Protein Biomarkers Method Development & Validation by LCMS, HRMS and Hybrid LBA/LCMS: Recent Advancements

Regulated Bioanalysis Interest Group (RBIG)

5:45 – 7:00 pm, Tuesday, June 4th 2019

Presiding:
Dian Su (Genentech), Jian Wang (Bristol-Myers Squibb) and Fabio Garofolo (Angelini Pharma)

Panelists:
Veronica Anania (Genentech), Timothy Sikorski (GlaxoSmithKline),
Huidong (Ryan) Gu (Bristol-Myers Squibb), and Hendrik Neubert (Pfizer)

Agenda

05:45-05:50pm – Introduction (Discussion Topics & Panelists)

05:50-05:55pm – ***Multiplexed MRM LC-MS/MS Biomarker Analysis***

-Veronica Anania (Genentech)

05:55-06:05pm – ***Protein Biomarkers Assays with LC/MS: Reagent Selection and Method Optimization Strategies*** **-Tim Sikorski** (GlaxoSmithKline)

06:05-06:10pm – ***Immunoaffinity-LCMS Strategies for Protein Biomarkers***

-Hendrik Neubert (Pfizer)

06:10-06:15pm – ***Novel In-Sample Calibration Curve Approach for Quantitative LC-MS/MS Bioanalysis of Protein Biomarkers*** **-Huidong (Ryan) Gu** (Bristol-Myers Squibb)

06:15-07:00pm – Panel Discussions

Introduction
(Selected Topics for Panel Discussion)

Recent advancement in method development, technologies and strategies

- **Immunoaffinity extraction**
  - Reagent selection and characterization
  - Capture reagent: generic vs. specific reagents
  - What are the strategies and current experience in using “poor” antiprotein-based capture to enrich target protein?
  - Competing vs. non-competing reagents in the measurements of drug and target, free and total, and drug-target complexes
  - Platforms: beads vs. tips/cartridges, automation
  - Protein vs. peptide immunocaptures

Introduction
(Selected Topics for Panel Discussion)

- Digestion
  - Digestion routes, signature peptide, and MRM optimization
- LC-MS
  - Chromatography: what are the merits of using micro/nanoflow technology?
    - Theoretical enhancement, technical challenges and practical considerations in robust quantitation
  - MS matrix effect and ion suppression/enhancement
  - Internal standard tracking/compensation

Introduction
(Selected Topics for Panel Discussion)

Assessing drug-target engagement in plasma and tissue with new hybrid LBA/LC-MS

- Design experiments: Is it practical to develop and validate a single multiplexed hybrid LBA/LCMS assay to quantify both drug and endogenous analyte at the same time?
- Multiplexing: drug and target, free and total, complexes
  - *Competing vs. non-competing reagents*
- Optimization of immunocapture: to overcome dissociation and to improve drug tolerance

Introduction
(Selected Topics for Panel Discussion)

- **Parallelism**
  - What is the best way to perform parallelism and matrix effect evaluations between the surrogate matrix and authentic biological matrix in hybrid LBA/LCMS?
- **What is the current status of reagent-free LCMS?**
- **Protein biomarker proteoforms by hybrid LBA/LCMS**
  - When is it important to develop proteoform specific biomarker assays?
  - Why is hybrid LBA/LCMS the ideal technology for decimating proteoforms (isoforms, phosphoforms, glycoforms, point of mutation and other PTMs)?
  - What is the best approach for calibration standards for isoform/PTM specific assays?

Introduction

(Selected Topics for Panel Discussion)

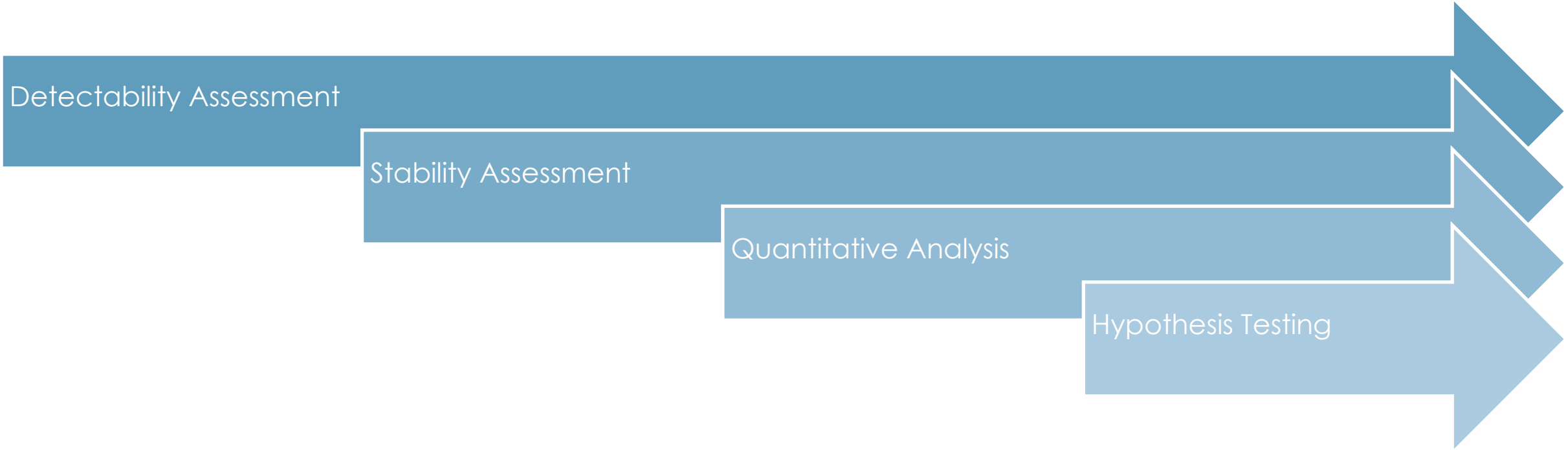
Progress in the recommendations for validation of protein biomarker assays by LC-MS and Hybrid LBA/LC-MS:

- ❑ What experiments should be conducted in the FFP method development and validations?
- ❑ 2018 FDA BMV Guidance/ 2019 ICH M10 draft Guidance
- ❑ Impacts, challenges and opportunities

Introduction

(Selected Topics for Panel Discussion)

- What are the new workflow and technical improvement in hybrid LBA/LCMS methodology?
- What new applications and assay formats for hybrid LBA/LCMS are emerging?



Multiplexed MRM LC-MS/MS Biomarker Analysis

Veronica Anania, PhD.

Genentech, Inc.

ASMS 2019

Genentech
A Member of the Roche Group

Biomarkers in Clinical Development

The Intended Use drives the discovery, validation process

Late Stage Research | Phase I | Phase II | Phase III | Life Cycle Mgmt.

Enrollment

Stratification

Prognostic

surrogate endpoint

Target Engagement

PK/PD – dose selection

Proof of Activity

Safety Monitoring

Pharmacodynamic

Surrogate Endpoint

CDx

Complimentary Dx

Predictive

MOA, Differentiation

Darker color reflects greater impact, requirement for validation (assay & clinical), regulatory, etc.

BEST (Biomarkers, EndpointS, & other Tools) Resource

NIH National Institutes of Health | FDA

Genentech
A Member of the Roche Group

Multiplexing with MRM: provide a faster and more successful translation of protein biomarkers to the clinic

Target Specific

Pathway Specific

Disease Specific

Proteomics Discovery (Literature)

Exploratory Studies

100's - 1000's of Candidates

Candidate Biomarker List (1000s-100s)

Targeted-MS assay

Candidate Biomarker List (100-10s)

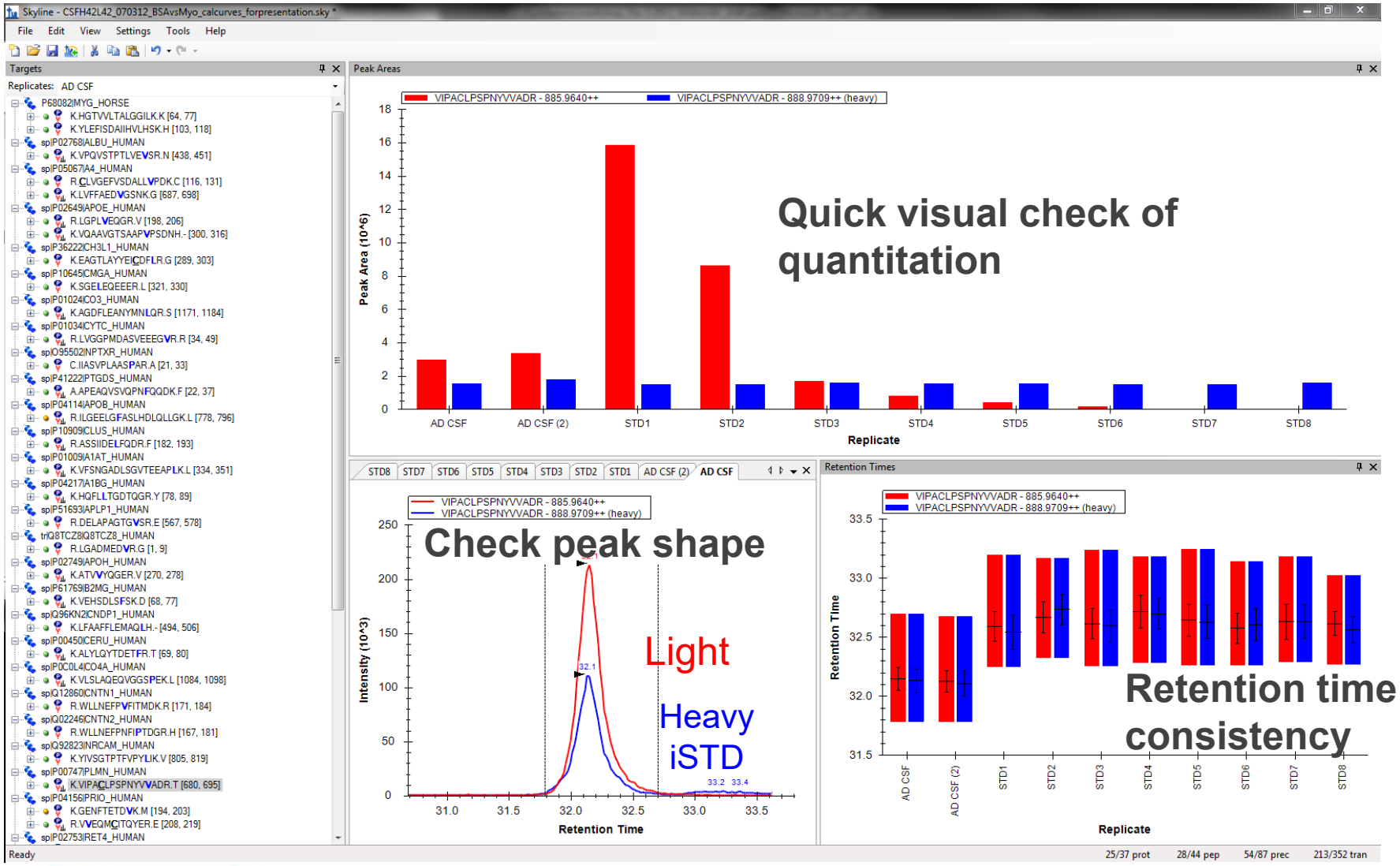
Targeted-MS assay or immunoassay

Clinical Marker(s)

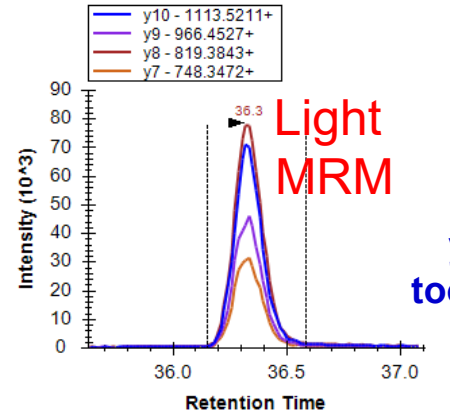
hypothesis-based focusing

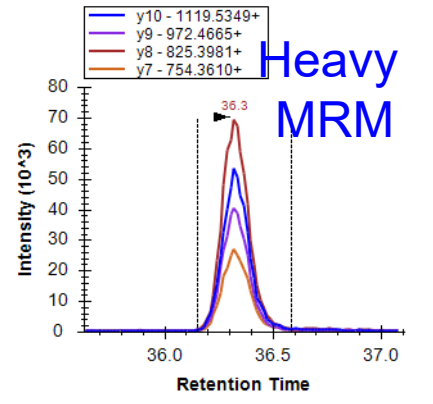
10's-100s of Biomarkers

Skyline enables MRM method development and data analysis



Monitor Matrix effects

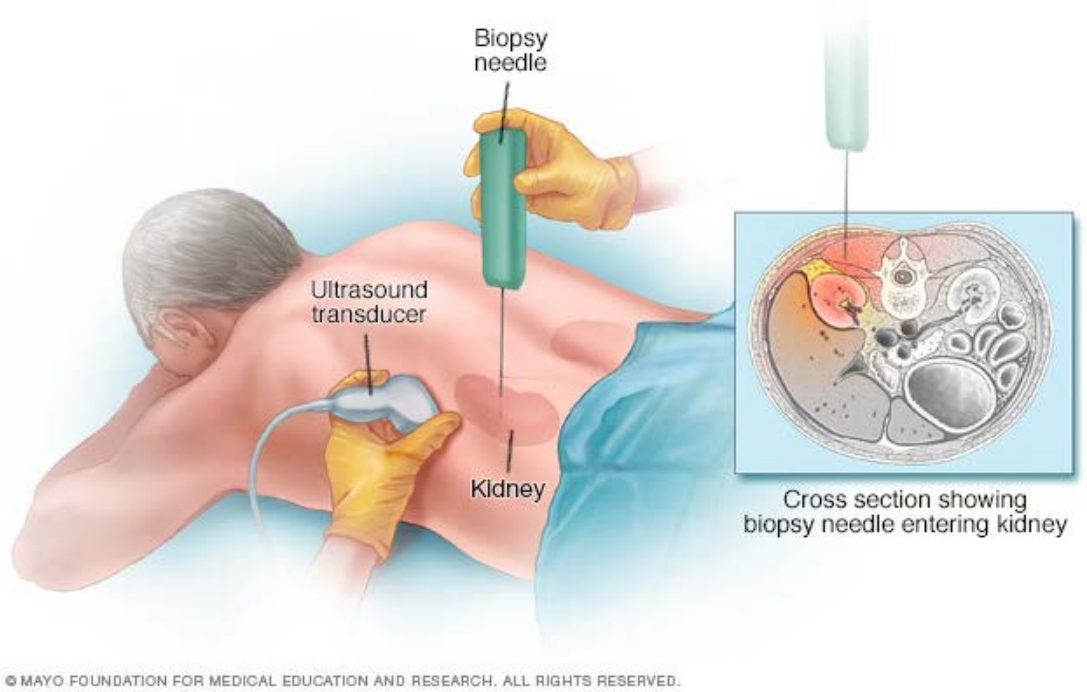




Genentech
A Member of the Roche Group

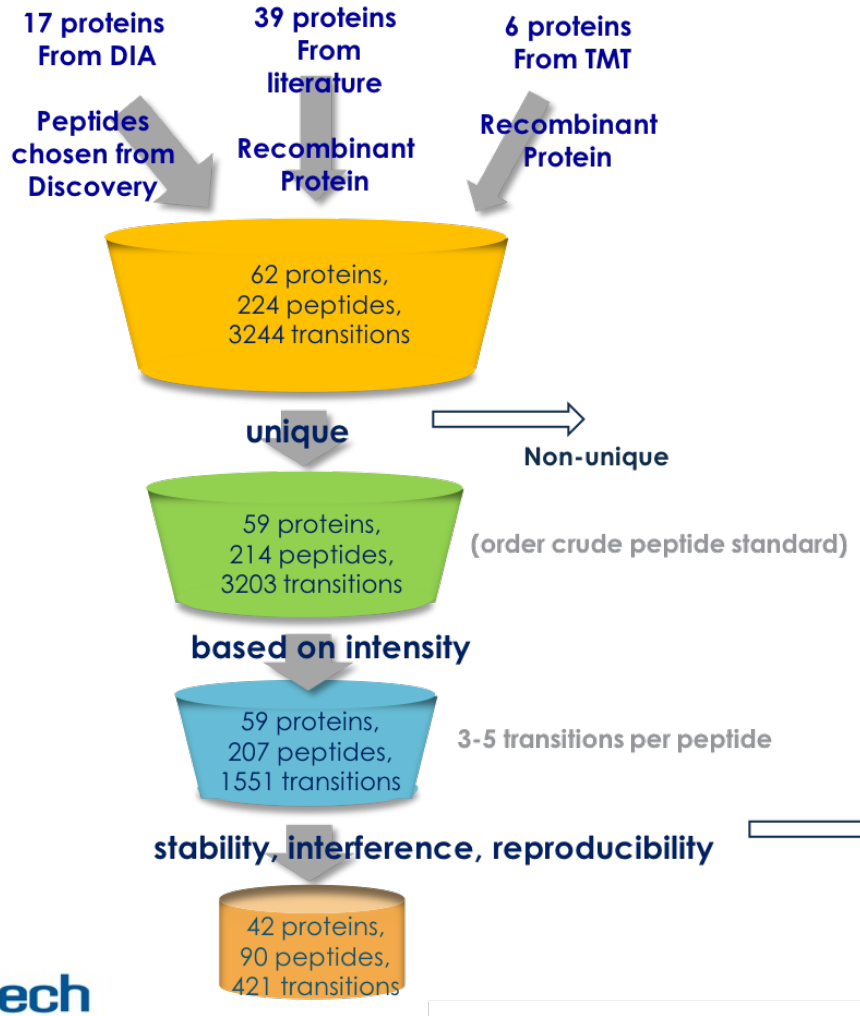
Development of an MRM to monitor disease activity in Lupus Nephritis

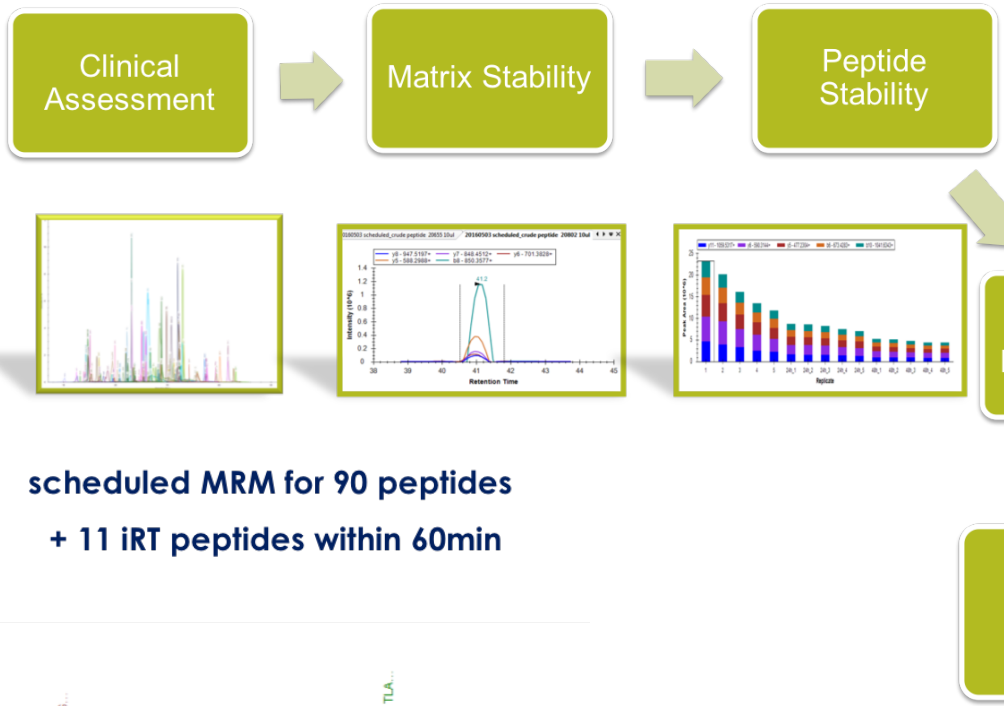
- Assessment of disease severity and activity requires a kidney biopsy, an invasive procedure with limited prognostic value

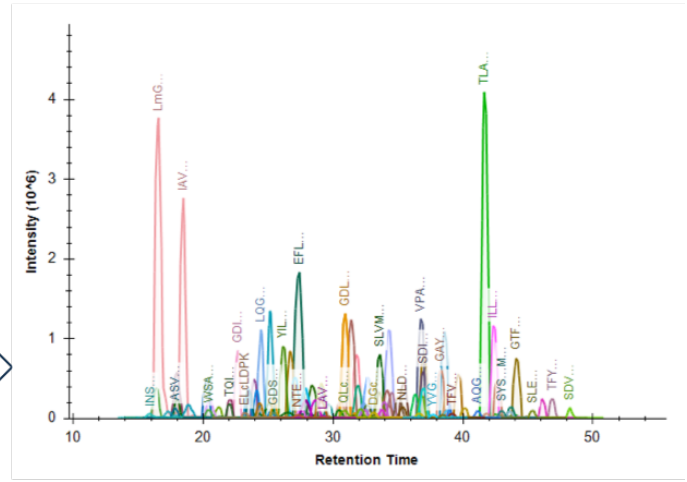


- A need remains for proximal, non-invasive biomarkers to help inform treatment decisions and to monitor disease activity and progression

Targeted MRM panel method development







Genentech
A Member of the Roche Group

Quantitative panel captures information from key inflammatory and disease-related pathways

Inflammation
Complement C3
Complement C5
IL-18
Osteopontin
PGDS
TNR1A
S100-A8
S100-A9
S100-A12
Annexin A2

Lymphoid
VCAM-1
ICAM-1
IgJ
IgG4 HC
IgG1 HC

Myeloid
IL-10
CSF-1
MCP-1
NGAL
CXCL16
DNaseI

Renal Disease
Collagen III
EGF
Vimentin
Adiponectin
EPCR
Hepcidin
Podocin
TINAL
Calreticulin

GBM Integrity
Carboxypeptidase N
a-1-acid glycoprotein 1
a-1-antitrypsin
a-2-macroglobulin
Haptoglobin
Afamin
Carbonic anhydrase-1
Serotransferrin
Ceruloplasmin

* indicates candidate came from proteomics experiments

Development and analysis of a novel targeted proteomics urinary biomarker panel in lupus nephritis

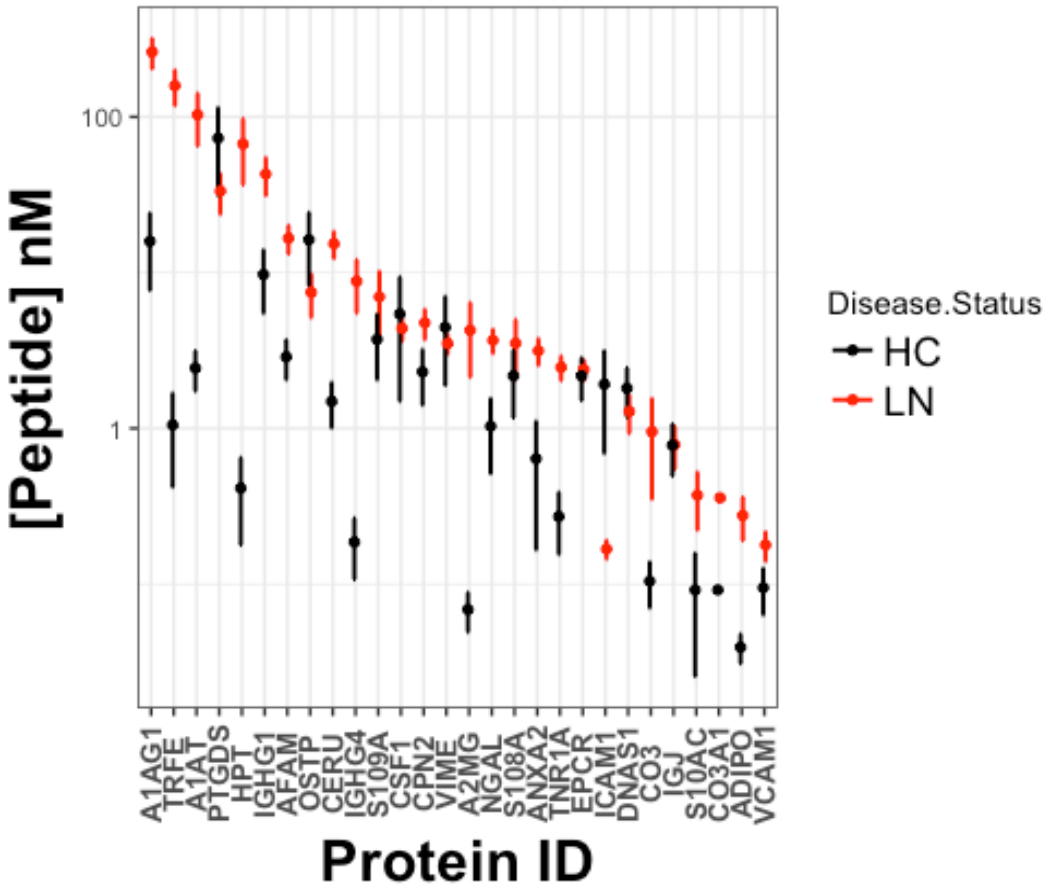
Summary:

We developed and assessed robustness of a multiplexed MRM method to monitor peptides from 42 urinary proteins

The majority of peptides are increased in LN urine compared to HC including lymphoid and inflammatory markers

MRM panels can be used to interrogate disease biology for 10-100 protein biomarker candidates in one assay

Ongoing initiatives in our group include comparisons to DIA methods which would expand biomarker candidate capacity into the 1000s and dramatically reduce method development time



Genentech
A Member of the Roche Group

Genentech

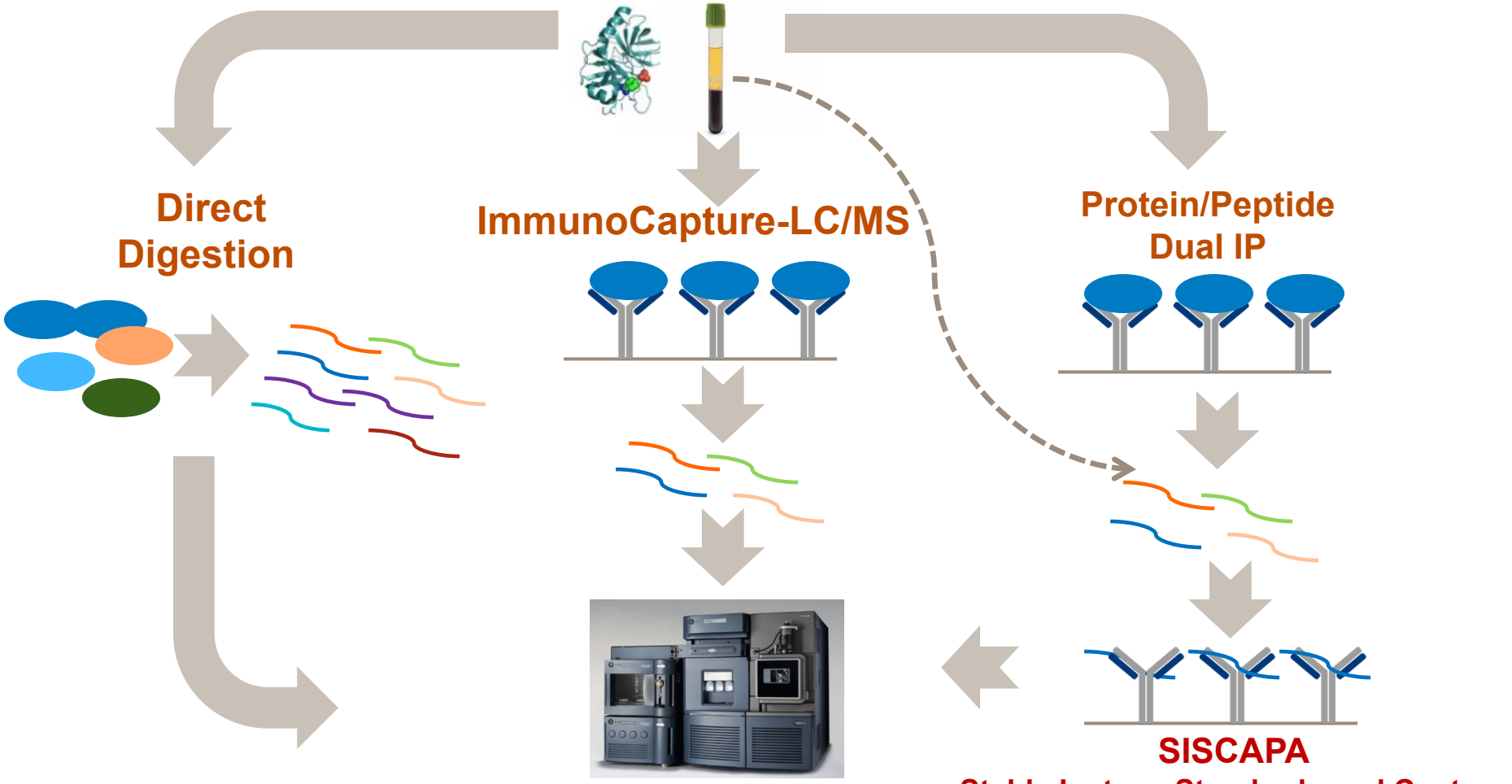
A Member of the Roche Group

Protein Biomarkers Assays with LC/MS: Reagent Selection and Method Optimization Strategies

Tim Sikorski, Ph.D.

GlaxoSmithKline
Bioanalysis, Immunogenicity & Biomarkers
IVIVT

Targeted Protein Analysis Workflows: Choosing The Right Fit

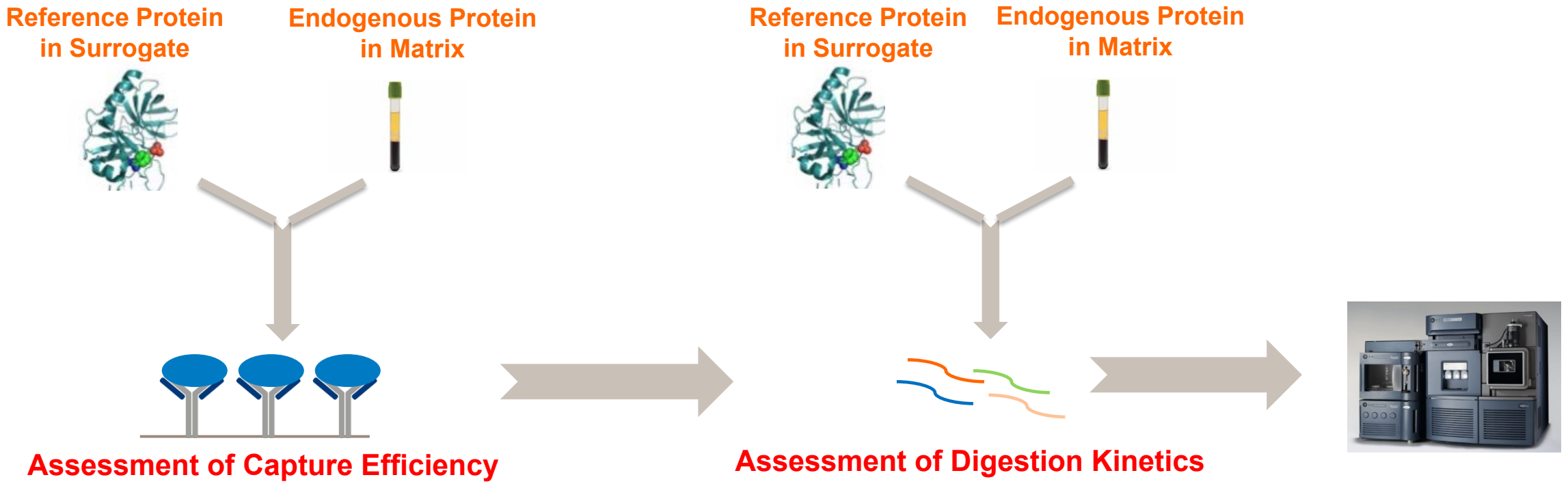


Increasing Sensitivity

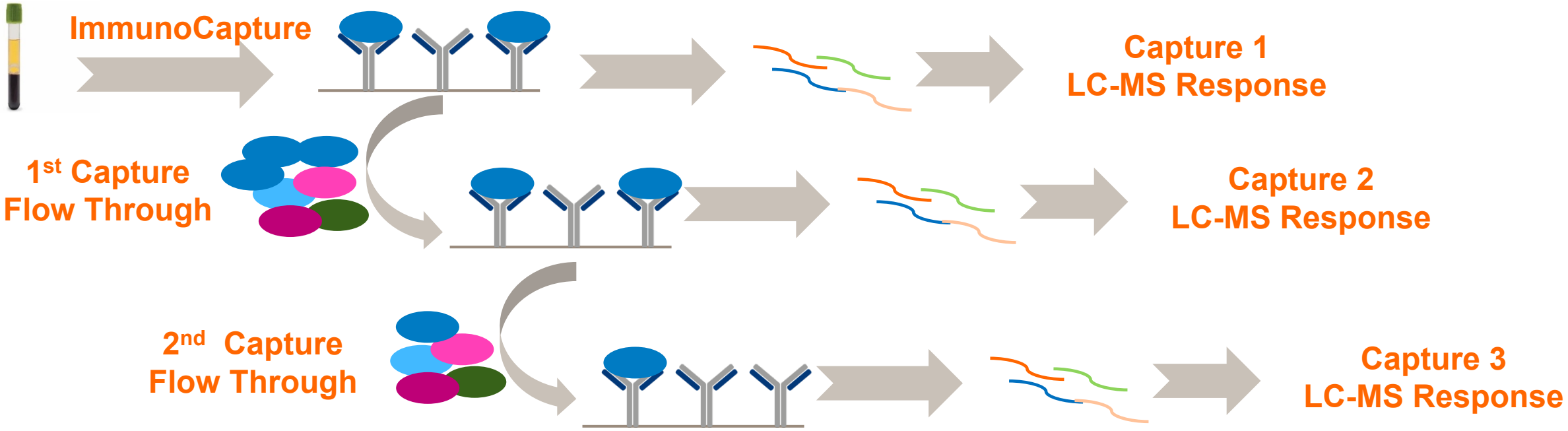
Increasing Assay Complexity and Analysis Time, and Cost

How Well Does the Reference Standard Represent the Endogenous Analyte?

- **Reference Standards for Protein Biomarkers**
  - Single Isoform
  - Post Translational Modifications dependent on enzymes in production host cell
  - Protein folding state may be dependent on chaperones in production host cell
  - Usually produced as a single protein rather than a protein complex

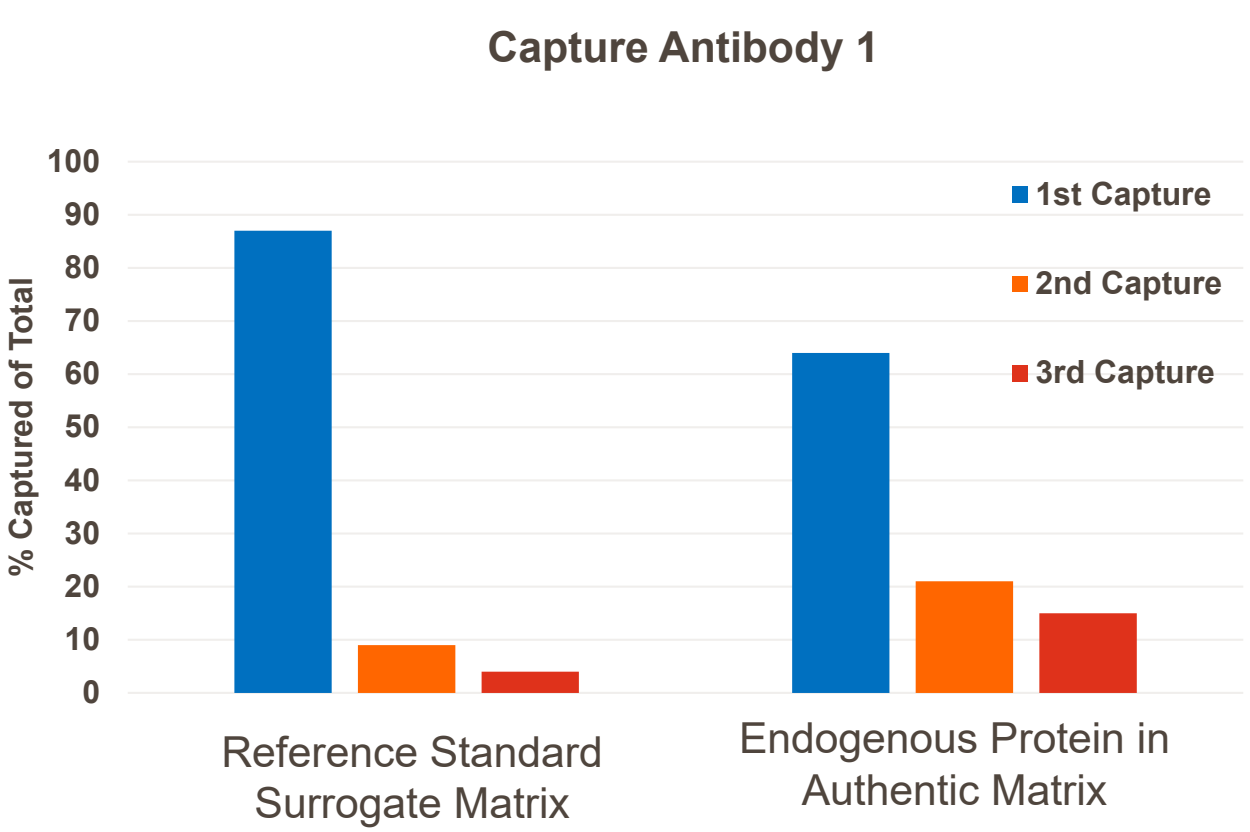


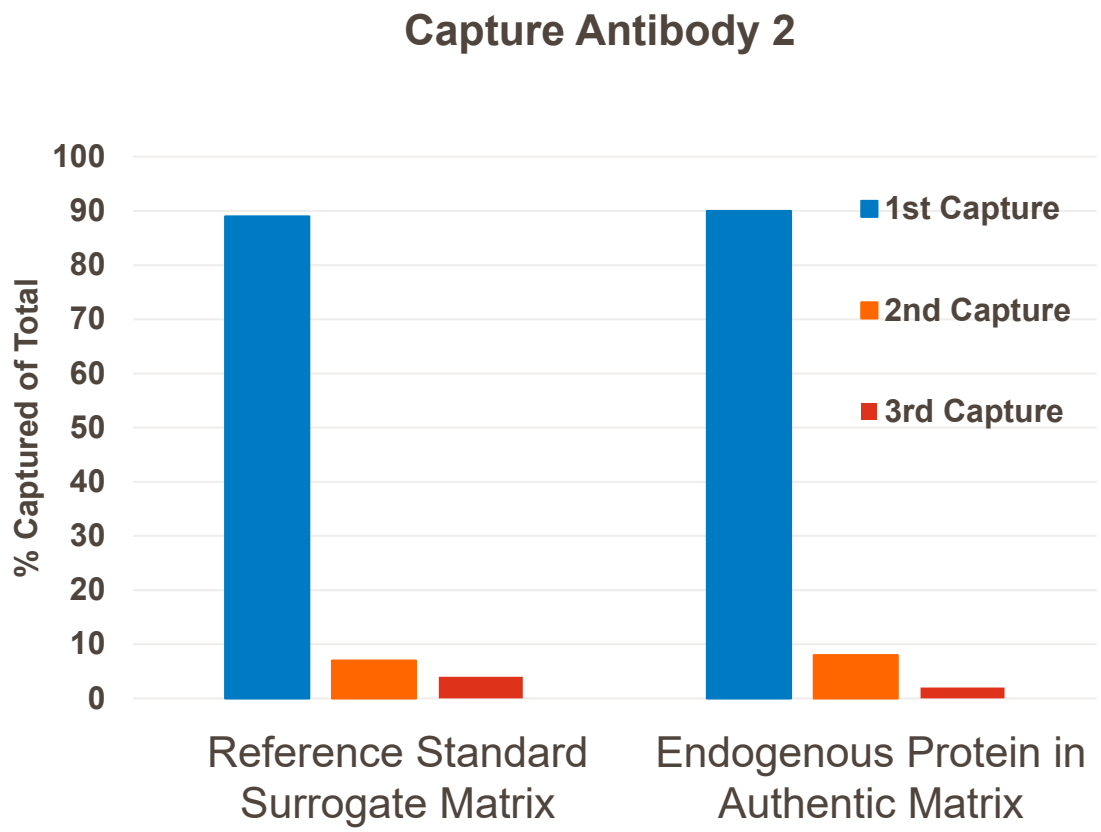
Sequential Enrichment Strategy to Assess Antibody Capture Efficiency



Important: Complete with both Reference Standard in Surrogate Matrix and Endogenous Analyte in Authentic Matrix Samples

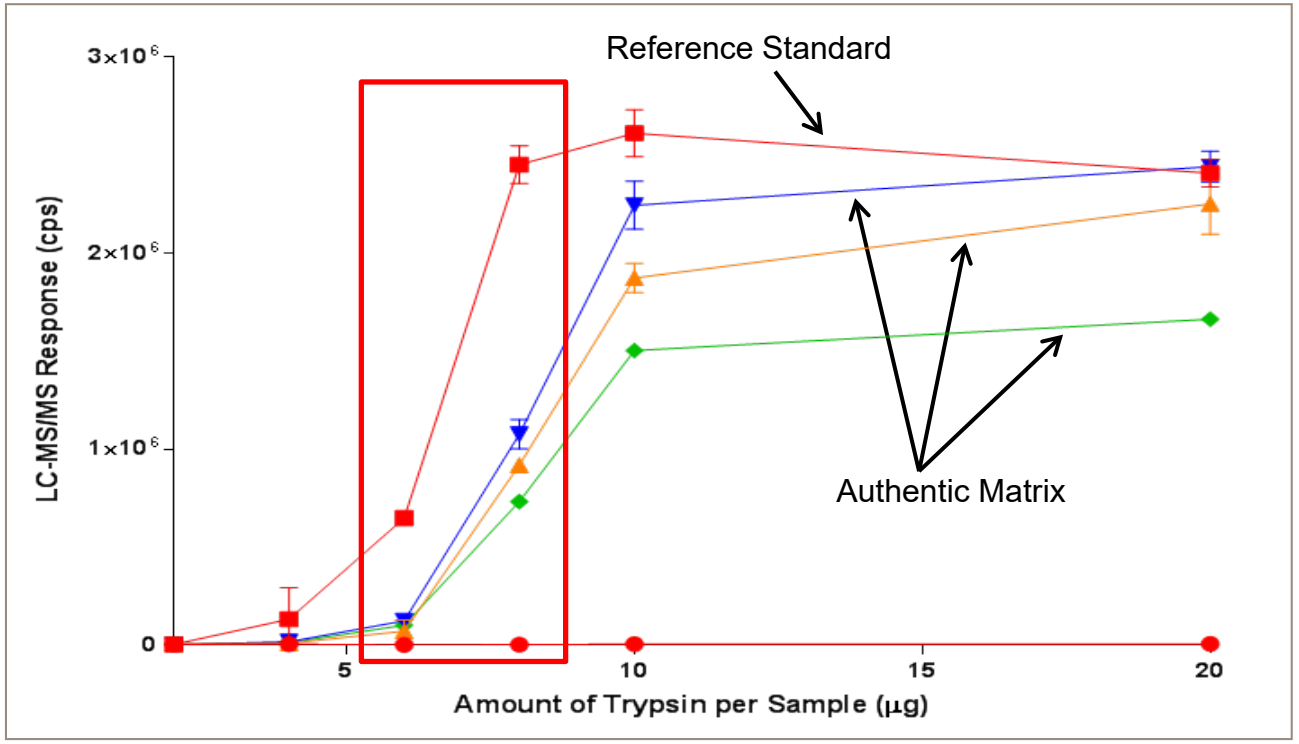
Immunocapture Differences of Reference Standard and Endogenous Proteins





- **Sequential immunocapture reveals Capture Antibody 1 suffers incomplete capture of authentic samples**
- **Using Capture Antibody 1 in assay could lead to inaccurate quantification**

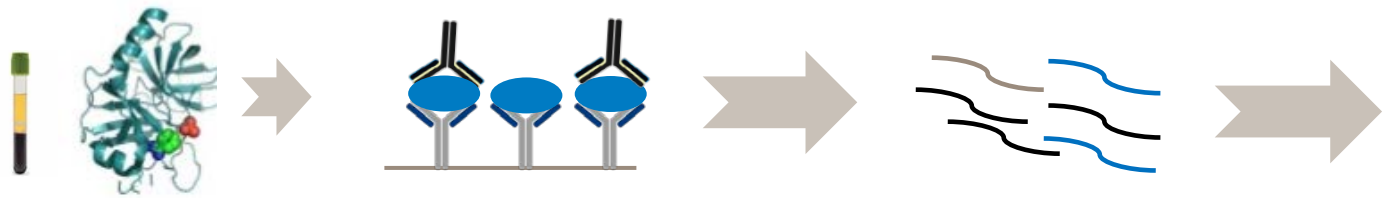
Digestion Efficiency Differences of Reference Standard and Endogenous Protein



- Endogenous protein more resistant to digestion than reference standard
- Need to optimize digestion conditions for both reference standard and endogenous protein

Biomarker Targets in the Presence of Biopharmaceuticals: The Right Assay for the Right Biological Question

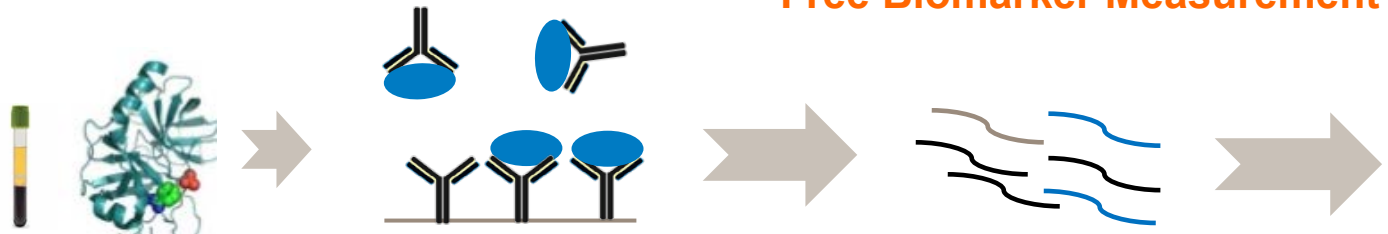
Total Biomarker Measurement



Capture at Different Epitope From Drug

Measure Biomarker Surrogate Peptide(s)

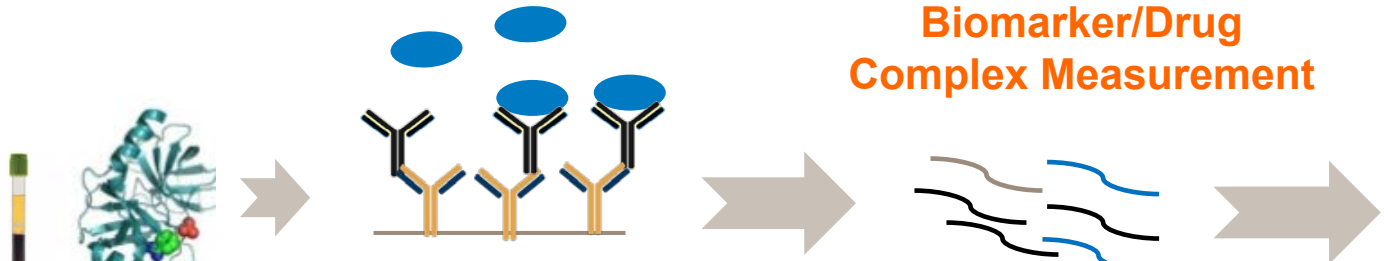
Free Biomarker Measurement



Capture with Drug

Measure Biomarker Surrogate Peptide(s)

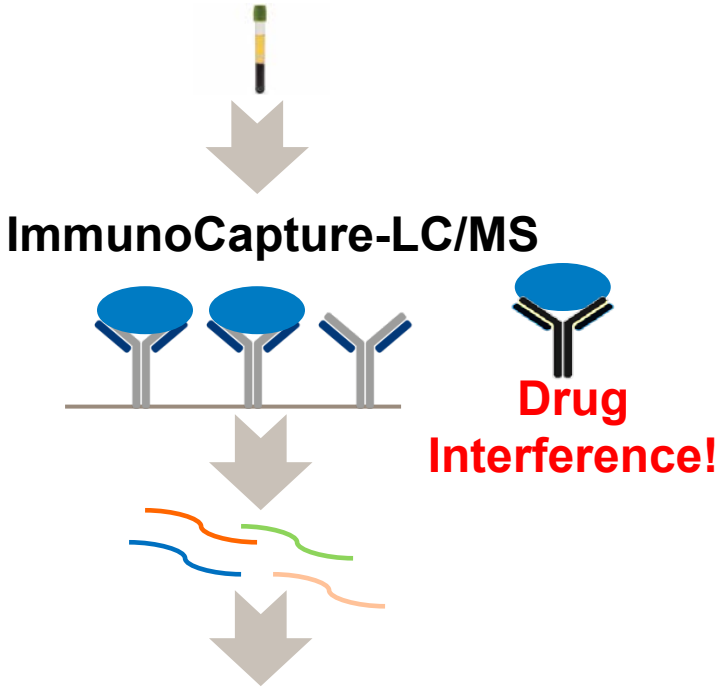
Biomarker/Drug Complex Measurement

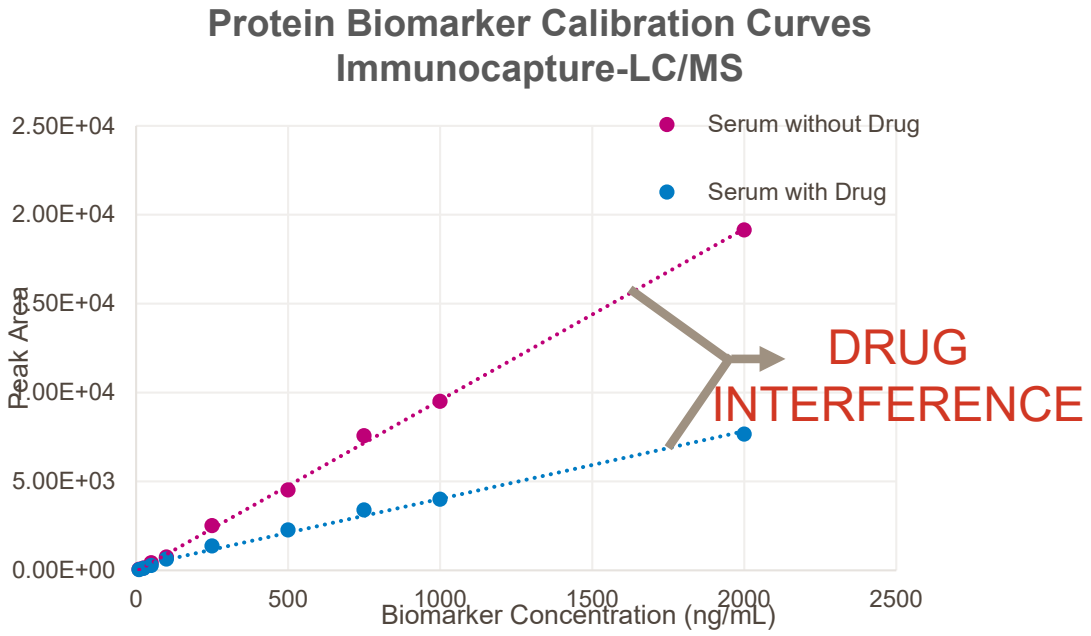


Capture Drug with Anti-Human Fc or Anti-idiotypic Antibody

Measure Biomarker Surrogate Peptide(s)

ADC Drug Interference Precludes Total Protein Biomarker Analysis of Dosed Patient Samples With Hybrid LBA/LCMS Assay

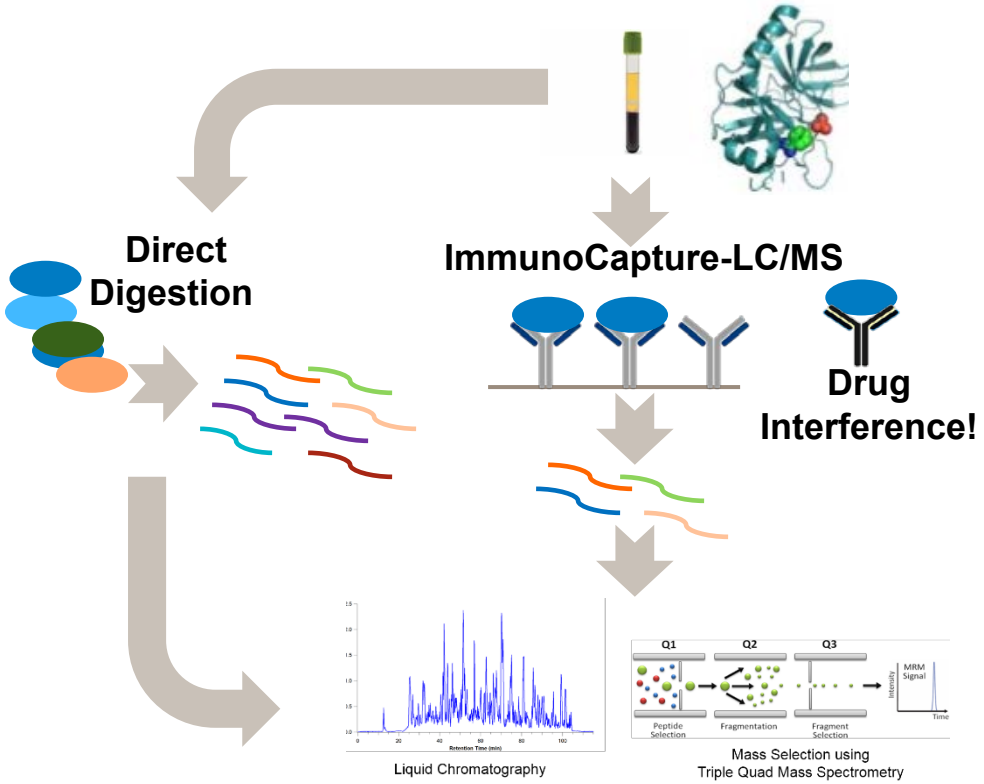


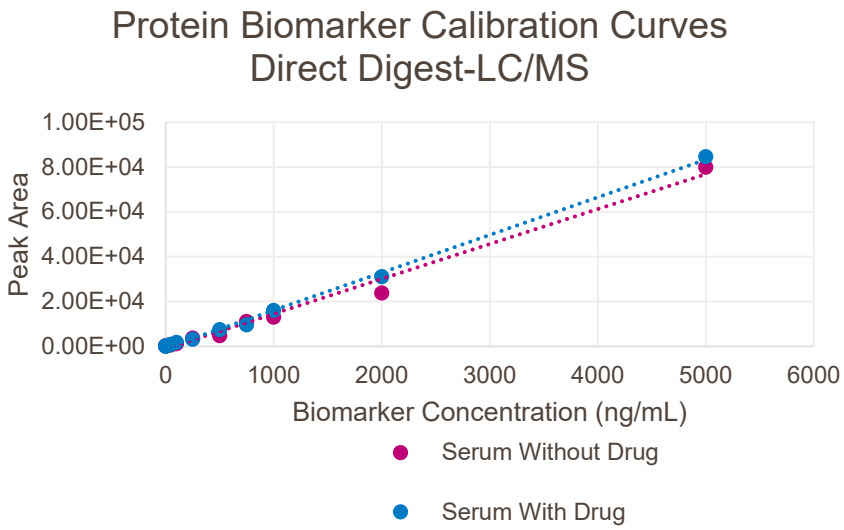


Serum spiked with Antibody Drug Conjugate at Expected Cmax
Assay Range: 10 ng/mL- 2000 ng/mL

- **Small biomarker protein (~6 kD)**
- **All available immunocapture reagents suffer from drug interference, confounding total protein quantification during dosing**

Overcoming Biopharmaceutical Drug Interference: Direct Digest or Custom Peptide Capture





Serum spiked with Antibody Drug Conjugate at Expected Cmax
Assay Range: 10 ng/mL- 2000 ng/mL

- **Antibody free based LC-MS methods overcome problems of drug interference for total biomarker assays**
  - **A growing need to employ proteomic technologies to reach sensitivity and selectivity requirements to measure protein biomarkers without specific enrichment**

Immunoaffinity-LCMS Strategies for Protein Biomarkers

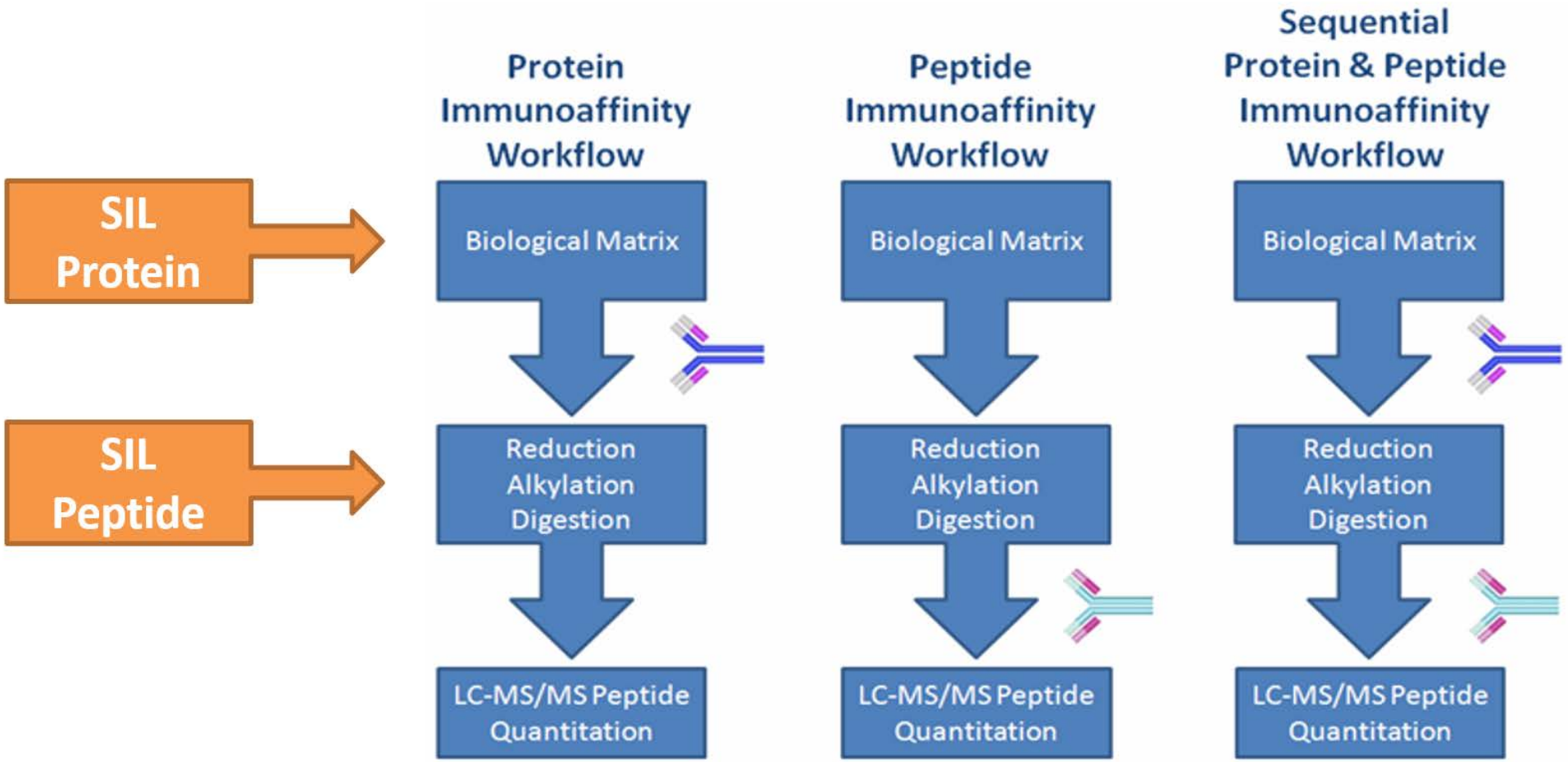
Hendrik Neubert PhD
BioMedicine Design; Pfizer

4 June 2019

Pfizer WORLDWIDE RESEARCH & DEVELOPMENT

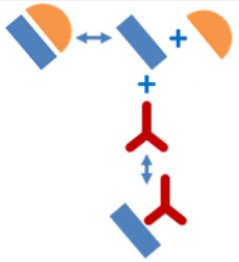
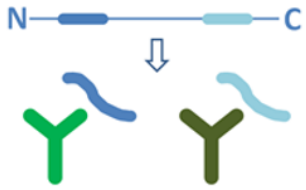
SCIENCE FOR LIFE-CHANGING IMPACT

Modes of Immunoaffinity LC-MS/MS



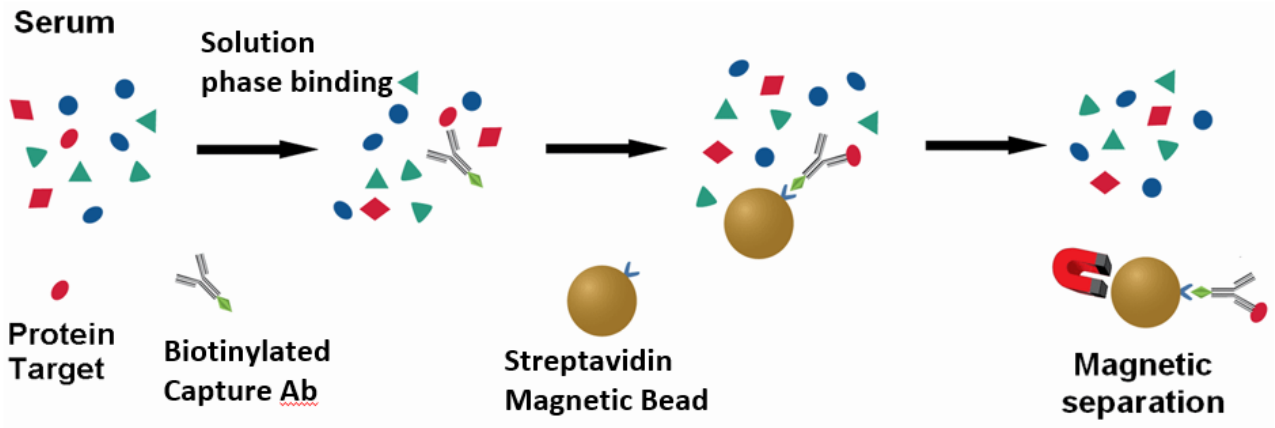
Current and Emerging IA-MS Applications

- New measurements previously unattainable
- Unique and creative workflows (tissue, membrane proteins, PTMs, occupancy)

	Biofluids: Soluble protein biomarkers	**Tissues:** All proteins including membrane and structural protein biomarkers			
Anti-protein	 Anti-protein	 Capture reagent directed against constant, unchanged region common to full length, truncated , splice variant or PTM containing form of protein	 Non-competing with binding protein, or biotherapeutic	 Competing with binding protein (endogenous binding partner or biotherapeutic)	 Co-immunoaffinity
Anti-peptide	 Anti-peptide	 Multiplexed anti-peptide IA capture	 bind multiple forms of a peptide including unmodified, sequence variants, neoepitopes, PTMs, or with covalent modifier	 anti-motif anti-peptide antibody	

Protein and Peptide Immunoaffinity Workflows

Offline Bead or Tip based **PROTEIN/PEPTIDE** Immunoaffinity Enrichment

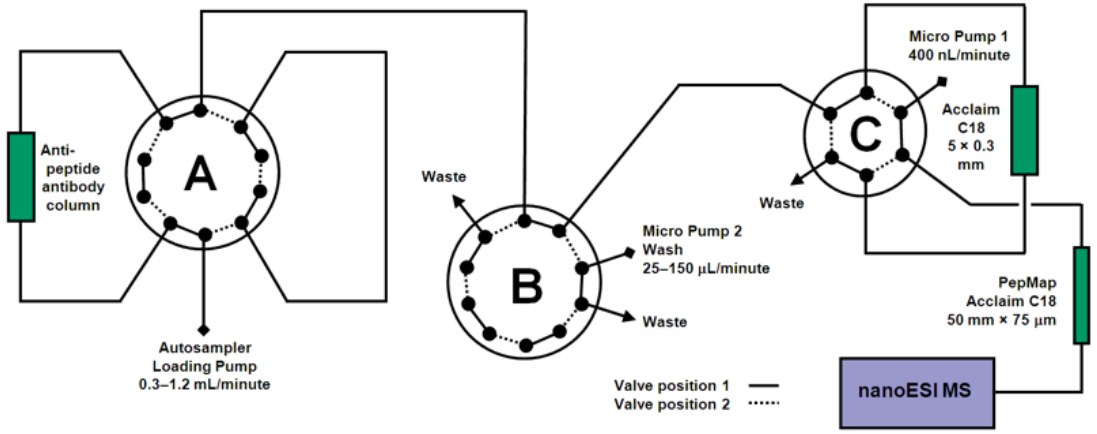


Robotic Liquid / Magnetic bead handling in 96w plates

Tip based IA methods

(1) Fernández Ocaña, Anal. Chem.; 2012;84(14):5959; **(2)** Neubert, Anal. Chem.; 2013;85(3):1719

Online Flow based **PEPTIDE** Immunoaffinity Enrichment



NanoSpray MRM or HRMS

(1) Neubert, Clin. Chem.; 2010;56(9):1413; **(2)** Fan, Anal. Chem.; 2016,88(8):4239

Novel In-Sample Calibration Curve Approach for Quantitative LC-MS/MS Bioanalysis of Protein Biomarkers

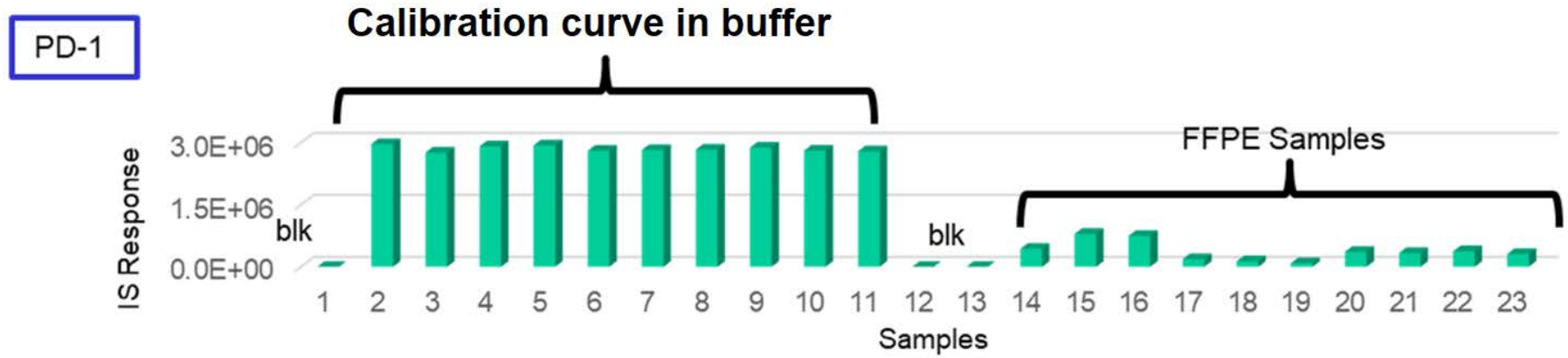
Huidong Gu
Bioanalytical Sciences
Bristol-Myers Squibb
Princeton, NJ

Protein Biomarkers Method Development & Validation by LCMS, HRMS and Hybrid LBA/LCMS: Recent Advancements
2019 ASMS RBIG Workshop
5:45-7:00 pm, Tuesday, June 4th, 2019

Bristol-Myers Squibb

Background

- Clinical protein biomarker measurement: tissue biopsy, formalin-fixed paraffin-embedded (FFPE) tissue and exosome samples
- No authentic matrices available
- External calibration curves in buffer
- Example: LC-MS/MS measurement of PD-1 and PD-L1 in FFPE tissues using anti-peptide immuno-capture



- Due to the severe matrix effect (or capture efficiency differences) in FFPE samples, the actual LLOQ in FFPE samples could be much higher than the LLOQ achieved in buffer
- Compromised accuracy and precision with external calibration curves in buffer
- Authentic matrix is not available (not possible)

Current solutions

AQUA (or PSAQ)

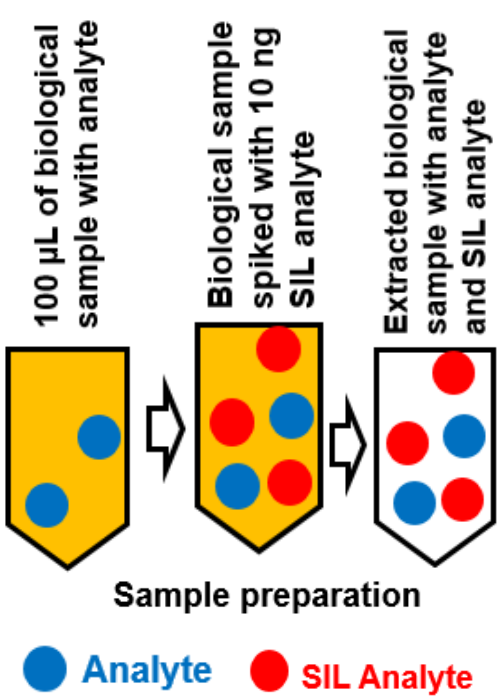
- Spike a known amount of a stable isotopically labeled (SIL) analyte into each study sample
- Widely used in quantitative proteomics
- One calibration point
- Not accurate for quantitative biomarker measurement

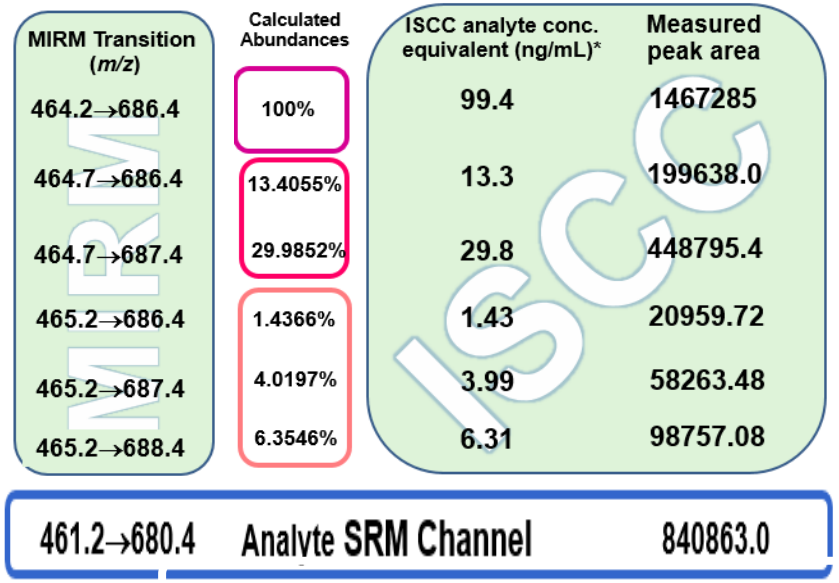
Calibration curve in study samples

- Pre-prepare a full range calibration curve using multiple differently SIL-analytes at different concentrations
- Spike the pre-prepared calibration curve into each study samples
- Accurate
- Multiple SIL-analytes are needed
- Reported by many groups for analysis of small-molecule and protein biomarkers, and quantitative proteomics

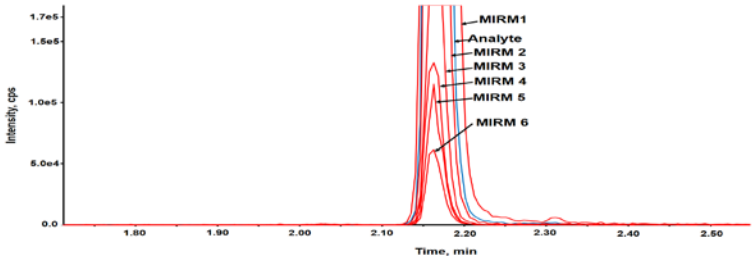
Our solution: MIRM-ISCC-LC-MS/MS Bioanalysis

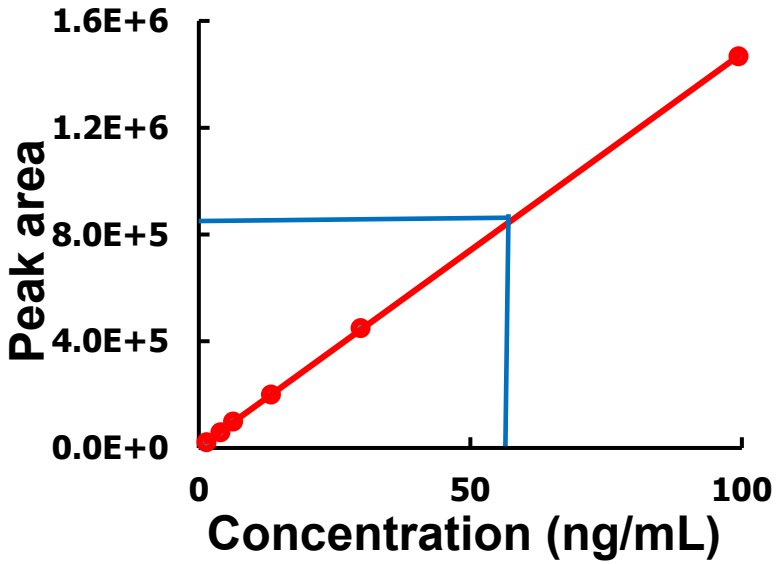
- **ISCC:** In-Sample Calibration Curve
- **MIRM:** Multiple Isotopologue Reaction Monitoring
- Spike a known amount of a SIL analyte into each study sample
- A full range multiple calibration points ISCC is constructed by MIRM of the SIL analyte in each study sample as the isotopic abundances in each MIRM channel of the SIL analyte can be calculated and measured accurately
- MIRM-ISCC-LC-MS/MS workflow:





MIRM-ISCC-LC-MS/MS Analysis





Bristol-Myers Squibb

MIRM-ISCC-LC-MS/MS Bioanalysis: Advantages and Applications

Advantages:

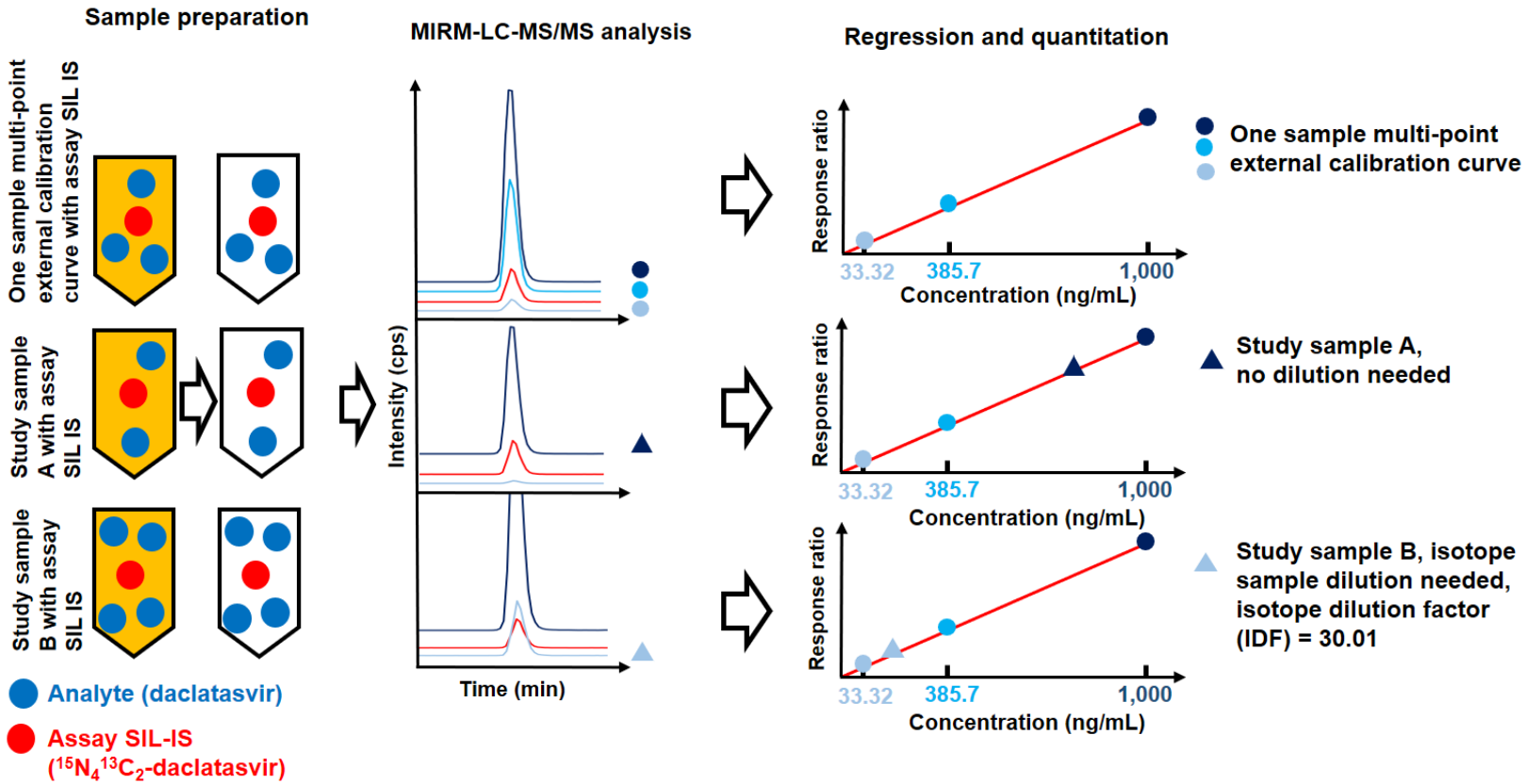
- Accurate full range ISCC constructed by using MIRM of one SIL-analyte
- Calibration curve in actual study sample
- No authentic matrices needed
- No calibration curve preparation needed
- No additional assay internal standard needed
- Matrix effect, dilution linearity and parallelism etc. are no longer issues
- Greatly reduces instrument time

Applications:

The methodology has wide application potential and it is particularly useful for cases

- where authentic matrices are not available (Biomarker measurement)
- where low throughput and long turnaround time are the main issues preventing the use of LC-MS/MS technique (Clinical diagnostic laboratories)
- where external calibration curve preparation is cumbersome (Fresh frozen and FFPE samples, as well as DBS samples etc.)
- some quantitative proteomics applications currently using isotope labeling approach, such as tandem mass tag (TMT), and the throughput is limited by the number of reagents with different labels

Eliminating Multi-Sample External Calibration Curve and Dilution of Study Sample using MIRM of an Analyte



Advantages:

- ❑ Improved assay robustness and throughput with no change on traditional external calibration curve workflow
- ❑ Especially useful for cases where physical sample dilution is difficult or impossible
- ❑ Automated sample preparation without the needs of calibration curve preparation and study sample dilution

References

1. **MIRM-ISCC-LC-MS/MS methodology:**

   Huidong Gu, Yue Zhao, Marissa DeMichele, Naiyu Zheng, Yan J. Zhang, Renuka Pillutla and Jianing Zeng, “In-Sample Calibration Curve Using Multiple Isotopologue Reaction Monitoring of a Stable Isotopically Labeled Analyte for Instant LC-MS/MS Bioanalysis and Quantitative Proteomics”, **Analytical Chemistry, 91(2019)2536-2543, and ASMS 2019, Poster TP 072**

2. **Application of MIRM-ISCC-LC-MS/MS methodology in biomarker measurement for FFPE tissues:**

   Naiyu Zheng, Kristin Taylor, Huidong Gu, Rasa Santockyte, Xi-Tao Wang, Yan J. Zhang, Renuka Pillutla and Jianing Zeng, “Evaluation on LC-MS/MS Assay Using Anti-Peptide Immunocapture to Quantify PD-1 and PD-L1 as Clinical Biomarkers in FFPE Tissues for Immuno-Therapy Development”, **ASMS 2019, Poster TP 069**

3. **Calculation of isotopic abundances in MIRM channels:**

   Huidong Gu, Jian Wang, Anne Aubry, Hao Jiang, Jianing Zeng, John Easter, Junsheng Wang, Randy Dockens, Marc Bifano, Richard Burrell and Mark Arnold, “Calculation and Mitigation of Isotopic Interferences in Liquid Chromatography-Mass Spectrometry/Mass Spectrometry Assays and Its Application in Supporting Microdose Absolute Bioavailability Studies, **Analytical Chemistry, 84(2012)4844-4850**

4. **Eliminating preparation of multi-sample external calibration curves and dilution of study samples in LC-MS/MS analysis :**

   Huidong Gu, Yue Zhao, Marissa DeMichele, Naiyu Zheng, Yan J. Zhang, Renuka Pillutla and Jianing Zeng, “Eliminating Multi-Sample External Calibration Curve and Dilution of Study Sample using MIRM Technique in Quantitative LC-MS/MS Bioanalysis”, **Analytical Chemistry,** Just Accepted (June 10, 2019).

Acknowledgement

Yue Zhao

Marissa DeMichele

Naiyu Zheng

Kristin Taylor

Craig Titsch

Jian Wang

Jianing Zeng

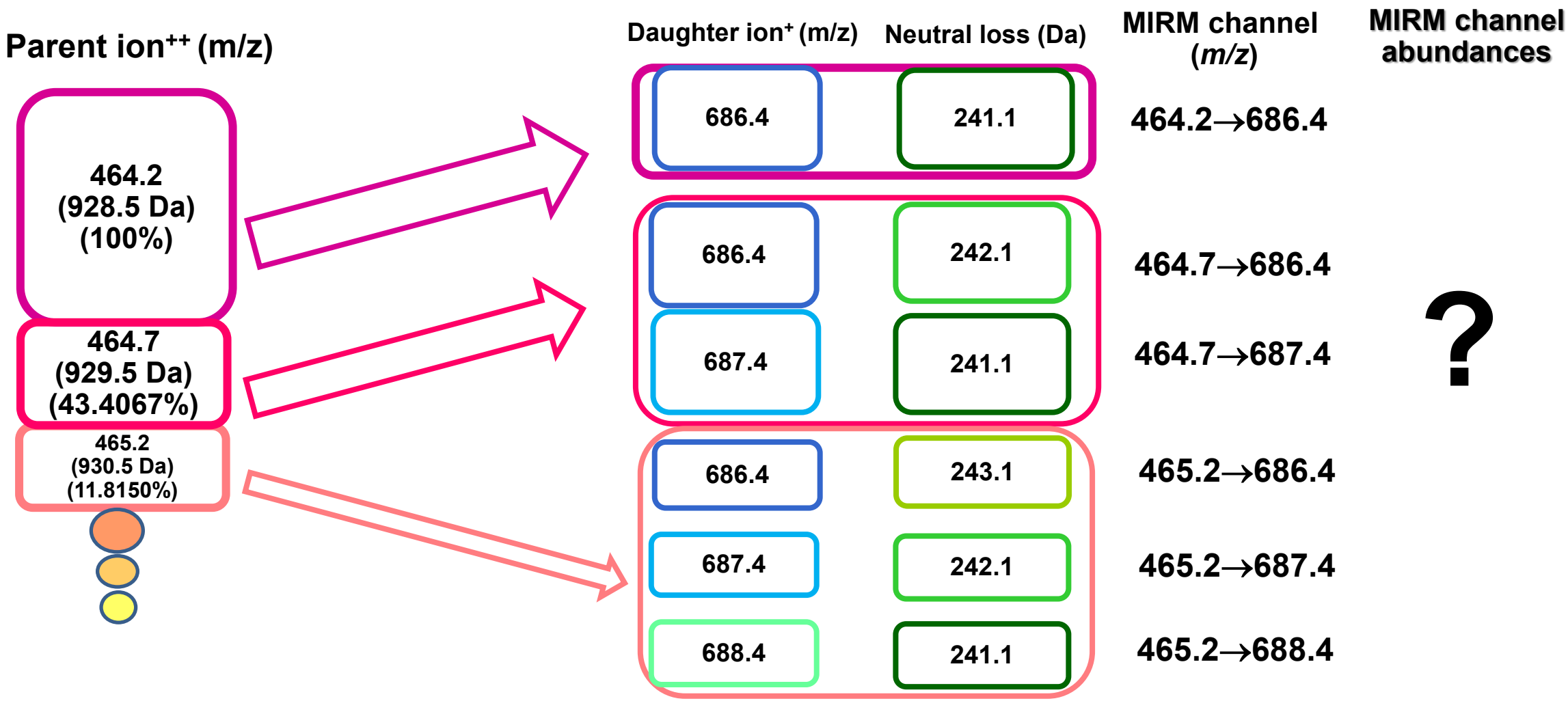
Yan J Zhang

Renuka Pillutla

Backup slides

Calculation of isotopic abundances in MIRM channels

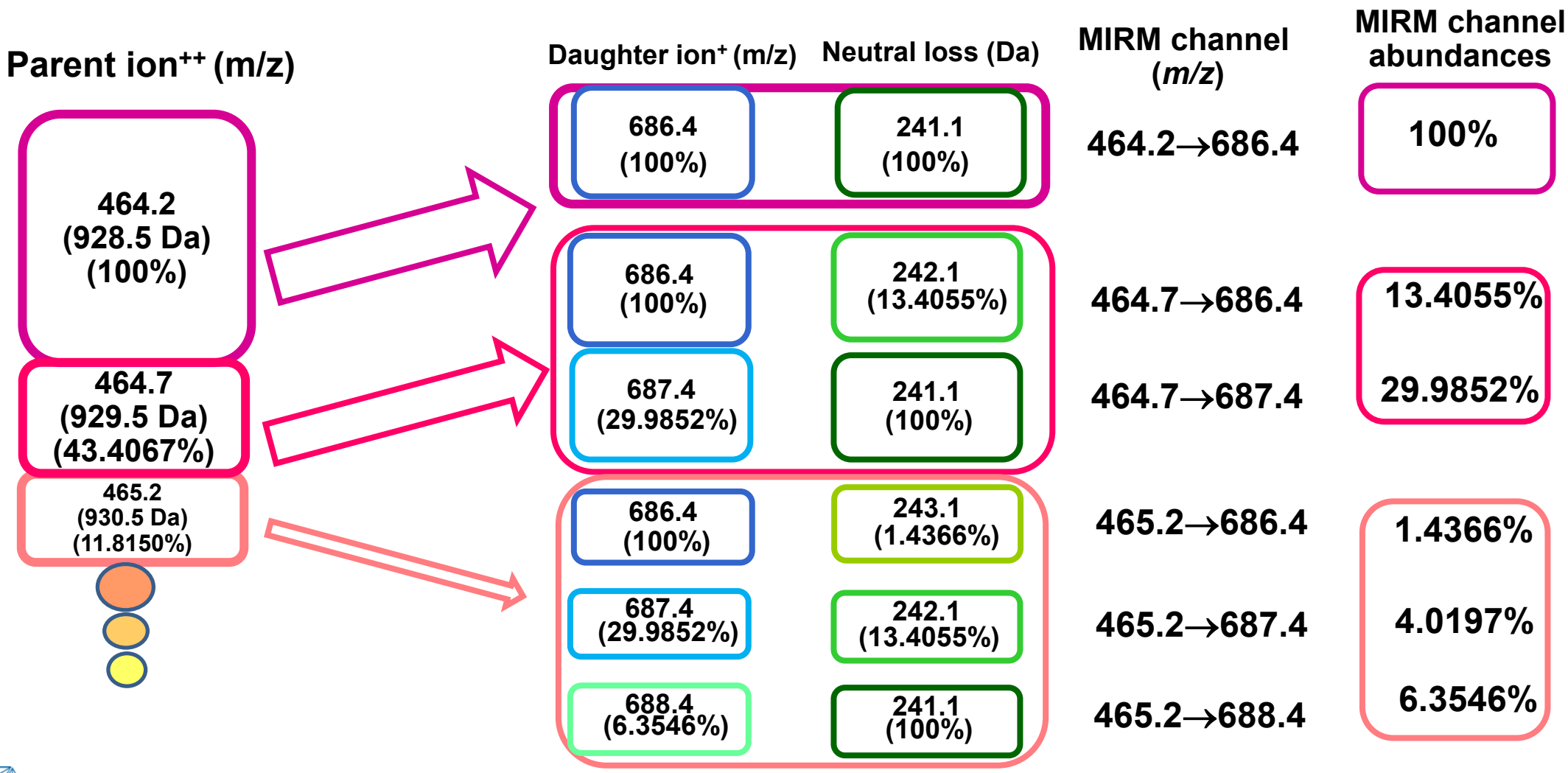
PD-L1 fragmentation: LQDAG[Val($^{13}C_5$, ^{15}N)]YR^{++} → DAG[Val($^{13}C_5$, ^{15}N)]YR^{+} ($^{13}C_5{}^{15}NC_{35}H_{66}N_{11}O_{13}$) → ($^{13}C_5{}^{15}NC_{24}H_{46}N_8O_{10}$)



Isotopic distributions for neutral loss ($C_{11}H_{19}N_3O_3$) and daughter ion([[$^{13}C5^{15}NC_{24}H_{46}N_8O_{10}]^+$) for stable isotopically labeled peptide LQDAG[Val($^{13}C_5$, ^{15}N)]YR

Mass shift for neutral loss – additional neutrons(α-β)	Lost in collision cell (neutral loss)		Mass shift for daughter ion – additional neutrons: β	Daughter ion (y6 ion)	
	$C_{11}H_{19}N_3O_3$			$[^{13}C5^{15}NC_{24}H_{46}N_8O_{10}]^+$	
	Mass (m/z)	Abundance (%)		Mass	Abundance (%)
0	241.1	100	0	686.4	100
1	242.1	13.4055	1	687.4	29.9852
2	243.1	1.4336	2	688.4	6.3546
3	244.1	0.112	3	689.4	1.007
4	245.1	0.0069	4	690.4	0.1322
5	246.1	0.0003	5	691.4	0.0146
			6	692.4	0.0012

Calculated isotopic abundances in MIRM channels of SIL-LQDAG[Val($^{13}C_5$, ^{15}N)]YR



Measured isotopic abundances in MIRM channels of SIL-LQDAG[Val($^{13}C_5$, ^{15}N)]YR

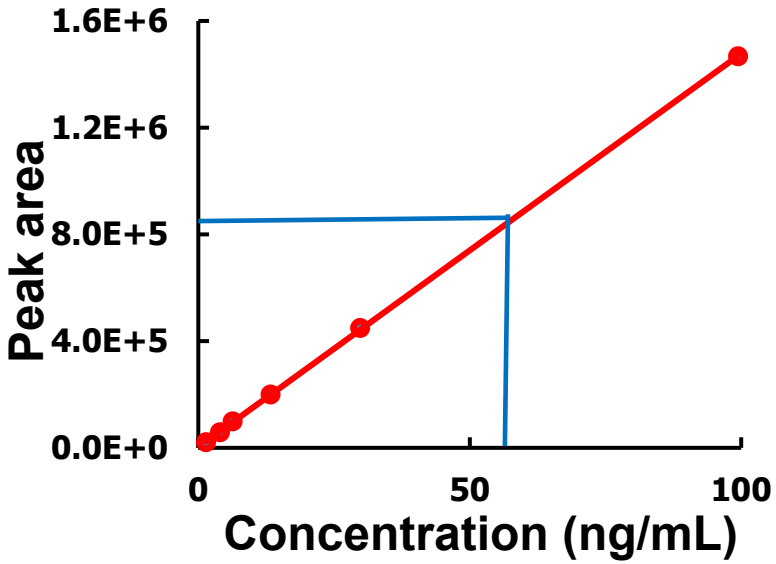
Daughter ion$^+$ (m/z)	Neutral loss (Da)	MIRM channel (*m/z*)	Calculated abundances	Measured Abundances	Dev%
686.4 (100%)	241.1 (100%)	464.2→686.4	100%	100%	
686.4 (100%)	242.1 (13.4055%)	464.7→686.4	13.4055%	13.6059%	1.5
687.4 (29.9852%)	241.1 (100%)	464.7→687.4	29.9852%	30.5868%	2.0
686.4 (100%)	243.1 (1.4366%)	465.2→686.4	1.4366%	1.4285%	-0.8
687.4 (29.9852%)	242.1 (13.4055%)	465.2→687.4	4.0197%	3.9708%	-1.2
688.4 (6.3546%)	241.1 (100%)	465.2→688.4	6.3546%	6.7306%	5.9

MIRM-ISCC-LC-MS/MS Bioanalysis of PD-L1

Additional neutrons on parent ion	MIRM Transition (*m/z*)	Calculated Abundances	ISCC analyte conc. equivalent (ng/mL)*	Measured peak area
0	464.2→686.4	100%	99.4	1467285
1	464.7→686.4	13.4055%	13.3	199638.0
1	464.7→687.4	29.9852%	29.8	448795.4
2	465.2→686.4	1.4366%	1.43	20959.72
2	465.2→687.4	4.0197%	3.99	58263.48
2	465.2→688.4	6.3546%	6.31	98757.08

MIRM ISCC

461.2→680.4	Analyte SRM Channel	840863.0



* Assume 10 ng of SIL-peptide was spiked into 100 μL of sample for analysis of the peptide, the equivalent conc. for the SIL peptide in the sample is 100 ng/mL, which can be further converted to analyte conc. equivalent = SIL-peptide concentration * (peptide molecular weight of 921 / SIL-peptide molecular weight of 927)