches native protein sequence
- ☑ High incorporation of stable isotopes
- ☐ Similar PTM to native protein (ie, glycosylation)
- ☐ Digestion kinetics same as native protein
- ☐ Similar enrichment or fractionation as native protein

SIL Protein Characterization: PTM's

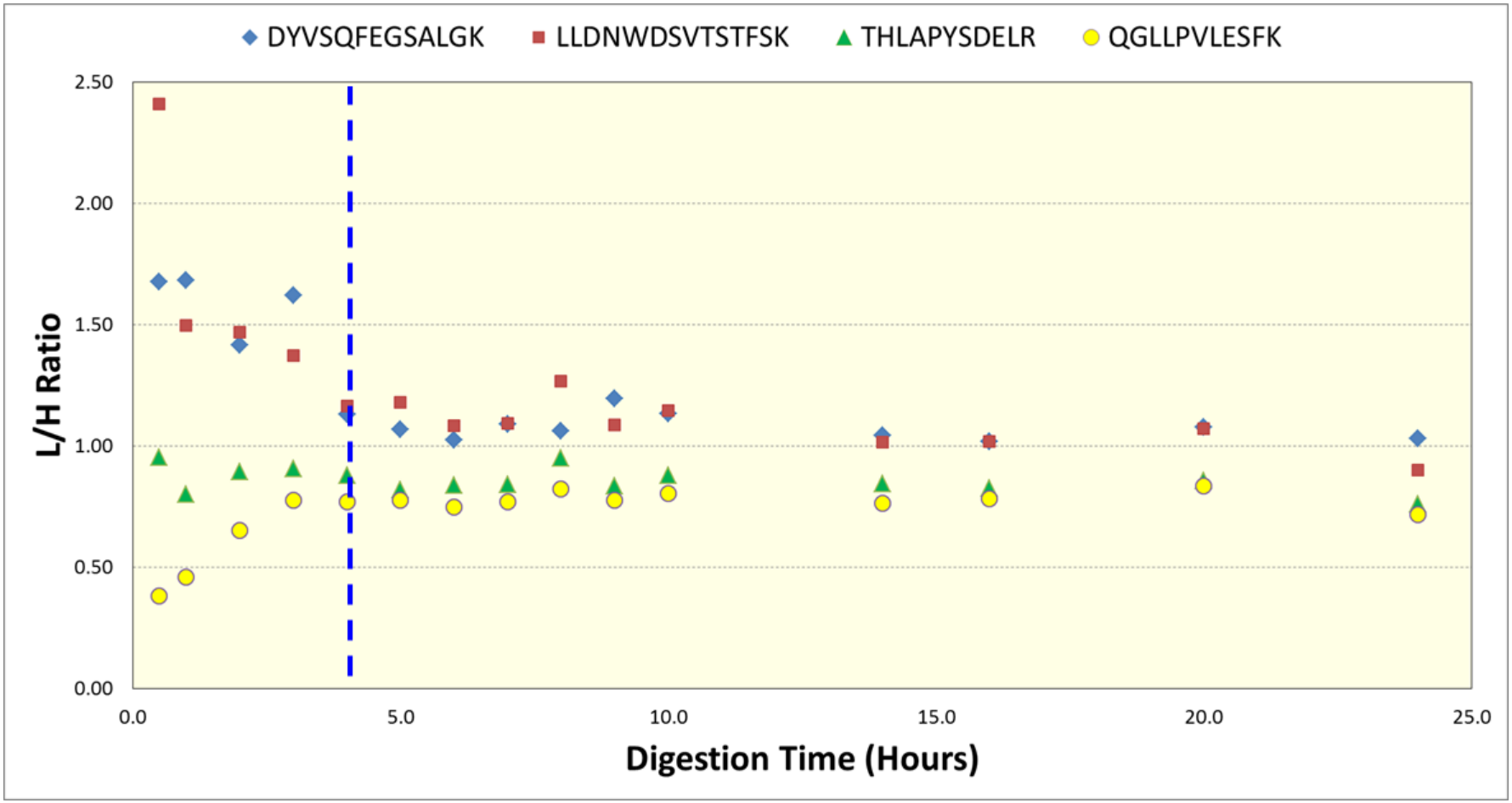
Glycosylation of SIL Thyroglobulin



Glycosylation similar to native human protein

SIL Protein Digestion Kinetics

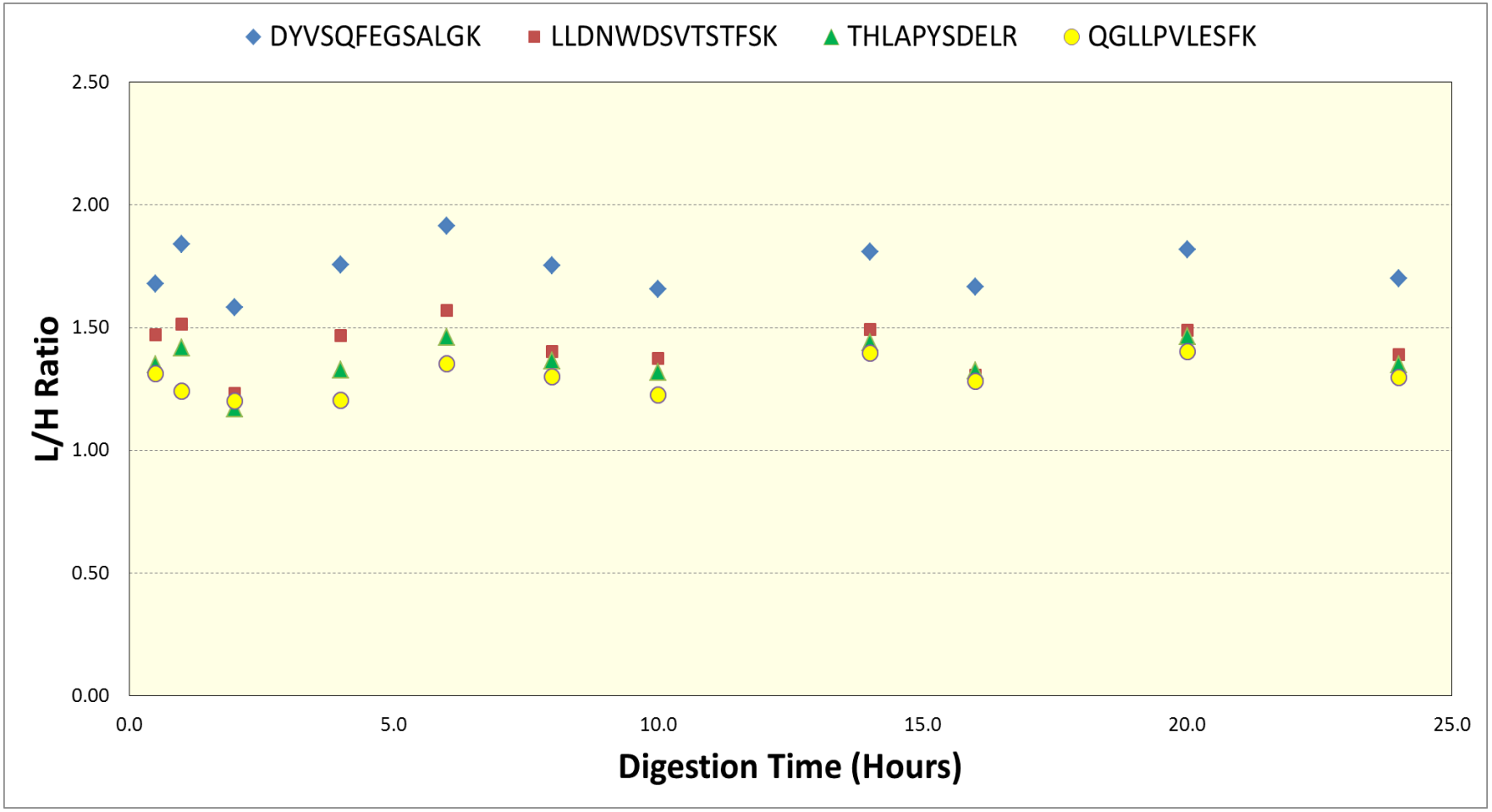
APOA1 in Human Serum, TFE Denaturation



Kinetics are similar after 4 hours of digestion

SIL Protein Digestion Kinetics

APOA1 in Human Serum, Urea Denaturation (FASP)



Kinetics are similar after denaturation

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Protein Internal Standard Workflow

Protein Fractionation
- 1D or 2D Gels
- Abundant Protein Depletion
- **Antibody Enrichment**

Sample Preparation

Dissolution/ Denaturation

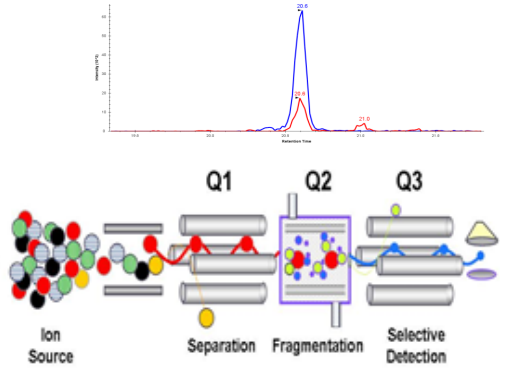
Protein Extract

Digestion

Enzymatic Chemical

Peptide Digest

LC-MS



QQQ Mass Spectrometer

Peptide Fractionation
- Anti-peptide antibodies (SISCAPA)
- Cation-exchange LC

Add SILuMab or SIL Protein

SIL protein should normalize enrichment variability

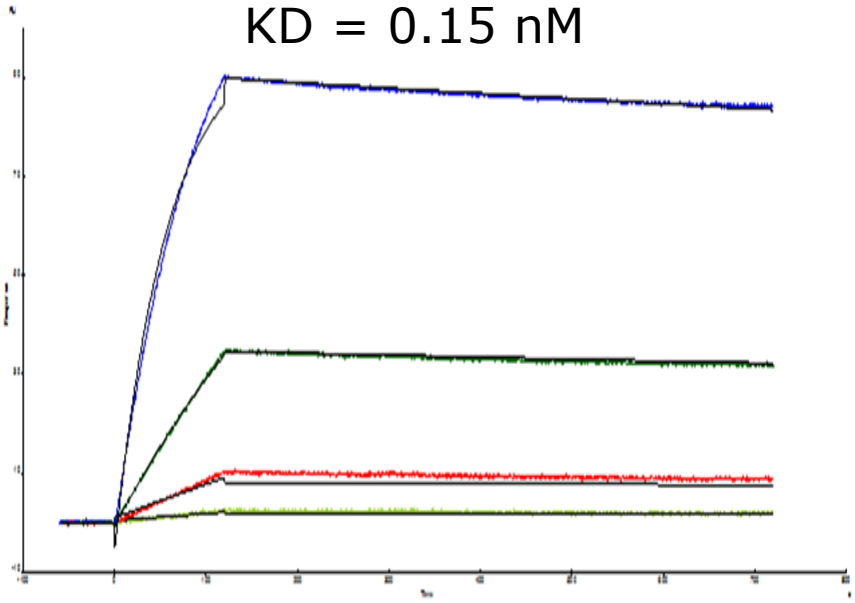
SIL Protein Characterization: Ligand Binding Affinity

SIL-Infliximab vs Remicade on Biacore

TNF-α sensorgrams

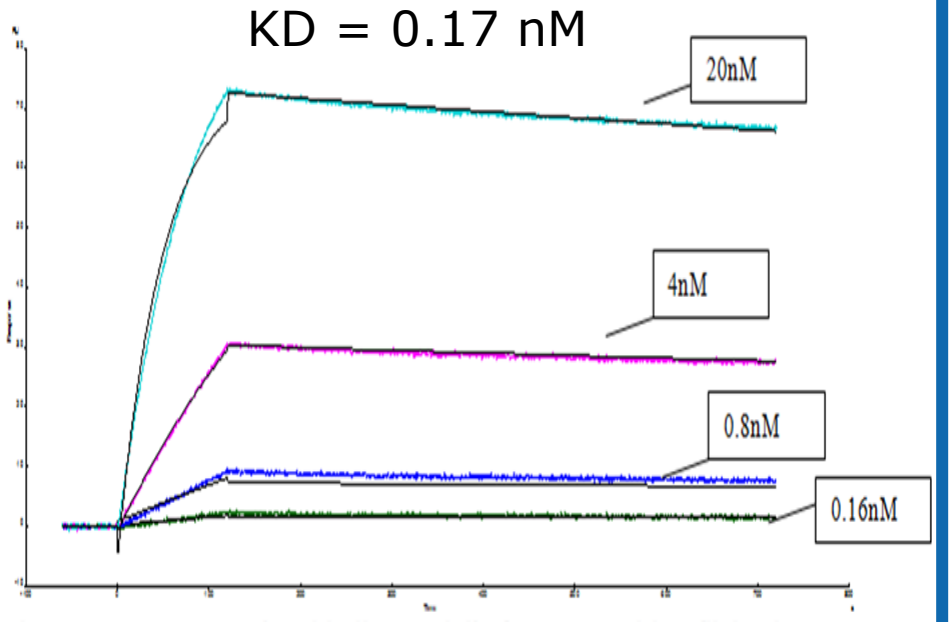
SIL-Infliximab

KD = 0.15 nM



Remicade

KD = 0.17 nM



Ligand binding equivalent to therapeutic antibody

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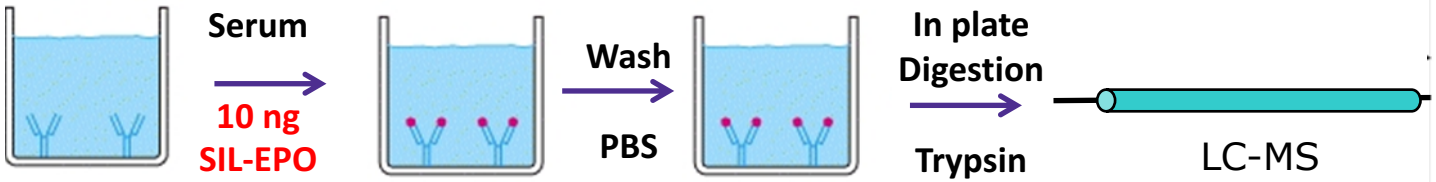
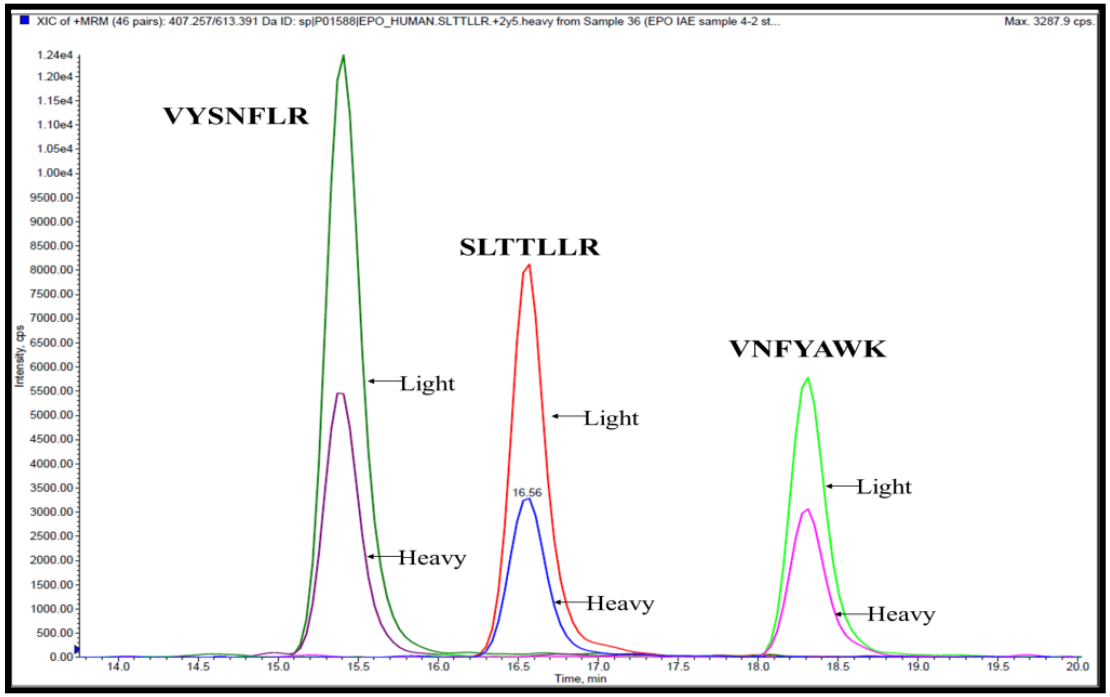
Immuno-affinity enrichment LC-MS assay

Erythropoietin (EPO) in dog serum



Anti hu-EPO
Capture Plate

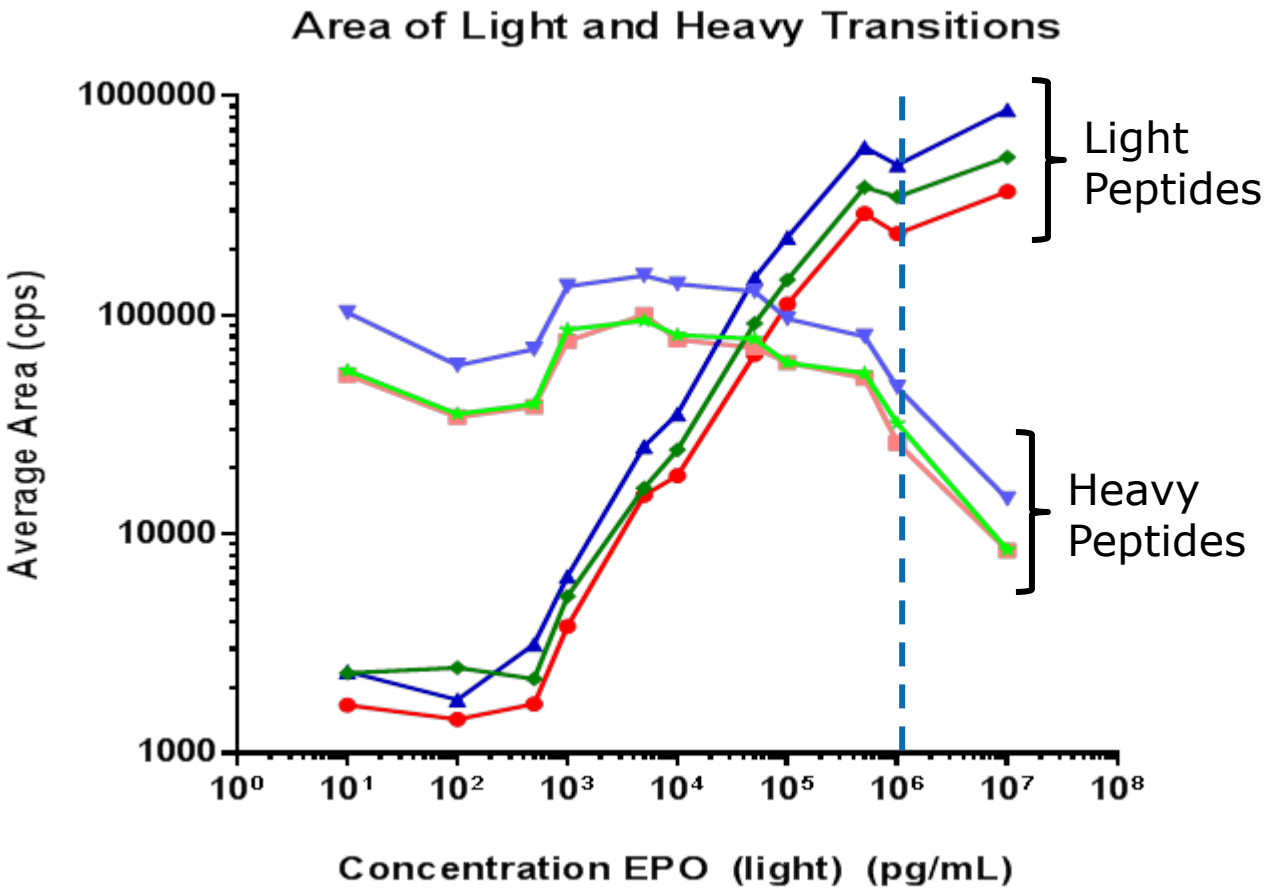
QQQ Mass
Spectrometer



Beagle serum with h-EPO at 1 pg/ml to 10 ug/ml, 50 ng/ml SIL-EPO

Immuno-affinity enrichment LC-MS assay

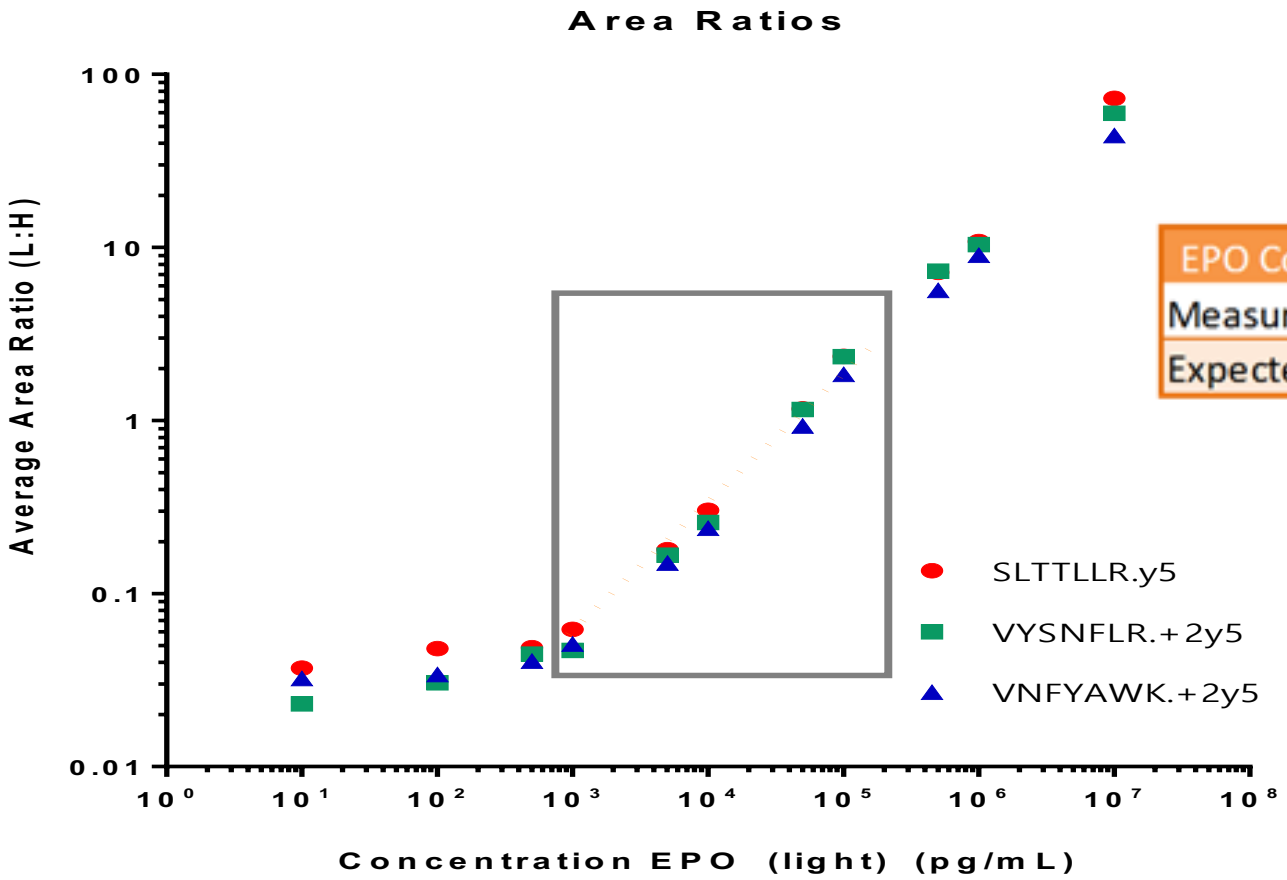
Erythropoietin (EPO) in dog serum



Variable capture efficiency, saturation above 1 ug/ml

Immuno-affinity enrichment LC-MS assay

Erythropoietin (EPO) in dog serum

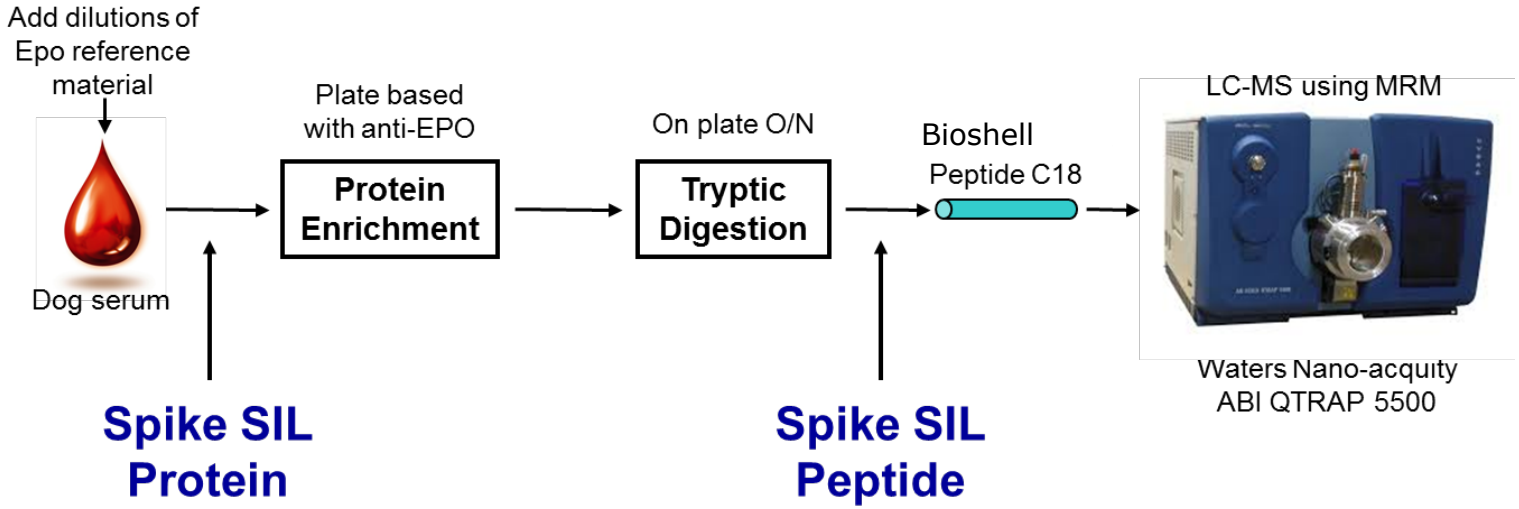


EPO Control 2 (ALPCO ELISA Kit)	
Measured	1.6 ng/mL
Expected	1.1-1.8 ng/mL

Response normalized by SIL protein ISTD

Comparison of Peptide and Protein Internal Standards

Erythropoietin (EPO) in dog serum



Accuracy Table

[EPO] ng/mL	Protein IS			Peptide IS		
	VNFYAWK	SLTTLLR	VYSNFLR	VNFYAWK	SLTTLLR	VYSNFLR
5	88.8	88.6	80.7	66.2	80.6	88.4
10	107.6	99.6	108.6	194.4	143.5	133.5
50	109.8	113.6	118.6	117.5	122.5	118.7
100	93.1	101.2	93.1	124.1	136.3	127.1
250	100.7	97.1	93.1	85.3	81.3	85.7

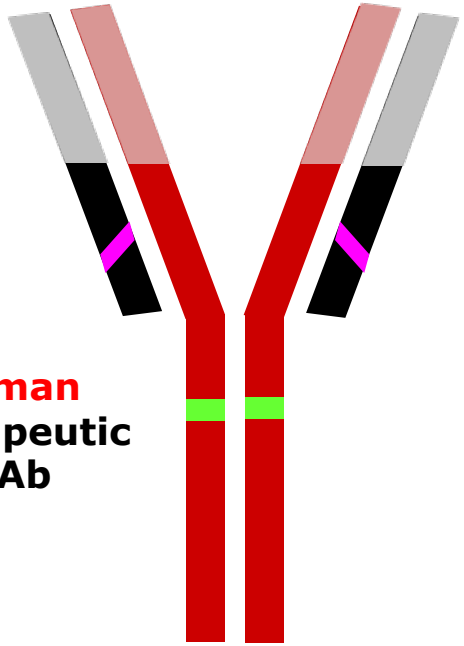
Red highlight:
>20% deviation from expected

Greater accuracy achieved with SIL protein ISTD

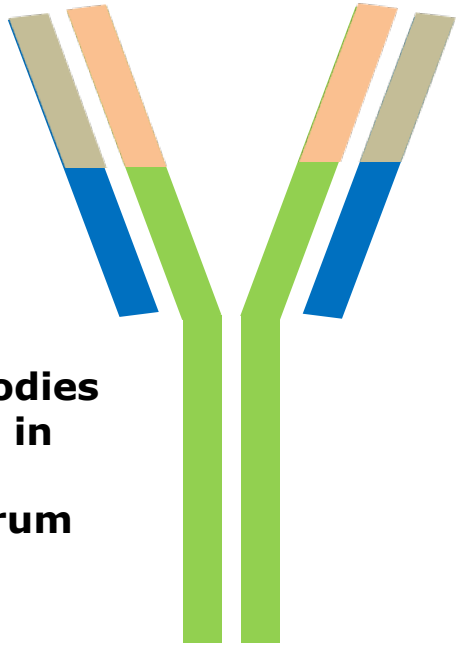
Universal Peptide Strategy

Quantification of Human MAb in Pre-Clinical Model Plasma

- Surrogate tryptic peptide from Fc region of human MAb
- Second tryptic peptide from light chain of human MAb



Human Therapeutic MAb

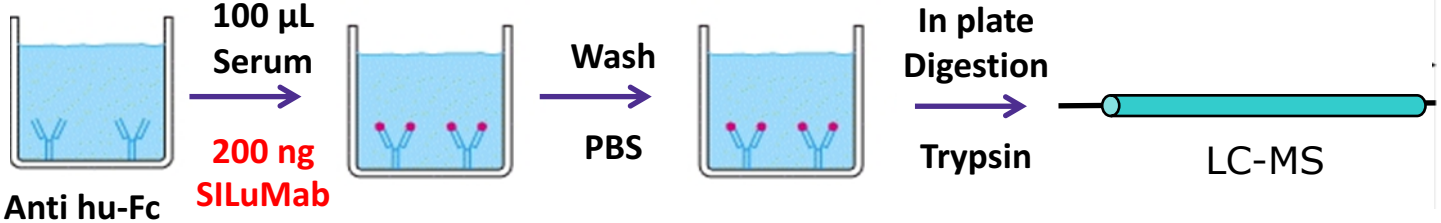


Native antibodies circulating in Animal Plasma/Serum

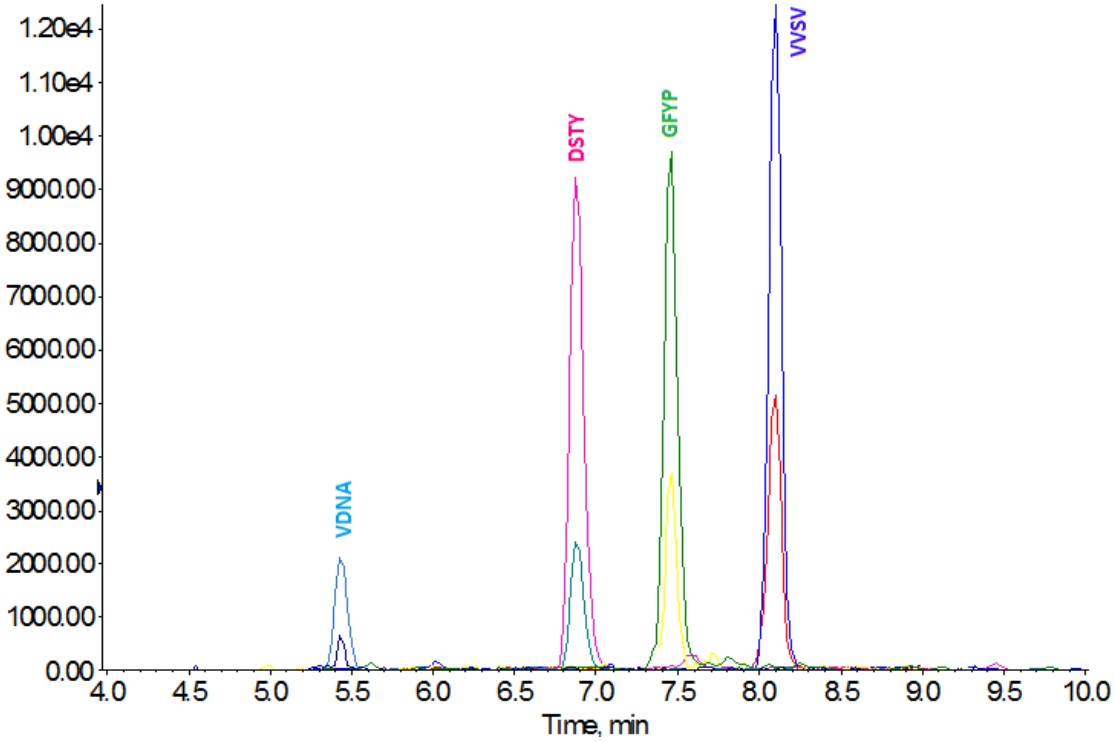
Generalized preclinical PK assay employing surrogate peptides from constant regions

Immuno-affinity enrichment LC-MS PK assay

Humira (adalimumab) in monkey serum



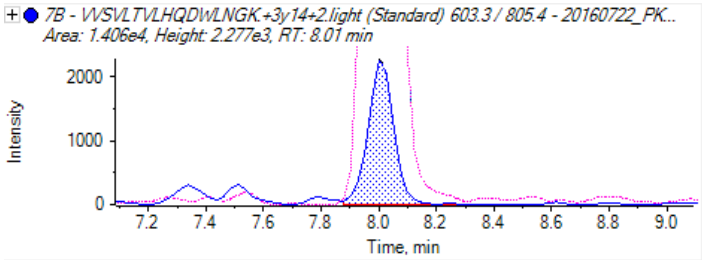
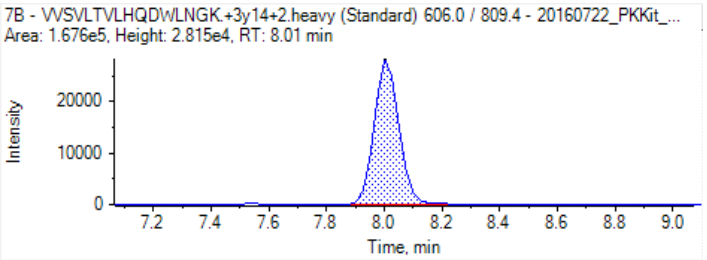
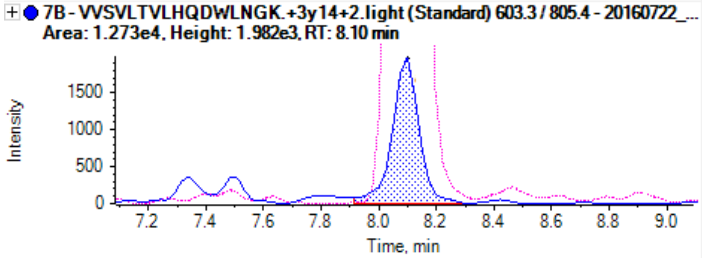
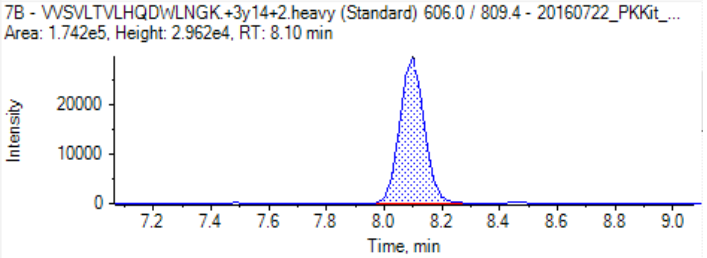
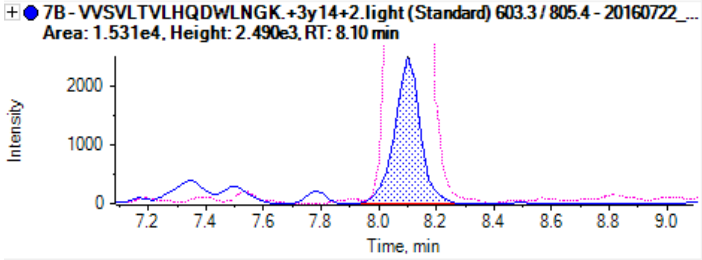
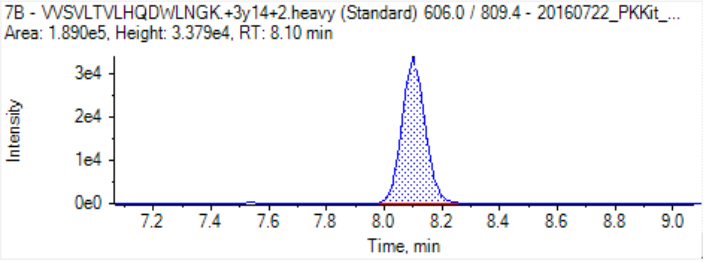
QQQ Mass Spectrometer



Monkey serum with ADA at 10 ng/ml to 50 ug/ml, 2 ug/ml SILuMab

Human Mab in Monkey Serum

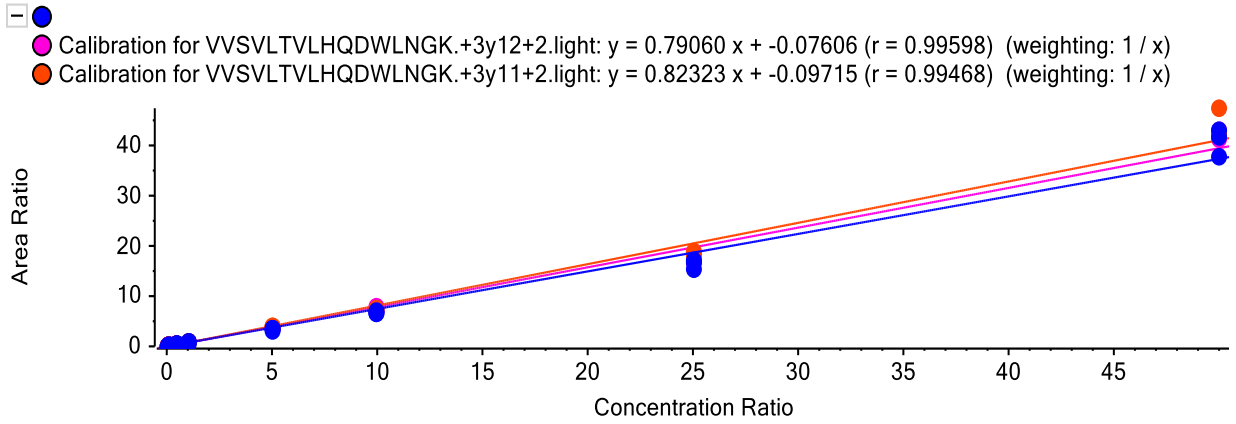
Peptide VVSV at LOQ

	Light peptide	***Heavy peptide***
Injection #1		
Injection #2		
Injection #3		

6% CV @ 100 ng/mL

Human Mab in Monkey Serum

Assay Statistics



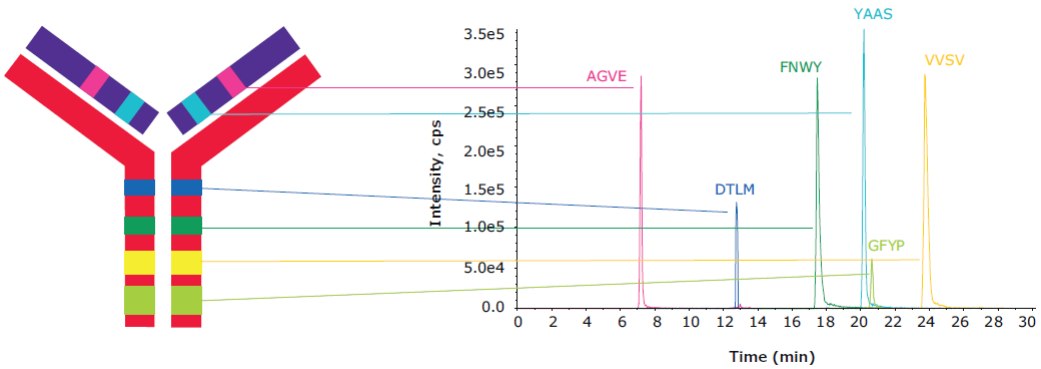
Row	Component Name	Actual Concentratio	Num. Val	Mean	Standard Dev	Percent CV	Accuracy	Value #1	Value #2	Value #3
1	VVSVLTVLHQDWLNGK.+3y12+2.ligh	0.00	0 of 9	N/A	N/A	N/A	N/A	N/A	N/A	N/A
2	VVSVLTVLHQDWLNGK.+3y12+2.ligh	0.01	0 of 3	N/A	N/A	N/A	N/A	N/A	~~N/A~~	N/A
3	VVSVLTVLHQDWLNGK.+3y12+2.ligh	0.05	0 of 3	N/A	N/A	N/A	N/A	~~1.012e-1~~	~~1.083e-1~~	~~6.893e-2~~
4	VVSVLTVLHQDWLNGK.+3y12+2.ligh	0.10	3 of 3	1.129e-1	7.104e-3	6.29	112.90	1.193e-1	1.141e-1	1.053e-1
5	VVSVLTVLHQDWLNGK.+3y12+2.ligh	0.50	3 of 3	5.154e-1	2.425e-2	4.71	103.07	4.954e-1	5.424e-1	5.084e-1
6	VVSVLTVLHQDWLNGK.+3y12+2.ligh	1.00	3 of 3	9.959e-1	7.764e-2	7.80	99.59	1.080e0	9.276e-1	9.797e-1
7	VVSVLTVLHQDWLNGK.+3y12+2.ligh	5.00	3 of 3	4.663e0	7.688e-2	1.65	93.25	4.574e0	4.701e0	4.713e0
8	VVSVLTVLHQDWLNGK.+3y12+2.ligh	10.00	3 of 3	9.501e0	4.117e-1	4.33	95.01	9.459e0	9.931e0	9.111e0
9	VVSVLTVLHQDWLNGK.+3y12+2.ligh	25.00	3 of 3	2.228e1	1.067e0	4.79	89.11	2.189e1	2.146e1	2.348e1
10	VVSVLTVLHQDWLNGK.+3y12+2.ligh	50.00	3 of 3	5.354e1	1.235e0	2.31	107.07	5.338e1	5.238e1	5.484e1

Accuracy: 85-115%, Precision: <10%

Range: 100 ng/mL to 50 ug/mL

SILuMab Standards

Product Name		Cat. No.
SILu™MAB Stable-Isotope Labeled Universal Monoclonal Antibody Standard	Human IgG1 – lambda	MSQC3
SILu™MAB K1 Stable-Isotope Labeled Universal Monoclonal Antibody Standard	Human IgG1 – kappa	MSQC6
SILu™MAB K4 Stable-Isotope Labeled Universal Monoclonal Antibody Standard	Human IgG4 – kappa	MSQC7
SILu™MAB Infliximab Stable-Isotope Labeled Universal Monoclonal Antibody Standard	Human IgG1 – kappa	MSQC9
SILu™MAB Mouse Stable-Isotope Labeled Universal Monoclonal Antibody Standard	Mouse IgG1 – kappa	MSQC10



sigma-aldrich.com/silutions

SILuProt Standards

Product Name	Cat. No.
SILu™Prot **APOA1** Apolipoprotein A-I	MSST0001
SILu™Prot **PTX3** Pentraxin-related protein	MSST0003
SILu™Prot **VEGFA** Vascular endothelial growth factor A	MSST0005
SILu™Prot **CLU** Clusterin	MSST0007
SILu™Prot **MAPK1** Mitogen activated protein kinase 1	MSST0009
SILu™Prot **ALB** Albumin	MSST0011
SILu™Prot **AMBP** Alpha-1 microglycoprotein	MSST0013
SILu™Prot **B2M** Beta-2-microglobulin	MSST0015
SILu™Prot **IL6** Interleukin 6	MSST0017
SILu™Prot **MAPK3** Mitogen activated protein kinase 3	MSST0019
SILu™Prot **CRP** C-reactive protein	MSST0021
SILu™Prot **APOA2** Apolipoprotein A-II	MSST0029
SILu™Prot **MAPT** Microtubule-associated protein tau-441	MSST0031
SILu™Prot **IFNG** Interferon Gamma	MSST0039
SIL-Thyroglobulin Certified Reference Material	T-109

Summary

- LC-MS can address the shortcomings of LBA's associated with long assay development time and specificity
- Immunoaffinity enrichment can be combined with LC-MS to improve sensitivity
- Stable isotope labeled SIL proteins have been produced in human and *e. coli* cells and characterized for quantitative MS applications
- Use of SIL proteins and SILuMab standards reduces error and variability associated with enrichment and enzymatic digestion

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