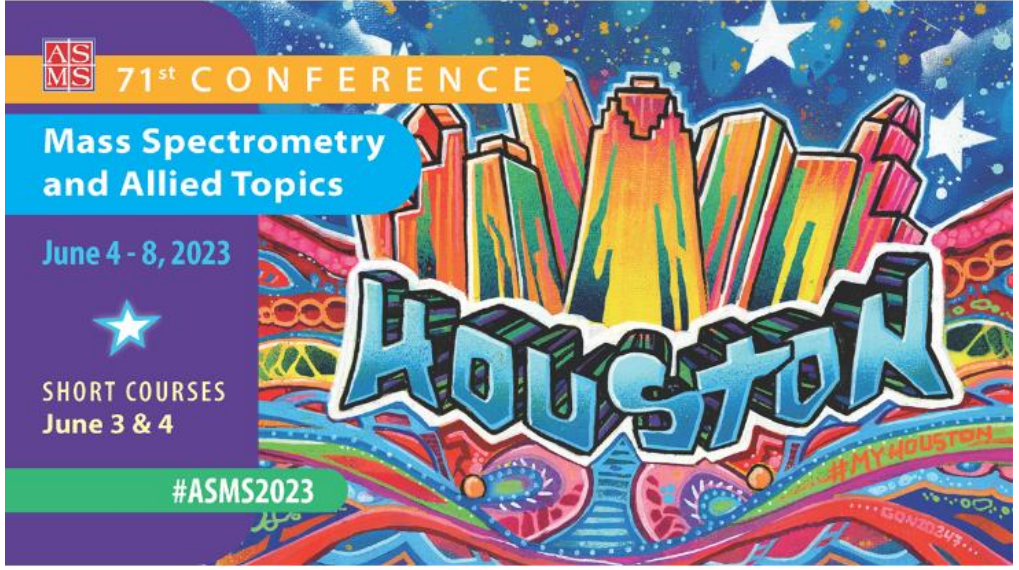


Biomarker Development: How Mass Spectrometry Is Changing the Field

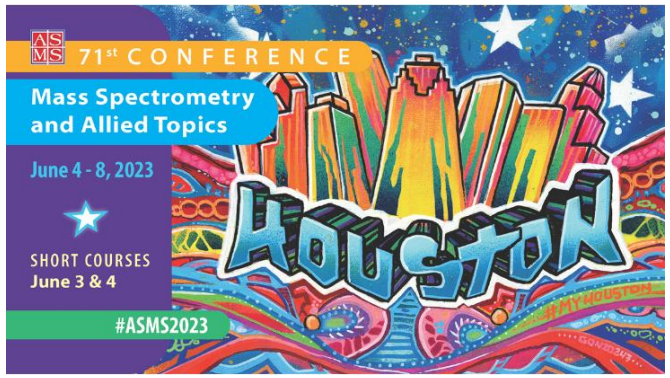
Regulated Bioanalysis Interest Group (RBIG)

5:45-7:00 pm, Wednesday, 07-June-2023

Presiding:
Wenkui Li (Novartis Institutes for BioMedical Research), Jian Wang (Crinetics Pharmaceuticals)

Panelists:
Long Geng, MS (Frontage Laboratories), Aiying Yu, Ph.D. (Genentech), Yifan Shi, Ph.D. (Janssen Research & Development), Yang (Thomas) Tang, Ph.D. (Genentech)

Agenda



05:45 - 05:50 pm – Introduction

*05:50 – 06:00 pm - **Using LC-MS for biomarker analysis: When? How? Why?***

- Long Geng, MS (Frontage Laboratories)

*06:00 – 06:10 pm - **Target occupancy in frozen and FFPE tissues using immunoaffinity capture 2D-LC-MS/MS***

- **Aiying Yu, Ph. D.** (Genentech)

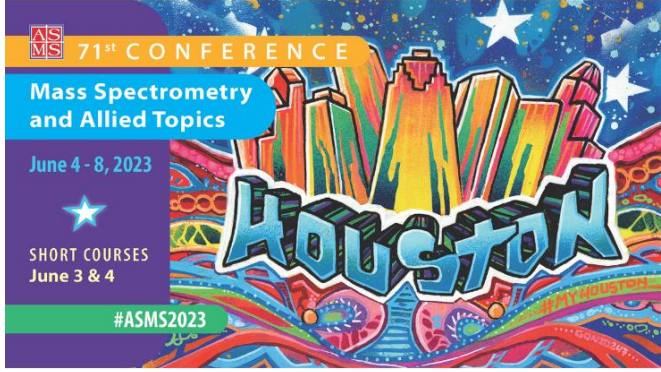
*06:10 – 06:20 pm -**Implementing antibody-free strategies for protein quantitation in monkey liver tissues using liquid chromatography coupled with mass spectrometry***

- Yifan Shi, Ph. D. (Janssen Research & Development)

*06:20 – 06:30 pm - **A universal surrogate matrix assay for urea measurement in clinical pharmacokinetic studies of respiratory diseases***

- Yang (Thomas) Tang, Ph.D. (Genentech)

06:30 – 7:00 pm - *Panel discussion*



Long Geng, M.S., Ph.D. Candidate

Lab Manager in Frontage Lab Biomarker Department since 2022. M.S. in Bioengineering/ Biomedical Engineering from New Jersey Institute of Technology. Ph.D. Candidate in Biochemical Engineering from Villanova University. Before joining Frontage Lab, he worked at Janssen Research and Development and Teva pharmaceuticals. 10 years of experience in both regulated and non-regulated PK/PD/Biomarker/ADA assay development using ligand binding, LC-MS/MS and hybrid platforms.

USING LC-MS FOR BIOMARKER ANALYSIS: WHEN? HOW? WHY?

Long Geng, Zhongping John Lin
Frontage Laboratories

71th ASMS Conference, RBIG Workshop
June 7th, 2023

Using LC-MS for biomarker analysis

When? How? Why?

- Bioanalysis assay platform considerations
  - Assay capability between phases
  - Assay challenges and complexity
  - Time and associated costs

- Biomarker assay platforms
  - Immunoassays (MSD, Simoa, ELLA, SMC, Olink)
  - Mass Spectrometry (LC-MS, GC-MS)
  - Genomics (PCR, NGS)
  - Imaging (X-ray, MRI)
  - Flow Cytometry

Using LC-MS for biomarker analysis

When? How? Why?

- High assay sensitivity
  - pg/mL to ng/mL level
- Less reagent variability
  - Lot to lot variation
- Less Matrix interference
  - Selectivity, Specificity
  - Different matrices
- Multiplexing
  - 10+ analytes can be analyzed simultaneously
  - Therapeutic target and ligands

Using LC-MS for biomarker analysis

When? How? Why?

Improvement of assay selectivity, specificity, and sensitivity

- **Case study 1**: Development and validation of an LC–MS/MS Method for the quantitation of heparan sulfate in human urine[1]

- **Case study 2:** A novel LC–MS/MS assay to quantify dermatan sulfate in cerebrospinal fluid as a biomarker for mucopolysaccharidosis II[2]
  - Rapid digestion, filtration, solid-phase extraction and chemical derivatization

- **Case study 3**: Development and validation of an LC-MS/MS method for the quantification of fascin proteins in human serum[3]
  - Immunocapture, protein precipitation, enzymatic digestion and solid phase extraction

1. *Kai Wang, Ming Li2, Yijin Xiao, Mark Ma2, Wei Hu, Tao Liang, Zhongping John Lin. (2018) Biomedical Chromatography.*
2. *Peng Pan, Mu Chen, Zhiling Zhang, Amauri Dalla Corte, Carolina Souza, Roberto Giugliani, Luying Pan, Yongchang Qiu, Lakshmi Amaravadi, Jiang Wu.(2018) Bioanalysis*
3. *Ke Li, Zhiling Zhang, Kai Wang, Xin-Yun Huang, Zhongping (John) Lin. (2022) Bioanalysis*

Using LC-MS for biomarker analysis

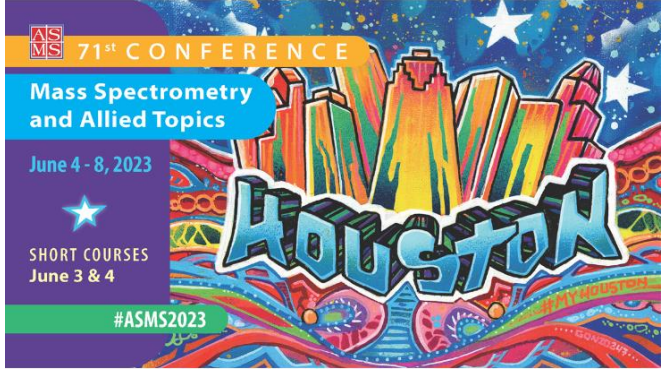
When? How? Why?

Multiplexing

- **Case study 4**: Development and Validation of an LC-MS/MS Method for the Simultaneous Quantitation of Fifteen Bile Acids in Human Serum[4]
  - Fifteen bile acids, including five major bile acids (cholic acid, deoxycholic acid, ursodeoxycholic acid, chenodeoxycholic acid and lithocholic acid), and their respective glyco- and tauro- conjugates were extracted by protein precipitation using methanol.

Analyte	Statistics	QC LLOQ	Intra-run QC Low	Intra-run QC Mid	Intra-run QC High	Analyte	Statistics	QC LLOQ	Intra-run QC Low	Intra-run QC Mid	Intra-run QC High	Analyte	Statistics	QC LLOQ	Intra-run QC Low	Intra-run QC Mid	Intra-run QC High
CDCA	Inter-run %CV	10	10	3.7	3.5	GDCA	Inter-run %CV	6	6.4	5.1	6.1	TCA	Inter-run %CV	7.7	6.1	4.8	8.3
	Inter-run %Bias	8	0	-3.1	-5		Inter-run %Bias	2	-5.9	-4.4	-6.4		Inter-run %Bias	0	-6.7	-4.5	-1.7
CA	Inter-run %CV	9.2	6.2	5.2	4.6	GLCA	Inter-run %CV	17.1	9.6	4.1	3.3	TDCA	Inter-run %CV	14.8	9.6	4.7	4.6
	Inter-run %Bias	2	-3.3	-3.8	-3.1		Inter-run %Bias	-3.5	-5.7	-3.8	-6.3		Inter-run %Bias	-2.8	-4.7	-3.6	-6
DCA	Inter-run %CV	9	6.1	4.1	4.8	GUDCA	Inter-run %CV	11	6.7	4.2	3.7	TLCA	Inter-run %CV	10	5.7	4.6	3.2
	Inter-run %Bias	-1.7	-1.3	-2.2	-4.2		Inter-run %Bias	-4	-4.3	-1.9	-6.8		Inter-run %Bias	-3.2	-3	-5.8	-7.2
GCDCA	Inter-run %CV	6.6	4.7	5.2	2.9	LCA	Inter-run %CV	8	4.8	3.3	3.8	TUDCA	Inter-run %CV	14.6	7.1	5.1	5.9
	Inter-run %Bias	-3.5	1.3	-2.4	-8.3		Inter-run %Bias	1	-1	-1	-3.2		Inter-run %Bias	-6	-3.7	-3.9	-4
GCA	Inter-run %CV	4.8	6.1	4.2	4.1	TCDCA	Inter-run %CV	7.8	6.6	3.6	6.2	UDCA	Inter-run %CV	6.1	5.3	5	4.6
	Inter-run %Bias	-1.5	-2.3	-1	-6.5		Inter-run %Bias	3.5	-3.7	-5.1	-4.9		Inter-run %Bias	1	1.8	4	-2.3

4. Kai Wang, Siliang Chen, Yafei Xu, Luca Matassa, Zhongping (John) Lin, Pamela Vig, Hassan Rashidzadeh, Hongmei (Karen) Cao and Marita Larsson Cohen (2019) The American Association of Pharmaceutical Scientists (AAPS)



Aiying Yu, Ph.D.

Senior Scientist in Department of BioAnalytical Sciences (BAS) at Genentech since 2022. Ph.D. in Analytical Chemistry from Texas Tech University in 2022. Expertise in comprehensive structural identification and quantification of proteins, glycans, and glycopeptides in various biological matrices. Hands-on experience in analyzing tissues from Alzheimer's, breast cancer, and kidney brush-border membranes. Current work is focused on developing assays to enable characterization and quantification of protein therapeutics and biomarkers in fixed tissue samples such as formalin-fixed paraffin-embedded (FFPE) tissues using innovative LC-MS based approaches.

Genentech
A Member of the Roche Group

Target Occupancy in Frozen and FFPE Tissues using Immunoaffinity Capture 2D-LC-MS/MS

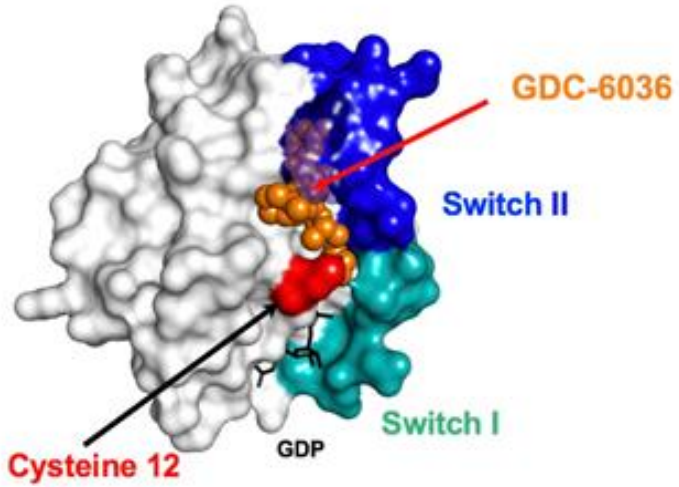
Aiying Yu, Lingyao Meng, Jintang He, Surinder Kaur, Keyang Xu

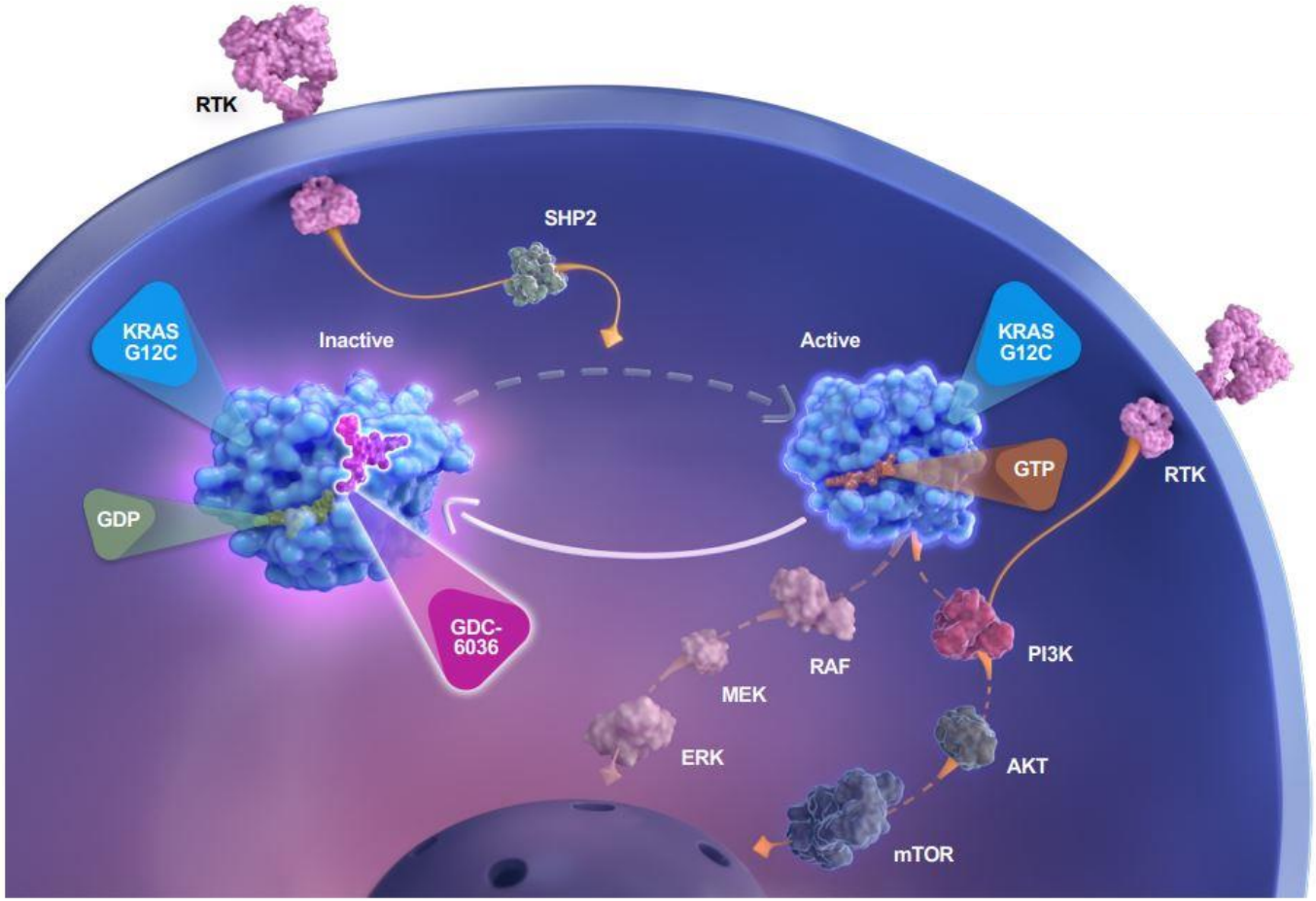
71th ASMS Conference, RBIG Workshop

June 7th, 2023

KRAS G12C and GDC-6036 Inhibitor

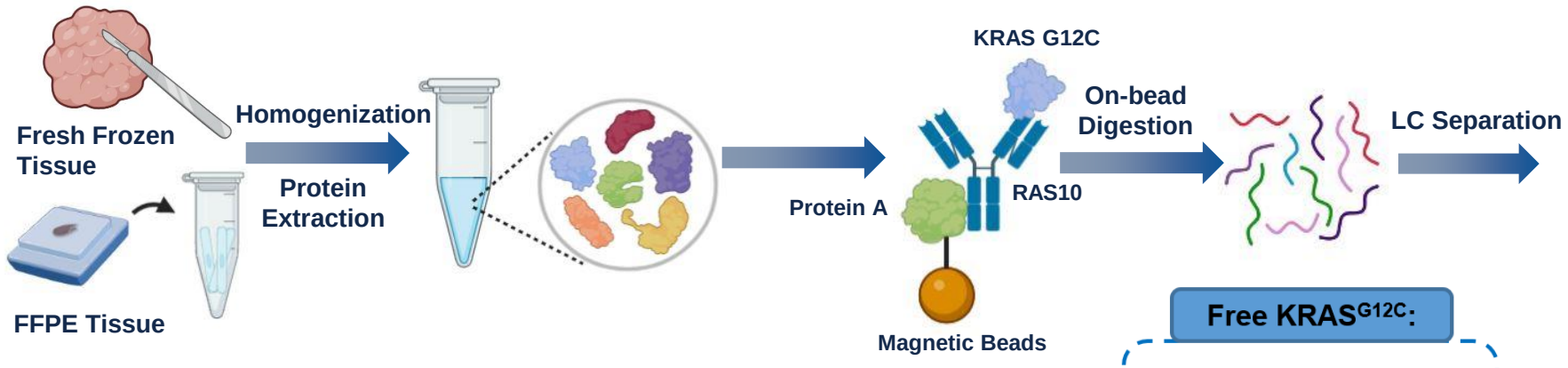
- KRAS G12C, a KRAS mutation favors the activated state of KRAS which results in uncontrolled cell growth and tumor formation.
- GDC-6036, an investigational KRAS G12C inhibitor that acts by irreversibly binding to the switch II pocket of KRAS G12C, blocking GTP binding and activation.

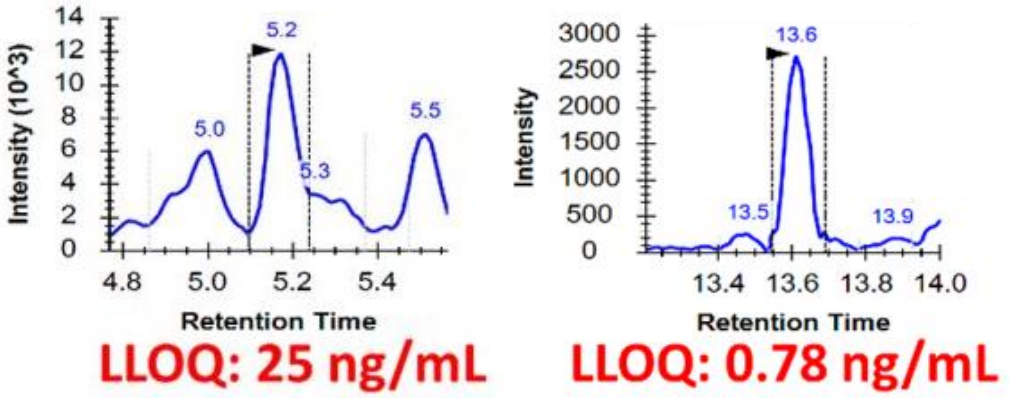




Purkey, Hans. Cancer Research 82.12_Supplement (2022): ND11-ND11.

Sensitive and Hybrid Assay (Immunoaffinity Capture + 2D-LC-MS/MS)





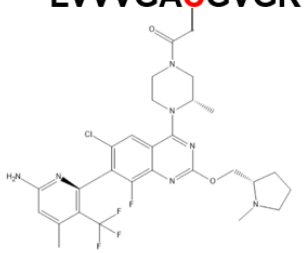
Free KRAS^{G12C}:

LVVVGAC(cam)GVGK

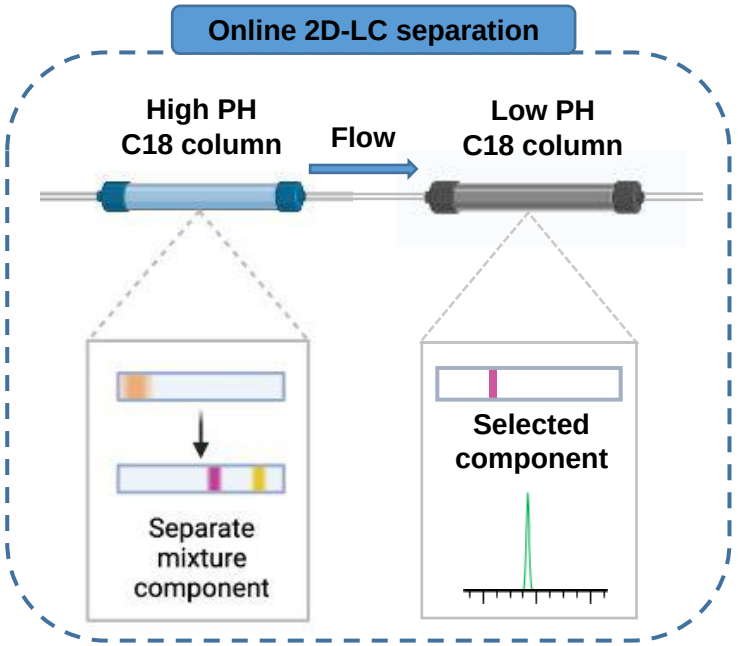
$[M+2]^{2+}$=529.8050

GDC-6036-bound KRAS^{G12C}:

LVVVGACGVGK



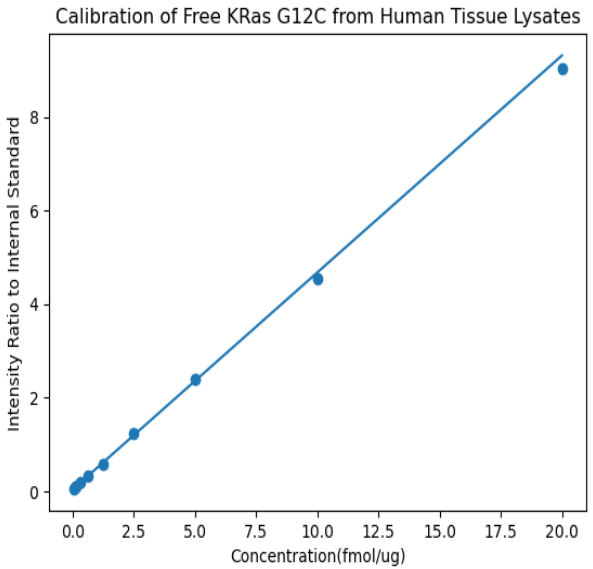
$[M+3]^{3+}$=541.9319



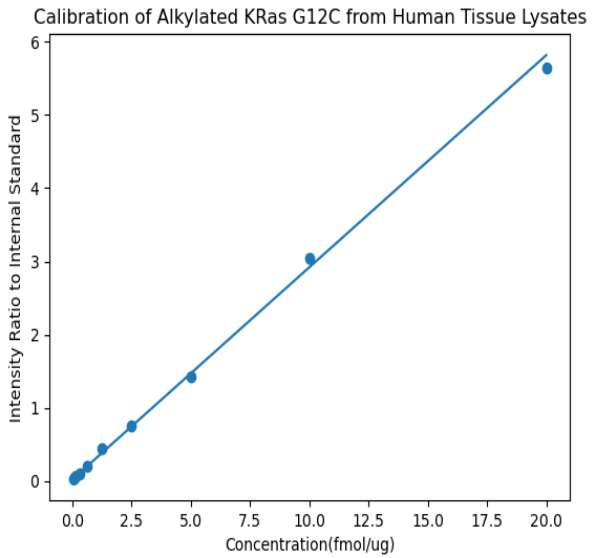
MRM

He, Jintang, Lingyao Meng, Jane Ruppel, *et al*.. Analytical Chemistry 92. 13 (2020): 9412-9420.
Lingyao Meng, Emily W. Chan, Carl Ng, *et al*.. Analytical Chemistry 2022 94 (37), 12927-12933

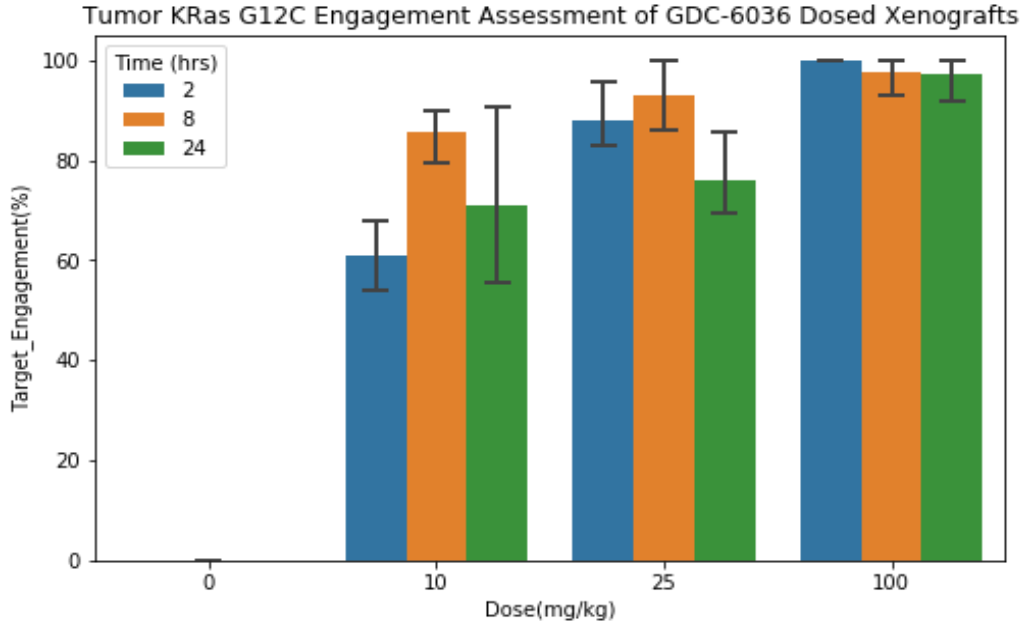
KRAS G12C Engagement of Xenograft Core Needle Tumor Biopsies



LLOQ: 0.04 fmol/µg
(equivalent to 400 pg/mL)
ULOQ: 20.0 fmol/µg
R-square: 0.9955



LLOQ: 0.04 fmol/ug
(equivalent to 400 pg/mL)
ULOQ: 20.0 fmol/ug
R-square: 0.9917

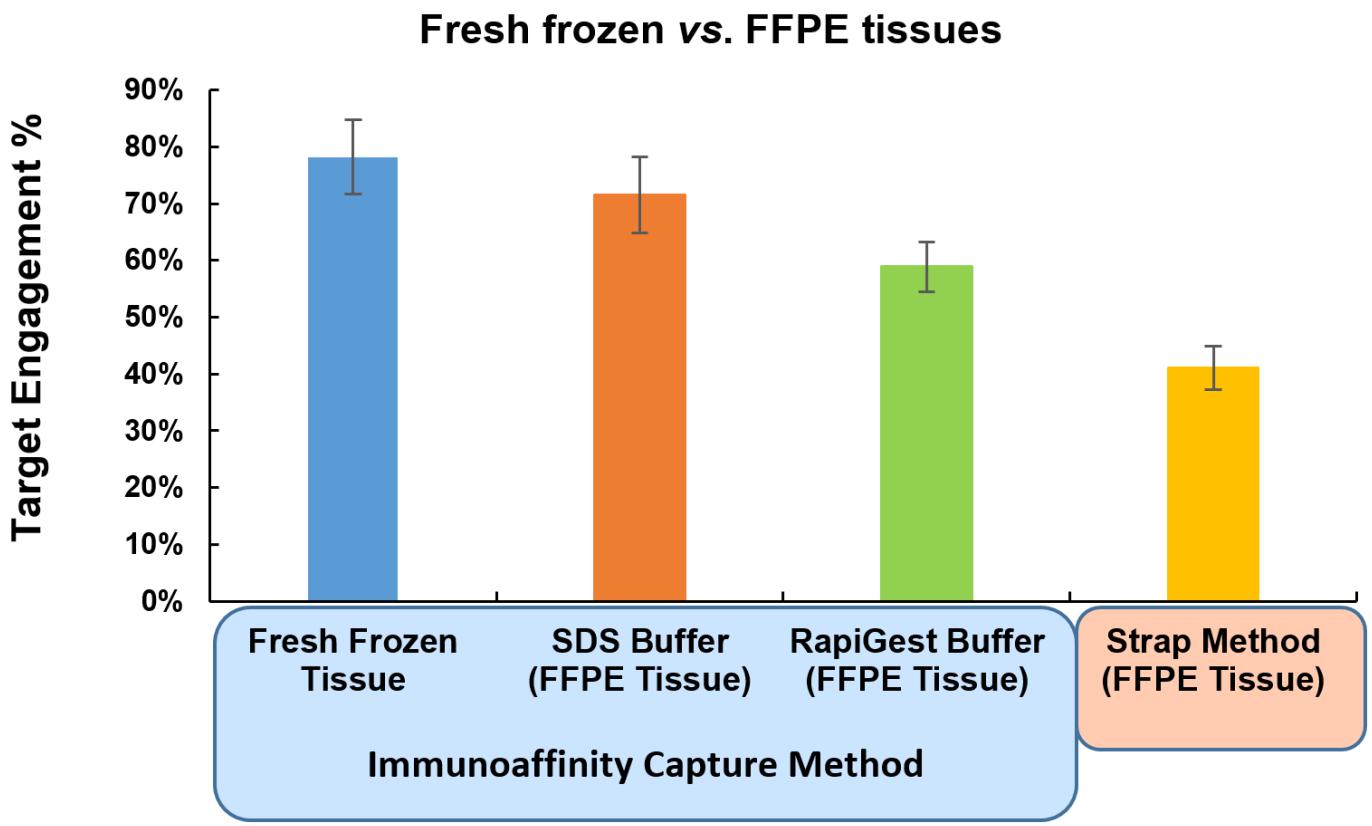


$$\text{Target Engagement \%} = \frac{\text{Alkylated}}{\text{Free + Alkylated}}$$

- Sub fmol/µg level sensitivity was achieved with 5 µg of total protein: ~ 5 - 10% of the core needle biopsy
- Increase trend of KRAS G12C engagement observed with dosing escalation

Lingyao Meng, Emily W. Chan, Carl Ng, *et al*.. Analytical Chemistry 2022 94 (37), 12927-12933

KRAS G12C Engagement of Mouse Xenograft FFPE Tissue



Summary:

- Several approaches were compared for assessment of KRAS G12C engagement in FFPE tissues.
- Several extraction buffers were evaluated and SDS buffer achieved the highest protein recovery.
- Early xenograft data indicates that target engagement in FFPE tissues comparable to that in frozen tissues.

Future work:

- Optimization for protein extraction and automation of sample preparation ongoing.
- Quantification of KRAS G12C engagement in FFPE tissues from clinical samples.

Acknowledgement

BAS-O3M

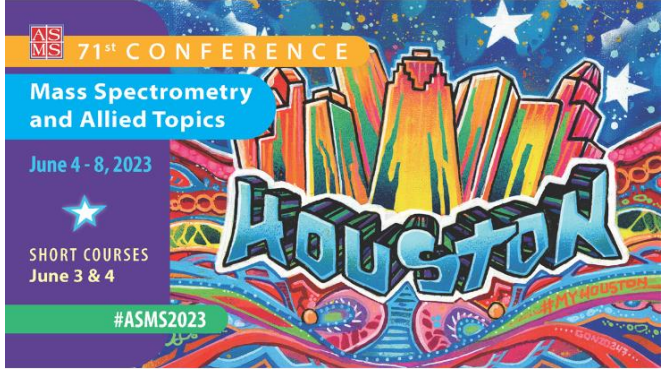
- Surinder Kaur
- Keyang Xu
- Jintang He
- Lingyao Meng
- Luna Liu
- Carl Ng
- Maoyin Li
- Ola Saad
- Randy Dere
- Violet Lee
- Suk Hyung
- Arun Taylor
- Helen Davis
- Sylvia Wong
- Jason Lamar

KRAS G12C Team

- Zhen Shi
- Emily W. Chen
- Mark Merchant

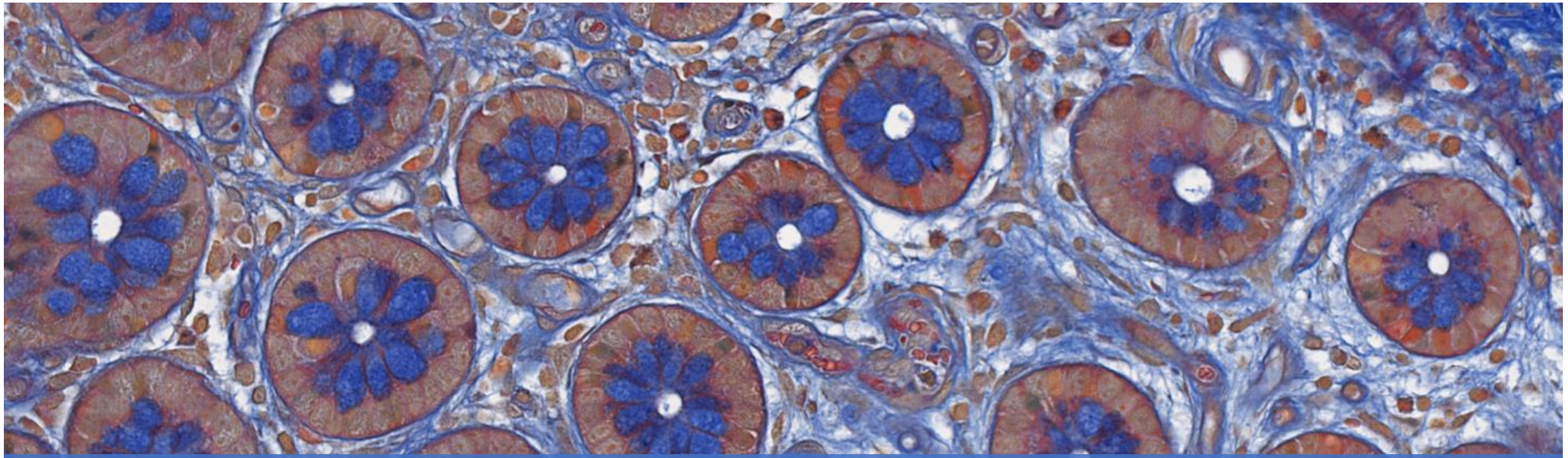
Pathology Labs

- Hartmut Koeppen
- Jian Jiang



Yifan Shi, Ph.D.

Senior Scientist in the Bioanalytical Discovery & Development Sciences department of Janssen R&D at Spring House, PA for 8 years. Associate director at Alliance Pharma (2011 – 2015). Staff scientist in Covance & Tandem Labs (2009 – 2011). PhD in chemistry from University of Utah. BSc in material chemistry from Peking University. Experience in small and large molecule quantitation, target engagement, and protein dynamics using various LC-MS platforms. Responsible for supporting drug discovery and development, including biomarker studies for TK and PK.



Implementing antibody-free strategies for protein quantitation using LC-MS

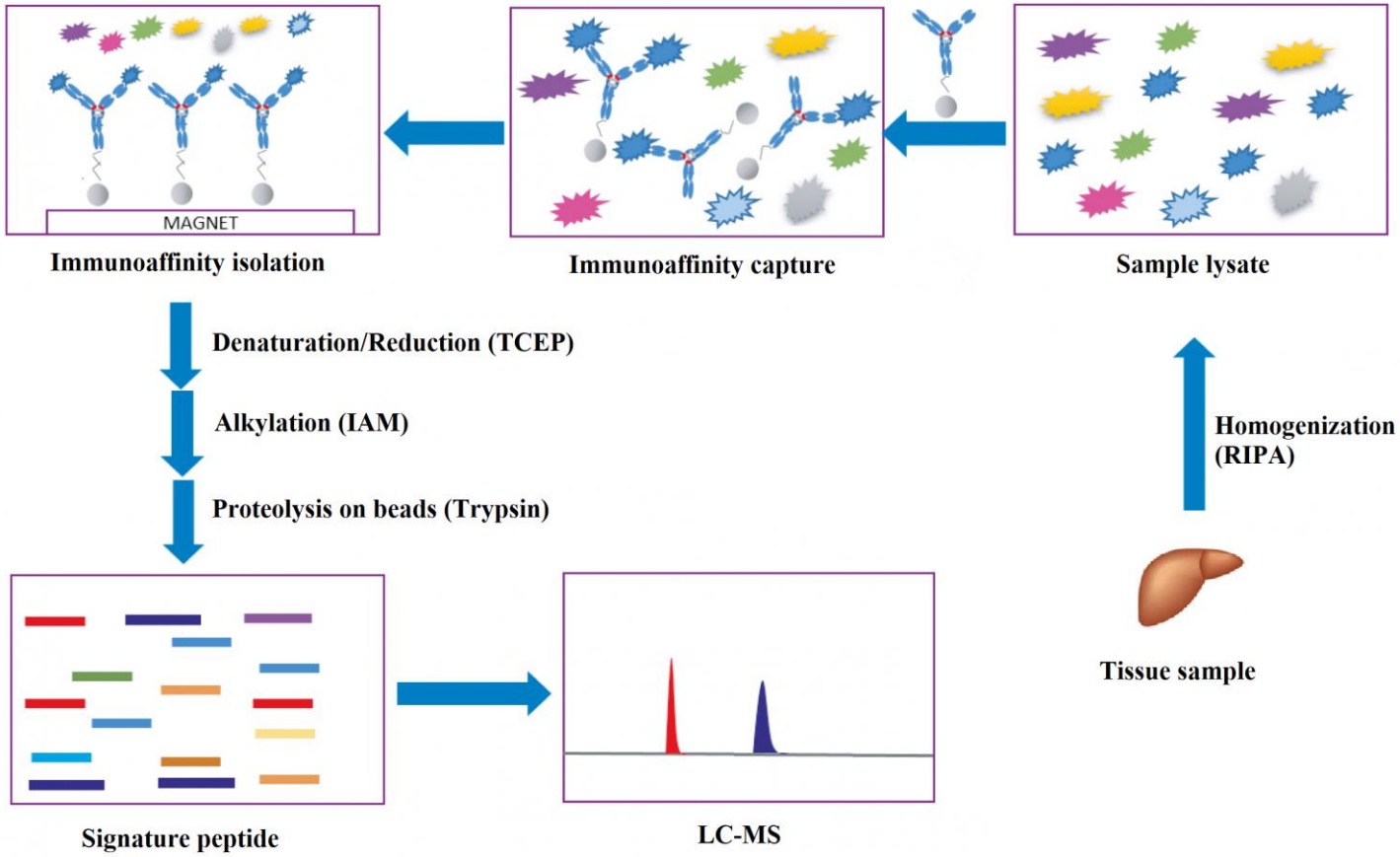
Yifan Shi, Ph.D.

Janssen R&D
Spring House, PA
June 7th, 2023

Colonic mucosa highlighted by Picro-Mallory trichrome special stain, from an exploratory study for Pulmonary Arterial Hypertension (PAH)

Credit: Vini Carreira, Pathology, Preclinical Sciences & Translational Safety

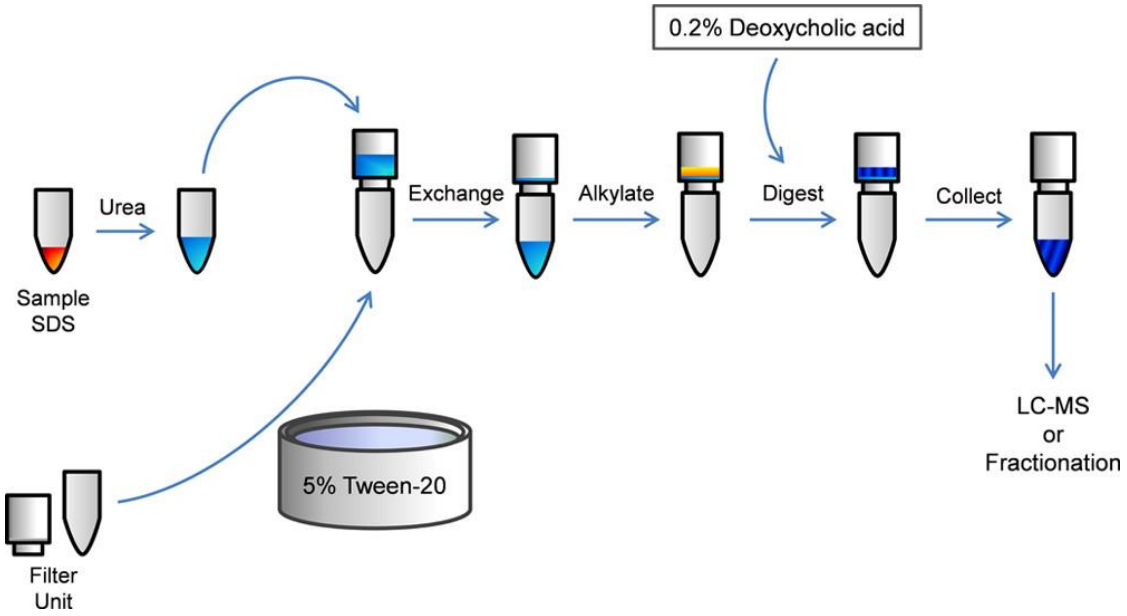
Workflow for Hybrid LC-MS Protein Quantitation



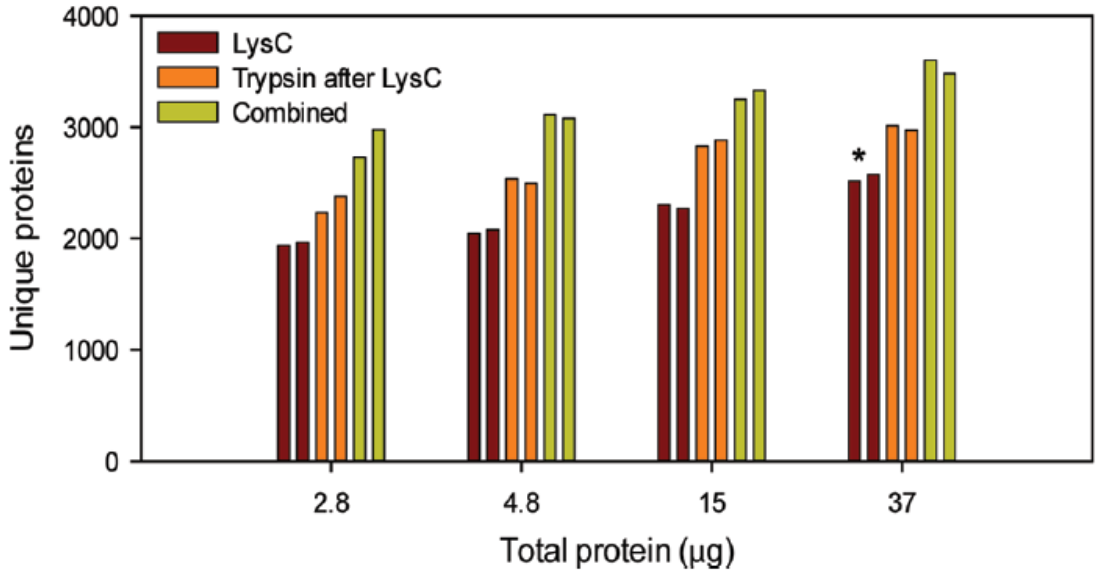
Magnetic beads based immunoaffinity LC-MS protein quantitation

- **Advantages**
  - High throughput
  - Relatively high sensitivity and dynamic range
- **Hybrid LC-MS assay requirement**
  - Recombinant protein
  - Capturing antibody
- **Potential issues**
  - Recombinant protein unavailable (or small fragments)
  - Unreliable antibody supply

Antibody-free Protein Extraction and Digestion



FASP (Filter-Aid Sample Preparation) workflow



High protein coverage for proteomics with FASP

FASP basic concepts and advantages

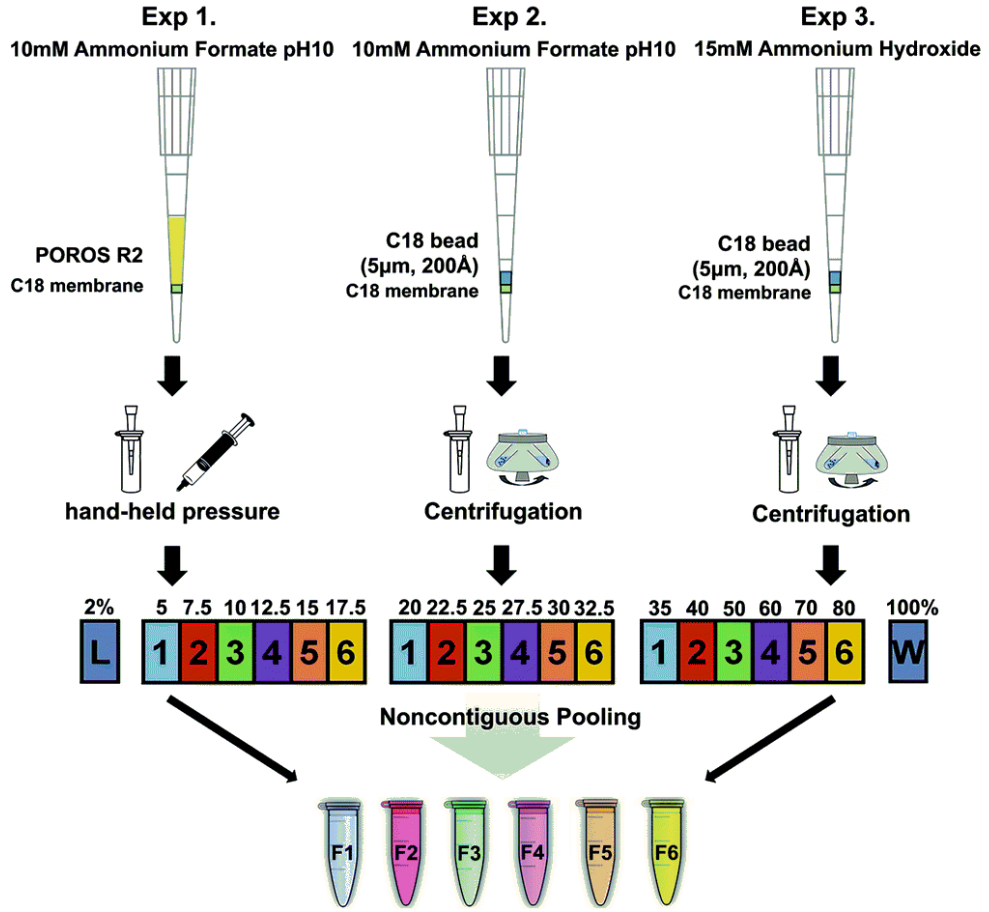
- Ultrafiltration device retaining proteins
- Compatible with strong detergent for tissue homogenization and extraction
- Antibody-free and multiplex

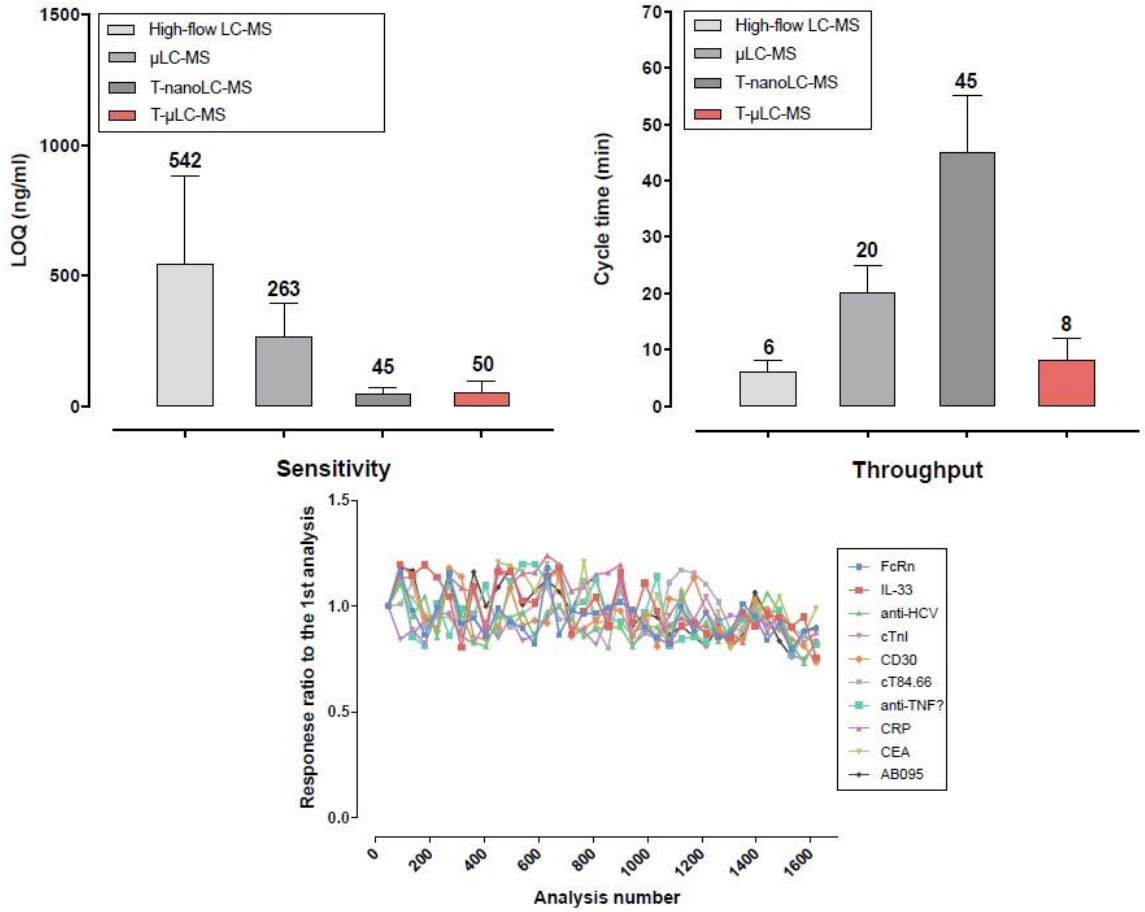
FASP method applications

- Effective extraction of cytosolic and membrane proteins
- Multiple enzyme digestion to increase protein sequence coverage
- Efficient digestion at low protein levels

Erde et al., J. Proteome Res. 13, 1885, 2014
Wisniewski et al., Anal. Chem. 84, 2631, 2012

Antibody-free Protein Biomarker Quantitation





High pH fractionation

- Orthogonal separation to identify low abundance target proteins
- Offline or online automation

Microflow HPLC and online trapping

- Improved assay sensitivity
- Robust system for BA sample analysis

Kim et al., Anal. Methods, 11, 4693, 2019
Zhang et al., Anal. Chem., 90, 1870, 2018

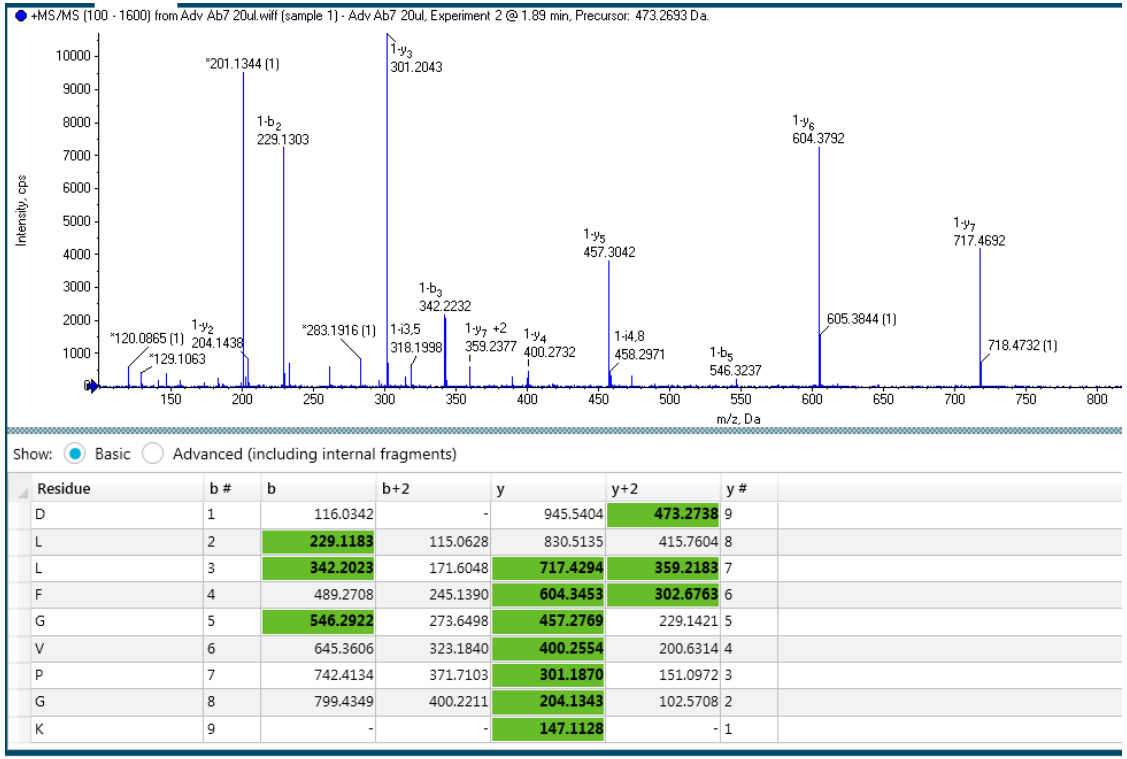
Case 1: FASP-Facilitated Method Development

Challenges

- Transmembrane protein with ~1300 AA
  - Recombinant full or fragment protein production failed at two vendors & Janssen internally
  - LC-MS method cannot be fully optimized without recombinant protein
- Low abundance in liver (<10 ng/g)
  - Difficulty in screening immunocapture antibodies using liver samples
  - No suitable reagent identified after testing 10 different antibodies

FASP for MS optimization & antibody screening

- Full length protein expression in a cell line
  - Adenovirus infection verified with mRNA detection
- FASP used for cell samples for LC-MS optimization
  - Highly sensitive LC-MS method developed for screening of antibody in liver homogenate for endogenous protein



Show: Basic Advanced (including internal fragments)

Residue	b #	b	b+2	y	y+2	y #
D	1	116.0342	-	945.5404	**473.2738**	9
L	2	**229.1183**	115.0628	830.5135	415.7604	8
L	3	**342.2023**	171.6048	**717.4294**	**359.2183**	7
F	4	489.2708	245.1390	**604.3453**	**302.6763**	6
G	5	**546.2922**	273.6498	**457.2769**	229.1421	5
V	6	645.3606	323.1840	**400.2554**	200.6314	4
P	7	742.4134	371.7103	**301.1870**	151.0972	3
G	8	799.4349	400.2211	**204.1343**	102.5708	2
K	9	-	-	**147.1128**	-	1

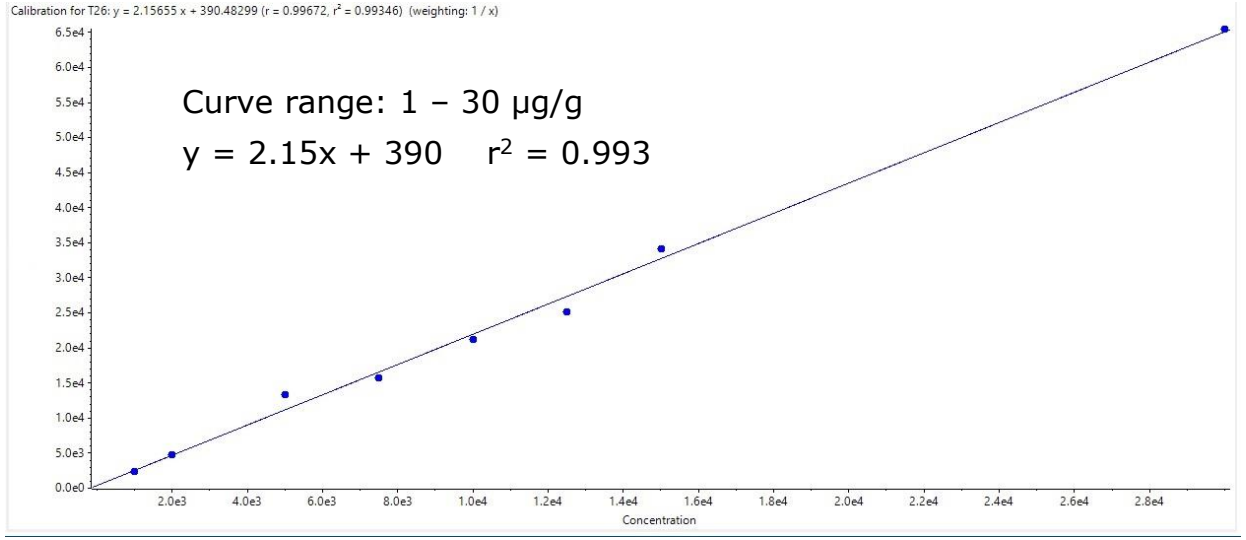
LC-MS method optimization with AAV overexpressed cell samples processed using FASP

Case 2: FASP for Target Protein Bioanalysis

Background

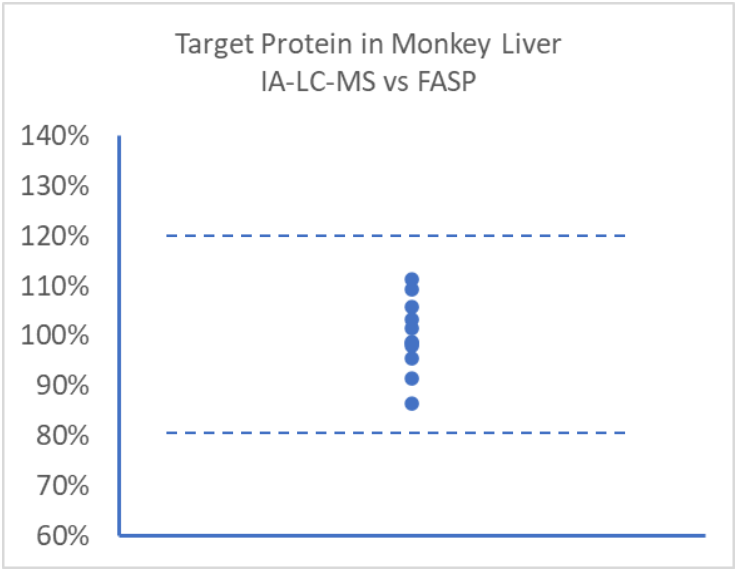
Hybrid LC-MS method established

- Endogenous protein conc. ~11 µg/g in liver
- Study samples analyzed successfully
- Antibody discontinued by vendor
- Other antibodies have poor recovery or linearity problems for tissue samples



FASP for protein bioanalysis in liver homogenate

- Antibody-free target protein quantitation
- High correlation between immunocapture & FASP
- Excellent reproducibility
- Adequate sensitivity
- Lower throughput



Protein conc. range in liver samples:

0.712 – 13.1 µg/g

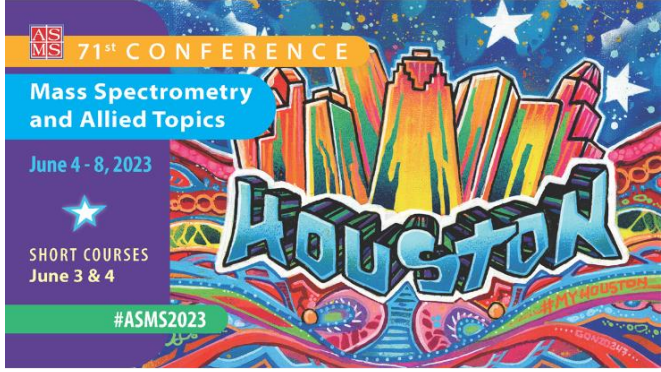
Hybrid LC-MS vs FASP

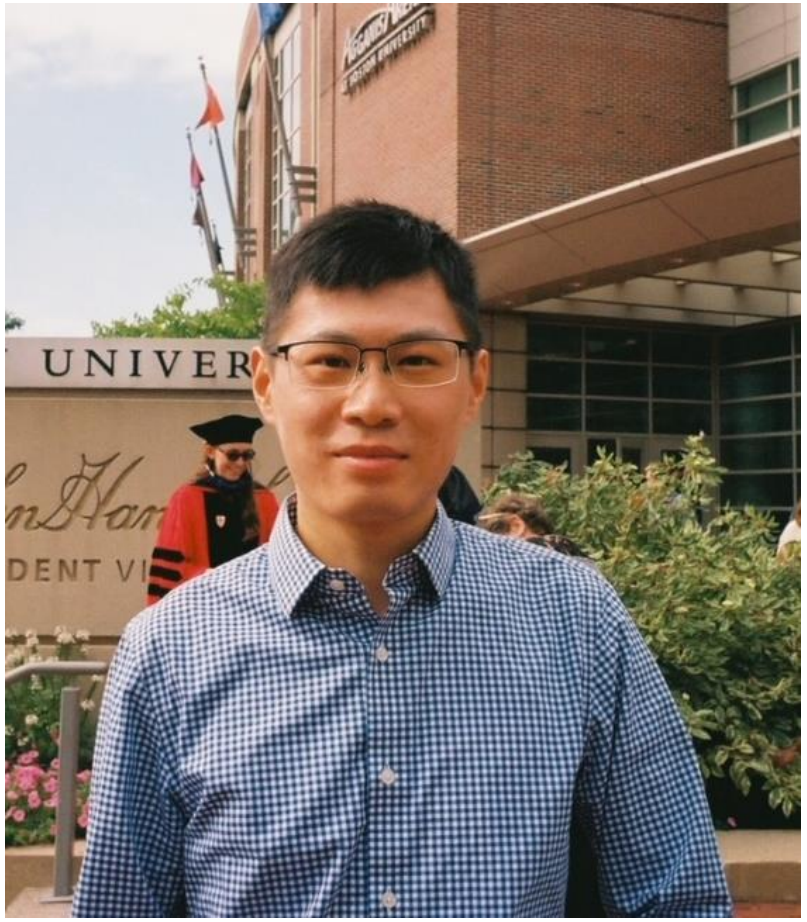
87% – 111%

Acknowledgement

- Bioanalytical Group
  - **Wenying Jian**
  - Lijuan Kang
  - Naidong Weng

- Therapeutic Discovery
  - **Sheng-Ping Wang**
  - **Fritz Kramer**
  - **Lifeng Wang**
  - Brian Rady





Yang (Thomas) Tang, Ph.D.

Principal Scientist in Regulated Small Molecule Bioanalysis, DMPK at Genentech since 2020. Ph.D. in Analytical Chemistry from Boston University (2015-2020). At Genentech, responsible for developing and validating LC-MS/MS assays for quantitation of small molecular drugs and biomarkers to support TK and PK studies in pharmaceutical industry. Additional expertise in detailed structure characterization of metabolites and glycans using radical-induced MS/MS fragmentation methods.

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A Universal Surrogate Matrix Assay for Urea Measurement in Clinical Pharmacokinetic Studies of Respiratory Diseases

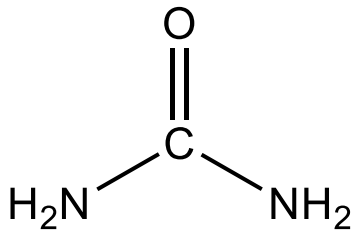
Yang (Thomas) Tang

Jun. 7th, 2023

Urea Bioanalysis

- Physiological matrices in respiratory diseases: Nasal secretion (NS), exhaled breath condensate (EBC), mucosal lining fluid (MLF), lung epithelial lining fluid (ELF) and plasma/serum.
- The measurement of drug concentration is challenging (Volume unknown).
- Urea as Dilution Marker to normalize liquid sample volumes

  [Urea] is consistent in most matrices from the same individual.



Urea

1. Diffuse freely and quickly throughout the body.
2. Low turnover rate.
3. Minimally affected by disease states.

$$V_{native} = (C_{buffer\ eluate}/C_{plasma}) \times V_{buffer\ eluate}$$

Urea quantitation by LC-MS/MS

- High specificity, sensitivity and easy accessibility.

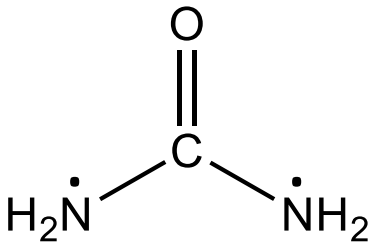
- Inherent difficulties:
  a) Vulnerable to interference.
  b) High background.
  c) Endogenous presence.

→

Approaches:

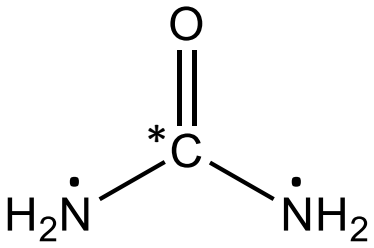
1) Chemical derivatization.
2) Surrogate analyte.
3) Surrogate matrix.

Surrogate Analyte vs Surrogate Matrix



[$^{15}N_2$]-urea

Analyte



[^{13}C,$^{15}N_2$]-urea

IS

Surrogate Analyte

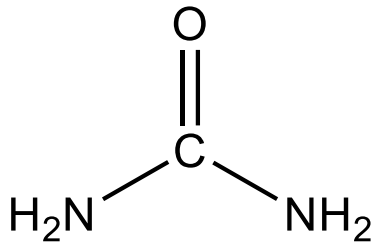
- Same biological matrix.
- [$^{15}N_2$]-urea as calibration curve.
- Signal ratio of urea and [$^{15}N_2$]-urea predetermined.

Inherent

Assay issues:

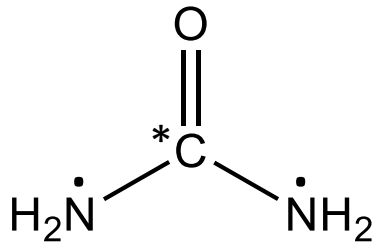
One assay per matrix

- Cumbersome data processing.
- Varied signal ratio.
- Endogenous QCs fail.



Urea

Analyte



[^{13}C,$^{15}N_2$]-urea

IS

Surrogate Matrix

- Alternative matrix.
- Urea as analyte.

Improvable

Assay Issues:

A universal assay for all matrices

- Poor LLOQ peak shape.
- High baseline.

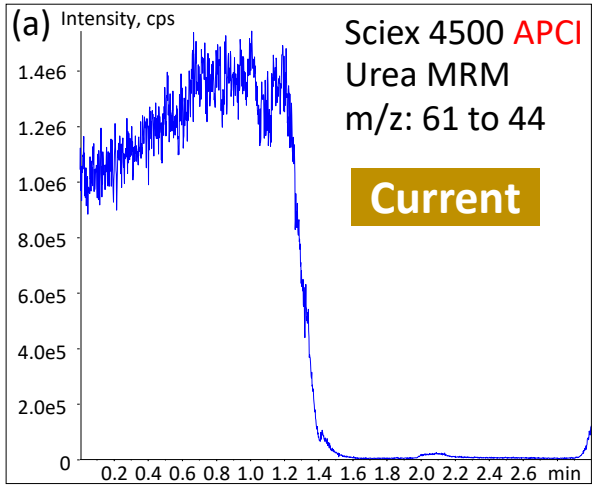
Comparison of Ionization Modes and Platforms

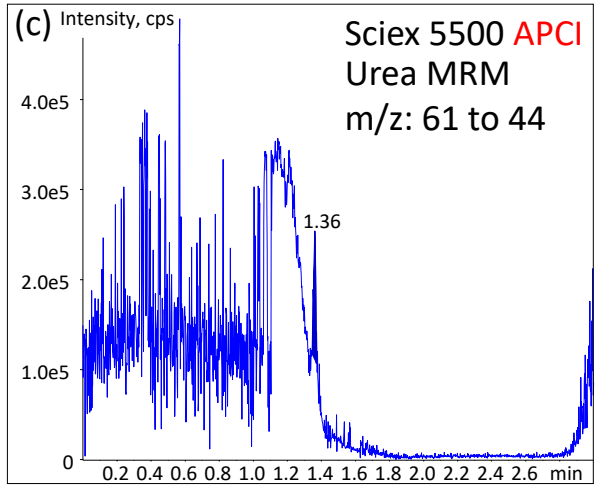
Sciex 4500

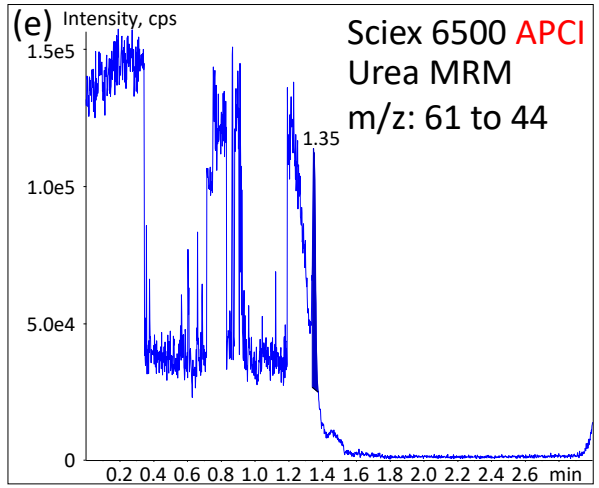
Sciex 5500

Sciex 6500

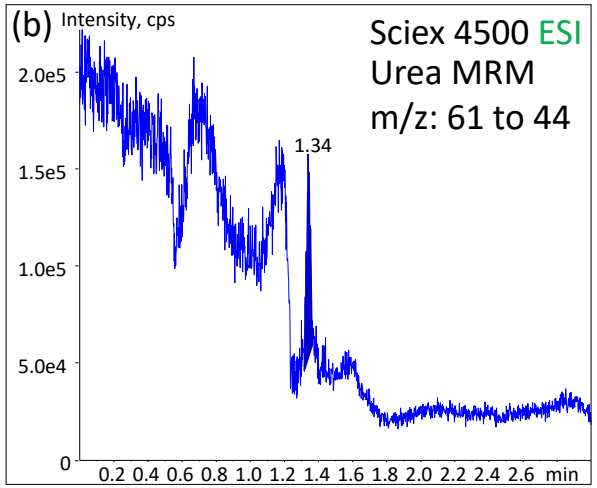
APCI

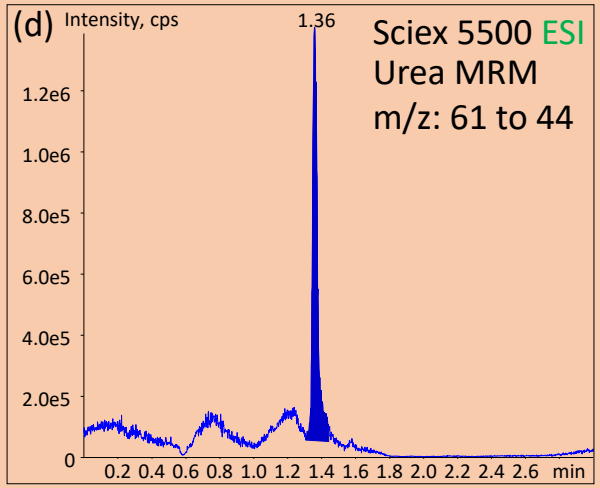


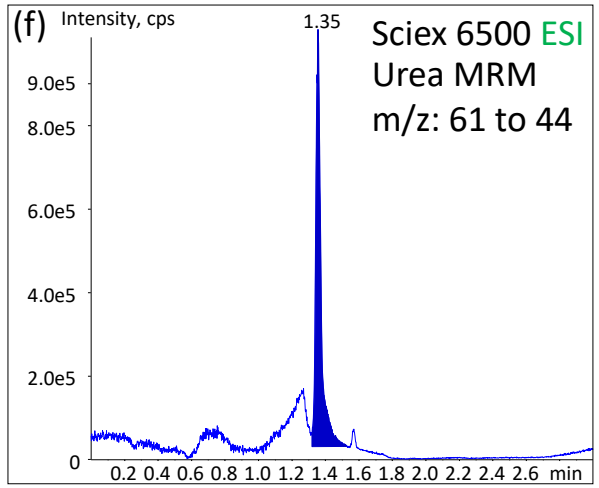




ESI







Neat solution injected

Surrogate Matrix Suitability

Surrogate matrix:

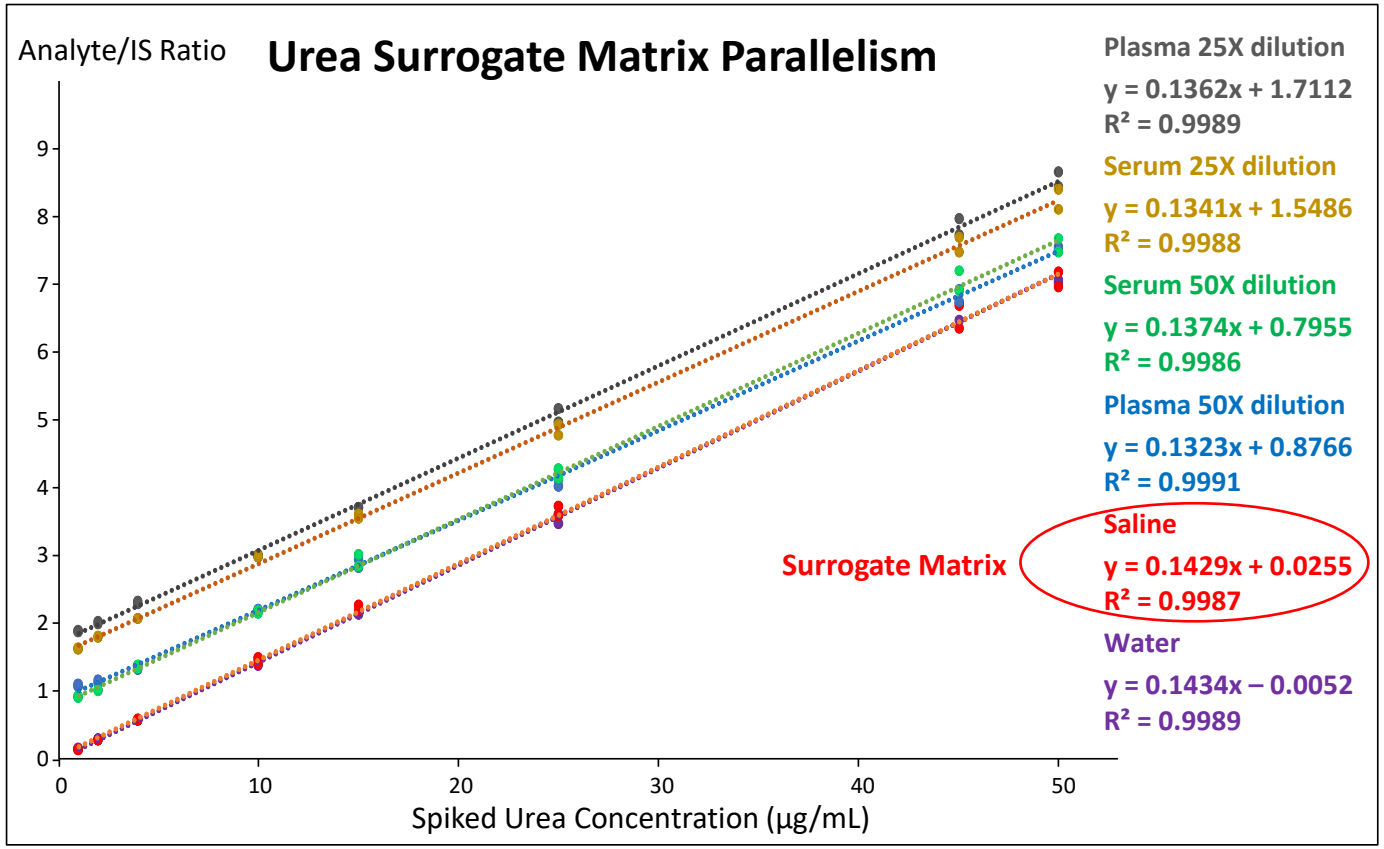
1) Contain no measurable endogenous analyte.
2) Lack matrix effects or interferences.

Parallelism

Parallelism of calibration curves between native and surrogate matrices.

Endogenous QCs

Quantitation accuracy of EQCs by surrogate matrix curve.



Minimal matrix effects have been confirmed by good parallelism between curves prepared in water, saline, diluted plasma and diluted serum.

Additional Tests

Accuracy and Precision

Sample	Theoretical (µg/mL)	Calculated (µg/mL)	RSD (%)	Accuracy (%)
Cal 1	1	1.01	10.2	101.3
Cal 2	2	1.94	2.0	96.8
Cal 3	4	4.05	4.9	101.2
Cal 4	10	10.04	4.1	100.4
Cal 5	15	14.54	1.4	96.9
Cal 6	25	26.06	2.8	104.2
Cal 7	45	45.02	2.9	100.0
Cal 8	50	49.57	1.5	99.1
LQC	3	2.91	3.0	97.1
MQC	12	12.03	2.2	100.2
HQC	40	39.97	3.7	99.9
DQC (20×)	300	307.01	3.1	102.3
Plasma ELQC (5×)	50	50.45	1.8	100.9
Plasma EQC (20×)	322	329.77	2.8	102.4
Plasma EHQC (50×)	1000	1045.59	1.6	104.6
Serum ELQC (5×)	50	48.39	1.8	96.8
Serum EQC (20×)	274	280.27	3.7	102.3
Serum EHQC (50×)	1000	1020.52	2.4	102.1

EQC: Endogenous QC
ELQC: Endogenous low QC
EHQC: Endogenous high QC

Surrogate Analyte vs Surrogate Matrix

		Surrogate Analyte		Surrogate Matrix	
Sample	Theoretical (µg/mL)	Calculated (µg/mL)	Accuracy (%)	Calculated (µg/mL)	Accuracy (%)
Plasma ELQC (5×)	50	53.07	106.1	50.45	100.9
Plasma EQC (20×)	322	337.74	104.9	329.77	102.4
Plasma EHQC (50×)	1000	989.35	98.9	1045.59	104.6
Serum ELQC (5×)	50	50.77	101.5	48.39	96.8
Serum EQC (20×)	274	267.45	97.6	280.27	102.3
Serum EHQC (50×)	1000	1011.39	101.1	1020.52	102.1

Comparable results demonstrate the satisfying performance of the newly developed surrogate matrix assay.

- **Recovery**
- **Sensitivity**
- **Benchtop Stability** (RT, 24 hr)

The newly developed assay is robust and reliable.

Conclusion

- A reliable LC-MS/MS surrogate matrix assay has been developed and qualified for urea measurement in plasma, serum, or BALF (bronchoalveolar lavage fluid).
- Sciex Triple Quad 5500 with ESI was identified as the most suitable platform.
- LC: isocratic elution with salt-free MP to give robustness and tolerability of interferences.
- MS: parameters were comprehensively optimized to maximize S/N.
- Saline was identified as the surrogate matrix. Good parallelism indicates minimal matrix effects.
- The assay demonstrated good precision and accuracy, dilution integrity (50×), sensitivity, recovery, and stability.
- The new surrogate matrix assay is easier to operate than the surrogate analyte assays, and more specific and accurate than colorimetric assays.

Acknowledgement

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- LabCorp FTE

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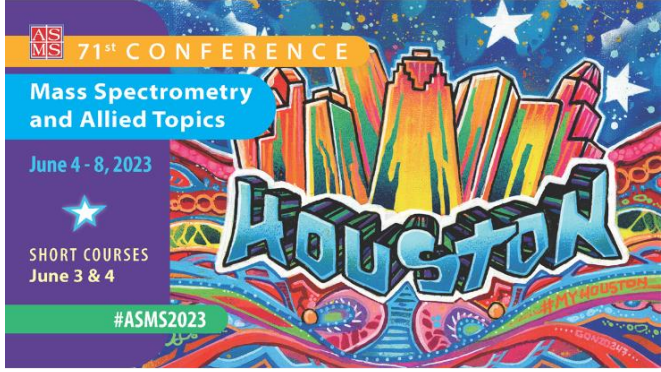
Alex Drelick

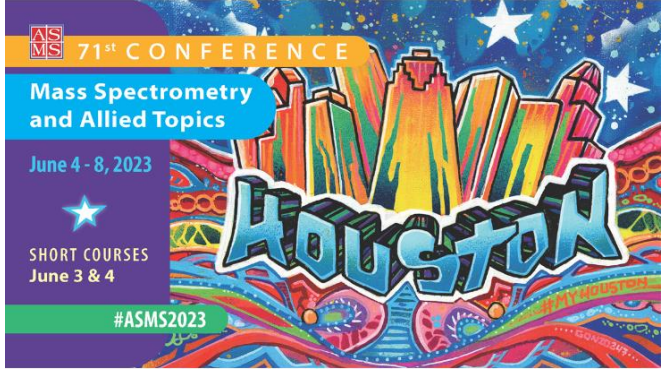
THANK YOU

Questions (1)



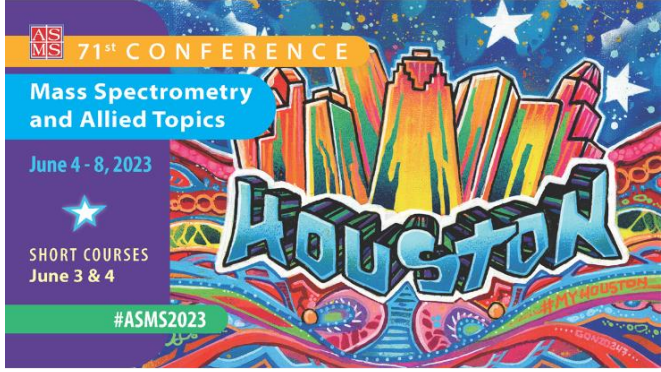
- For acceptance criteria of Multiplex Biomarker analysis, would keep the same as singelicate or different?
- For data analysis of Multiplex Biomarker, any existing or new tool that can be used to make the work more efficient?

Questions (2)



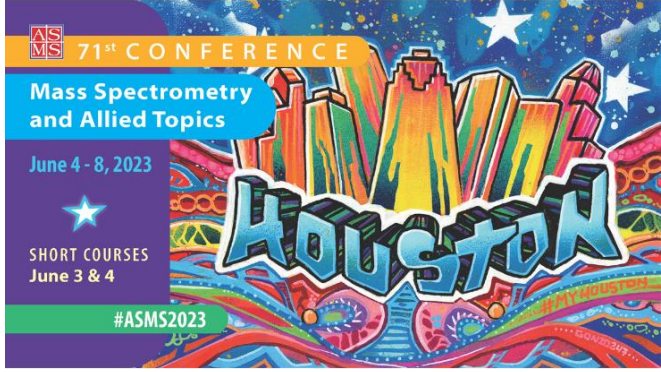
- What are the advantages to using the SDS buffer over MS-friendly buffers such as RapiGest for protein extraction in tissue samples?
- How to determine the retention time window for targeted peptides in a 2D-LC system?

Questions (3)



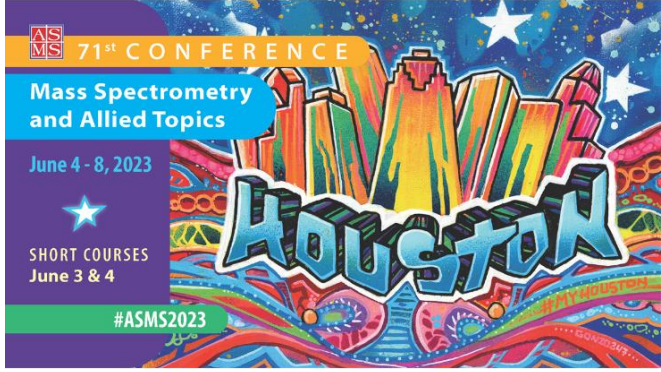
- What types of biological matrices have you used this approach for bioanalytical studies? How difficult is it to develop methods in different matrices for the same protein target?
- What are the considerations for selecting an internal standard for FASP methods?

Questions (4)



- What are the advantages and disadvantages of using surrogate analyte vs surrogate matrix in LC-MS biomarker analysis?
- In your organization, how many biomarker assays are qualified and how many are validated? What's the considerations in determining qualified or validated?
- In surrogate matrix assay development, when evaluating the parallelism between the surrogate matrix and native matrix, what is the typical acceptance criteria?
- Instrument platforms, Triple quadrupole vs. HR-MS?

Workshop Summary



- The workshop with ~70 attendees was featured by four excellent short presentations by the experts in the field of biomarker analysis using mass spectrometry, which was followed by panel discussions and Q&A on wide-range of important topics.
- The audience appreciated the topics, insights, opinions, and experiences shared by the panels and attendees, including experts (e.g., Hendrik Neubert, Dawn Dufield) in the field.
- Positive feedbacks were received from the audience on the quality and coverage of the presentations and discussions.