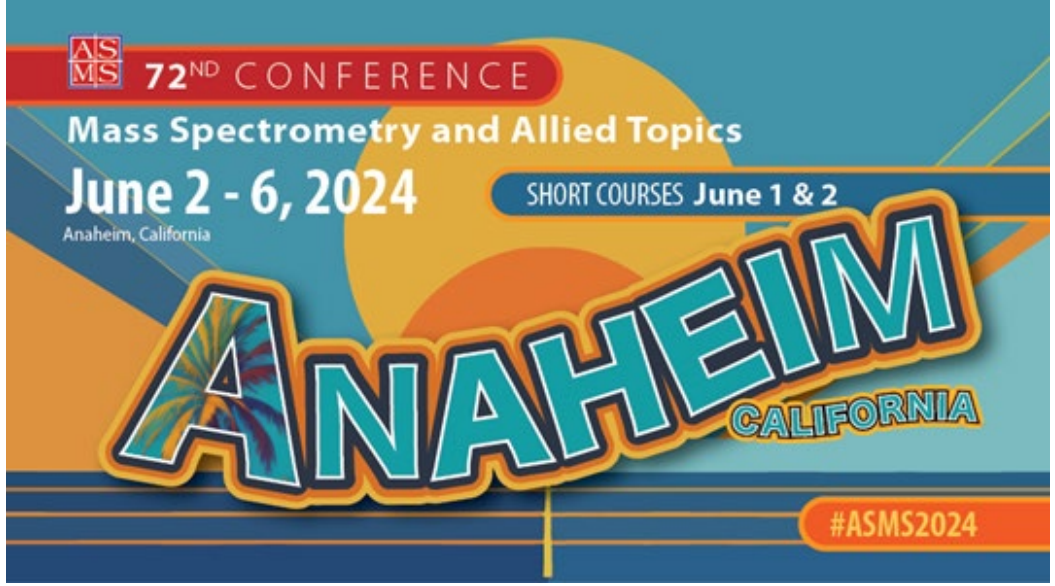


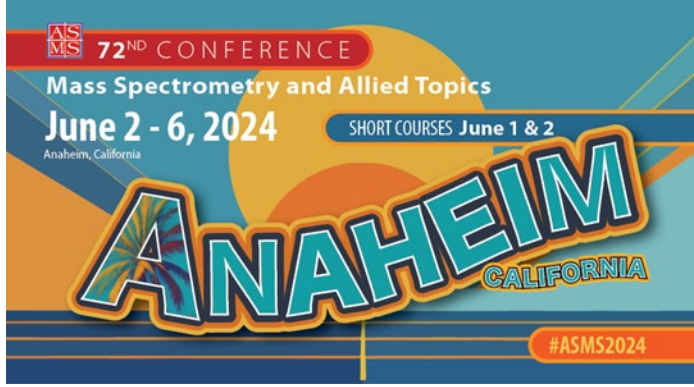
LC-MS Bioanalysis of Drug Conjugates

Regulated Bioanalysis Interest Group (RBIG) Workshop

5:45-7:00 pm, Wednesday, 05-June-2024

Presiding:
Jian Wang (Crinetics Pharmaceuticals), Wenkui Li (Novartis), Fabio Garofolo (BRI Biopharmaceutical Research Inc.)

Panelists:
Cynthia Chavez-Eng, Ph.D. (Merck), Kasie Fang, MS (GSK), Yipei Zhang, Ph.D. (Takeda), David Zuluaga-Rave, MS/Min Meng, Ph.D. (Resolian), Yang Xu, Ph.D. (Merck)



Abstract

Drug conjugates, or magic bullets, contain targeting agent (e.g., antibody), linker, and killing agent (e.g., payload). They have attracted widespread attention in the field of biomedical research and development due to their high specificity in targeting and killing cancer cells with no or minimum harm to normal cells. With the continuous advancement of biomedical engineering, conjugation chemistry and other techniques, in addition to well-known/studied antibody drug conjugates (ADC), a variety of new conjugation drug candidates have emerged for their clinical potentials, including antibody oligonucleotide conjugates (AOC), aptamer drug conjugates (ApDC), antibody fragment-drug conjugates (FDC), immune-stimulating antibody conjugate (ISAC), peptide (or polypeptide) drug conjugates (PDC), radionuclide drug conjugates (RDC), and small molecule-drug conjugates (SMDC), etc.

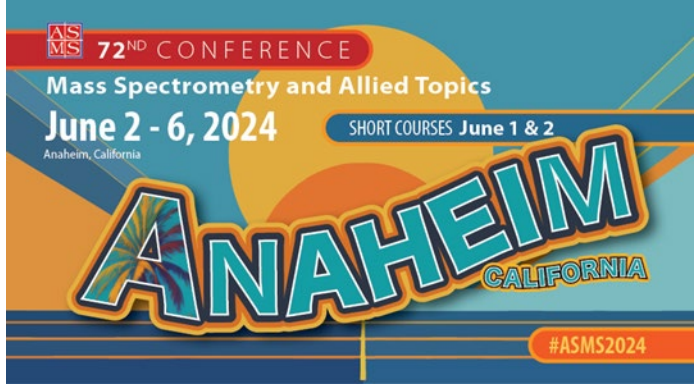
•

LC-MS is a powerful tool for quantitative and/or qualitative analysis of the various drug conjugates in support of their safety and efficacy assessment in preclinical and clinical development. This workshop is to be featured by 4 presentations, covering important aspects of LC-MS bioanalysis of drug conjugates.

•

This workshop will develop future discussions and consensus on regulated LC-MS bioanalysis of drug conjugates, including topics on sample preparation, mass spectrometric methods and data processing. Experts in the field will share their experience in this highly interactive workshop.

Summary of Workshop

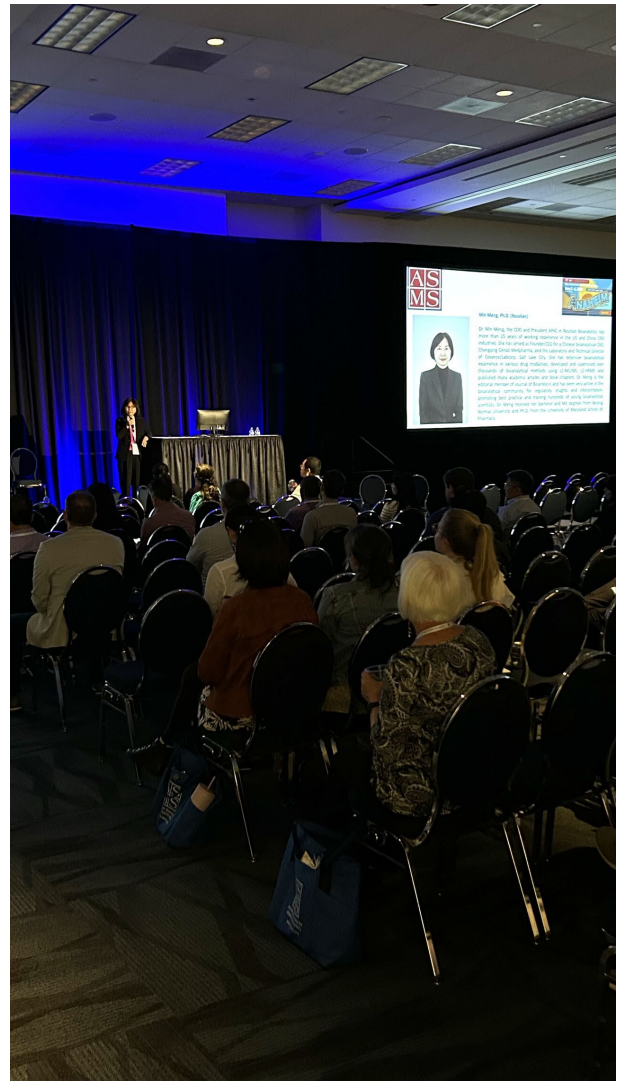


- The workshop was well prepared, went smoothly, and was well received. There were about 100-150 attendance in the workshop.
- Five panelists presented short overviews of their experience in bioanalysis of conjugates (i.e., ADC) covering variety of topics.
- The audiences were active in the Q/A session and appreciated the insights from the panelists.


Challenges in ADC Bioanalysis by LC-MS
Sample Preparation
LC-MS


ASMS
Kasie Fang, MS (GSK)


STimulator of Interferon Genes Catalyzes Cancer Immunity Cycle
STING agonist
Anti-hCCR2 IgG1
TAK-676


ASMS


ASMS


Q&A1
Choice of matrix and stabilization conditions
Q&A1
Period score
ASMS

Cynthia Chavez-Eng, PH.D. (Merck)

Senior Principal Scientist in Regulated Bioanalytics in the department of Pharmacokinetics, Dynamics, Metabolism, and Bioanalytics (PDMB), Merck & Co in West Point, PA. She has an extensive background in the pharmaceutical industry with over 32 years of experience conducting LC-MS/MS analysis for quantitation of small and large molecules. In her role, Cynthia's primary responsibilities involve the development of methods in support of non-clinical and clinical studies. She focuses on identifying and evaluating novel approaches and technologies to address complex assays and laboratory challenges. She earned her M.S. in Chemistry from the University of Southern Illinois, Carbondale, IL and later received her Ph.D. in Analytical Chemistry at Drexel University, Philadelphia, PA. Her research and expertise in the field of quantitative analysis using LC-MS/MS resulted in over fifty reviewed publications and fifty-six abstracts. Her scientific interests encompass various areas including analysis of unique matrices, unstable compounds, and high-throughput analysis in support of clinical trials.

Kasie Fang, MS (GSK)

Kasie is a principal scientist in Biomarker and Bioanalysis Department at GSK for 13 years. She is responsible for developing advanced LC-MS methodologies for the bioanalysis of NCE or large molecule drugs, metabolites and biomarkers to meet with rigorous regulatory requirements. Her research interests include ADC, Oligo, pro-drug, intracellular anabolite analysis as well as patient centric microsampling. Kasie obtained two master's degree in Polymer Science from China and Analytical Chemistry from California. She had also worked in a biotransformation group at Merck for 4 years prior to joining GSK.

Yipei Zhang, Ph.D. (Takeda)

Scientist II - Senior Scientist at Takeda Pharmaceuticals since 2022, working within the Bioanalytical Science & Immunogenicity group in the DMPK&M department. Previously, served as a Scientist - Senior Scientist at Kala Pharmaceuticals from 2018 to 2022. Holds a Ph.D. in Analytical Chemistry from the University of Massachusetts Lowell in 2018. At Takeda, specializes in developing LC/MS-based bioanalytical assays to characterize small and large molecule drugs in biological matrices to support PK and TK studies.

David Zuluaga, MS (Resolian)

Senior scientist and team lead of method development at Resolian, with an M.S. in biochemistry from the National Autonomous University of Mexico, focused on the quantitation of large molecules such as oligonucleotides, ADCs, and biomarkers within a regulated framework. Previously worked at Eurofins in the protein characterization group. With over 8 years of experience in the bioanalysis field.

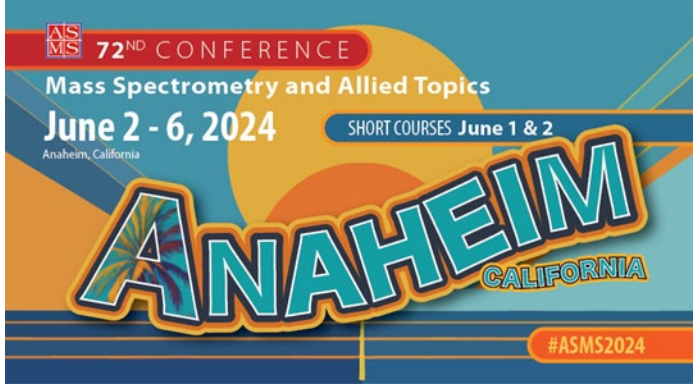
Yang Xu, Ph.D. (Merck)

Scientific Sr. Director and Lead of the Critical Reagent Group in Regulated Bioanalytics in PDMB at Merck, supporting both biologics and vaccine projects. Yang obtained her B.S. in Pharmaceutical Sciences from Beijing Medical University, M.S in Analytical Chemistry from Chinese Academy of Sciences, Ph.D. in Biochemistry from Wesleyan University, followed by postdoc fellowship in Molecular Biophysics and Biochemistry (MB&B) at Yale University. Since joined Merck in 2001, Yang developed key capabilities in Reg. BA, including microsampling implementation in GLP and clinical studies, immunoaffinity purification (IP) coupled with LC/MS for peptide/protein quantification, while her work using the hybrid IP-LC/MS/MS clinical PK assay enabled biosimilar approval of LusdunaTM NexvueTM. Outside of Merck, Yang serves as the co-chair for Critical Reagent Working Group in AAPS BA Community. She has over 50 publications in peer-reviewed journals and over 20 invited oral presentations at scientific meetings.

Min Meng, Ph.D. (Resolian)

Dr. Min Meng, the COO and President APAC in Resolian Bioanalytics, has more than 25 years of working experience in the US and China CRO industries. She has served as Founder/CEO for a Chinese bioanalytical CRO, Chongqing Denali Medpharma, and the Laboratory and Technical Director of Covance/Labcorp, Salt Lake City. She has extensive bioanalytical experience in various drug modalities, developed and supervised over thousands of bioanalytical methods using LC-MS/MS, LC-HRMS and published many academic articles and book chapters. Dr. Meng is the editorial member of Journal of Bioanalysis and has been very active in the bioanalytical community for regulatory insights and interpretation, promoting best practice and training hundreds of young bioanalytical scientists. Dr. Meng received her bachelor and MS degrees from Beijing Normal University and Ph.D. from the University of Maryland School of Pharmacy.

Agenda



05:45 - 05:50 pm – Introduction

*05:50 – 06:00 pm - **Challenges in ADC Bioanalysis***

- Cynthia Chavez-Eng, Ph.D. (Merck)

*6:00 – 06:10 pm - **Bioanalysis of Payload -- Challenges and Case Study***

- Kasie Fang, MS (GSK)

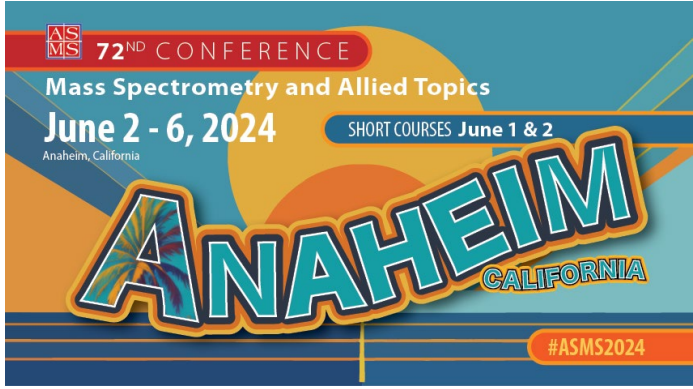
*06:10 – 06:20 pm -**A 2-in-1, two-cycle immunoaffinity enrichment LC/MS assay strategy for bioanalysis of an iADC***

- Yipei Zhang, Ph.D. (Takeda)

*06:20 – 06:30 pm - **Translational Challenges in ADC Bioanalysis: Preclinical Insights to Clinical Implementation***

- David Zuluaga, MS / Min Meng, Ph.D. (Resolian)

06:30 – 7:00 pm - *Panel discussion*



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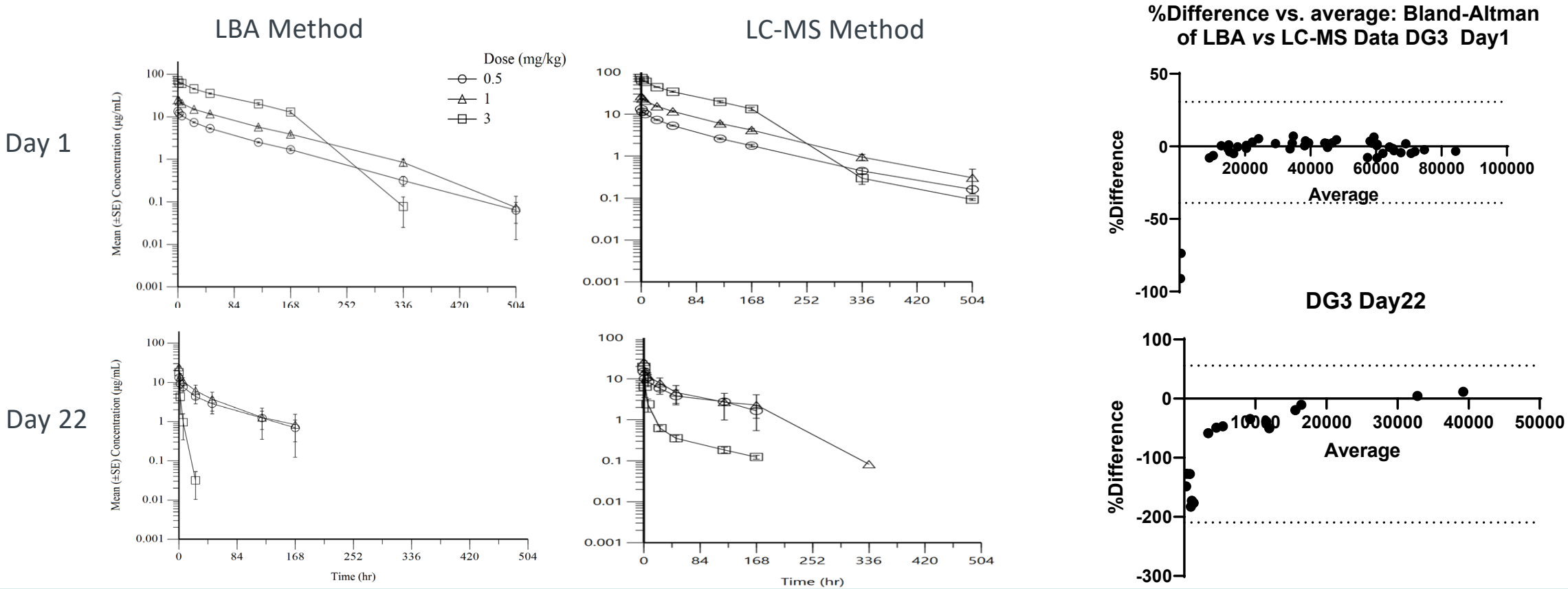
Challenges in ADC Bioanalysis by LC-MS

- **Multiple analytical assays to characterize TK/PK of ADCs**
  Multiple target analytes: total antibody, antibody drug conjugate, and free payload
  Impact of drug to antibody ratio (DAR) values
- **Sample Preparation**
  Complex extraction methods – Immuno capture, digestion
  Stability – integrity of the ADCs during sample collection and preparation
  ADA and/or soluble target interference with the assay
- **LC-MS**
  Resulting analytes (surrogate peptides) are more hydrophilic; lower MS ionization efficiencies
  Non-specific binding; low-retention on RP-LC
  Very low LLOQ required for free payload (low pg/mL)
- **Critical Reagents** – Takes time to generate capture reagent: Anti ID or target antigen; stable labeled internal standards (SIL-ADC), or SIL- surrogate peptide, SIL-payload

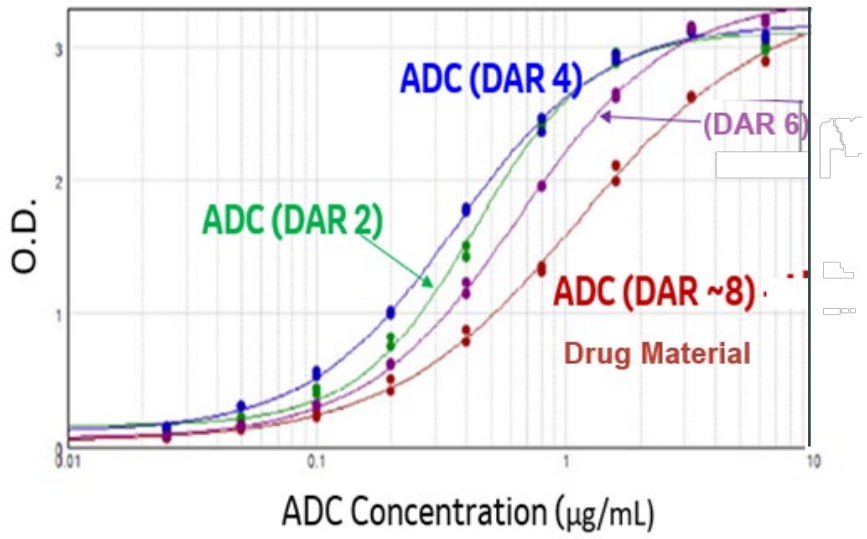
Case #1: Orthogonal technique (LC-MS/MS) Used to Assess Impact of ADA on TK of ADC-A Determined by LBA

- **LBA data (ADC Concentrations, µg/mL) corroborated by LC/MS/MS**



- **Fast Clearance mediated by ADA formation. ADA interference with LBA was limited to high ADA samples. No impact on overall study results and calculated exposure multiple.**
- **Switch PK assays to LC-MS/MS platform for future pre-clinical and clinical (FIH) study support**

Case #2: Assay DAR Sensitivity and Instability of ADC-B



- As payload is released in-vivo , DAR changes, hence for DAR sensitive assay –using a fixed DAR, *i.e.* 8 as calibrator, determined ADC concentrations of unknown samples are not likely to be accurate.
- Need to optimize method or switch to another assay platform to provide DAR insensitive assay.

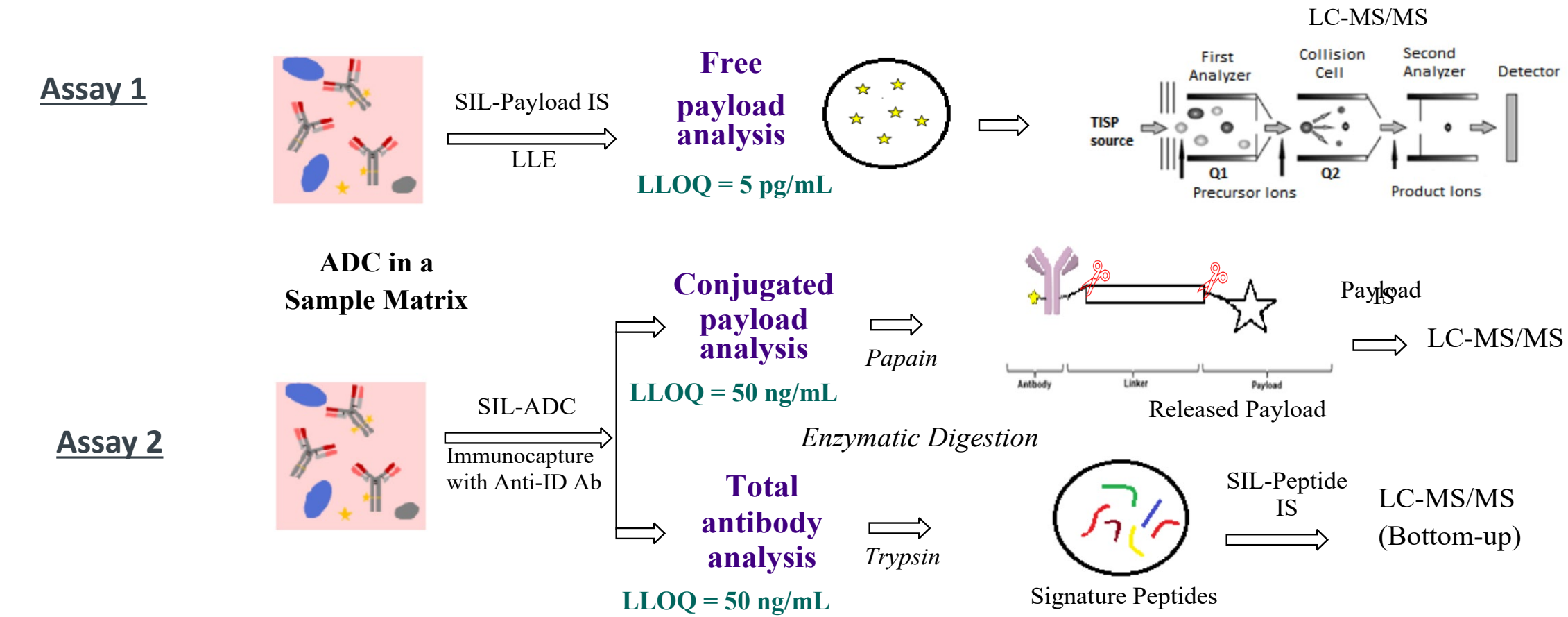
Instability of ADC-B

- Significant impact on measured free payload
  - *Ex-vivo release of payload will result in over-estimation of free payload and underestimation of ADC concentrations*
- Samples need to be stabilized that adds complexity in the clinic
- Required stability assessments

➢ Need for proper stabilization of ADC during collection/sample analysis for precise and accurate quantitation of analytes

➢ **Clinical Impact: DAR sensitive assay and inadequate stabilization of analytes during collection, storage, and sample processing will result in inaccurate characterization of PK, assessment of safety margin, and determination of efficacious dose.**

LC-MS based ADC Bioanalysis Strategy in Support of Clinical Studies



- **Critical Needs:**
  - Specific capture antibody (Anti-ID Ab)
  - SIL-ADC, SIL-payload
  - Sensitive Mass Spectrometer

- **Impact of Multiplexing:**
  - reduce sample volume, simplified collection a
  - minimize reagent consumption
  - increase sample throughput → quick data turn around time

Back-Up

Bioanalysis of ADCs with LCMS

- Increased specificity
  - Multiple aspects - sample preparation, LC separation, MS/MS
- Sensitivity
- Multiplexing - quantitate multiple analytes surrogate peptide (Ab), payload, and metabolite of payload if any
- Tool to assess *in vivo* structural modification, including deamidation, biotransformation, etc.
- Larger dynamic range compared to LBA assay
- Improved reproducibility, precision, and accuracy
- **Currently, Hybrid IP-LCMS bioanalysis of ADCs is our preferred approach for Clinical PK sample analysis**

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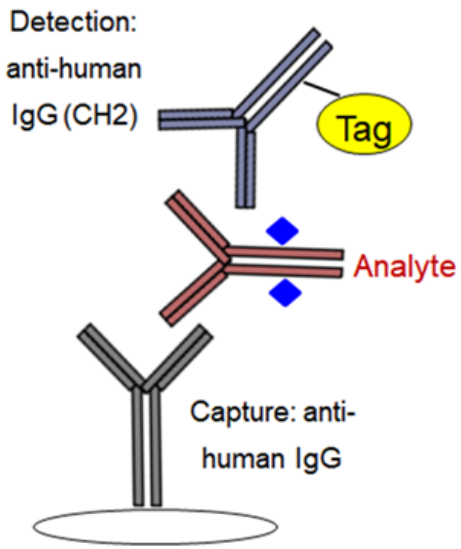
Case #1: Impact of ADA on TK of ADC-A in NHP Study

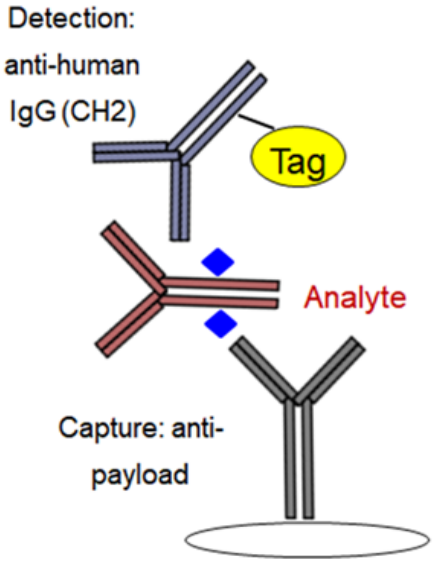
Study to determine potential toxicity and TK profile of ADC-A dosed IV every 3 weeks for 2 doses (administered on Day 1 and Day 22)

LBA Methods

Total antibody IgG (tAb)

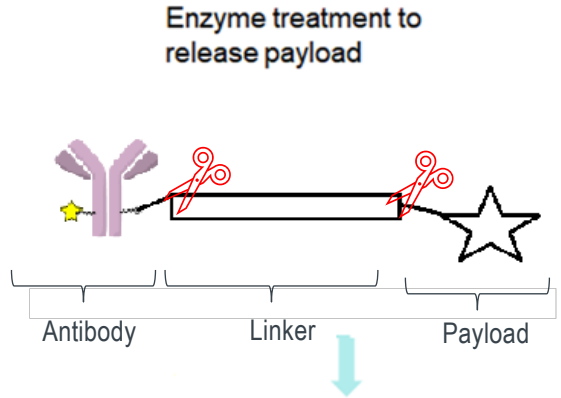


Conjugated antibody (cAb)



LC-MS Method

Conjugated drug (cDrug)



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Kasie Fang, MS (GSK)

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Bioanalysis of Payload -- Challenges and Case Studies

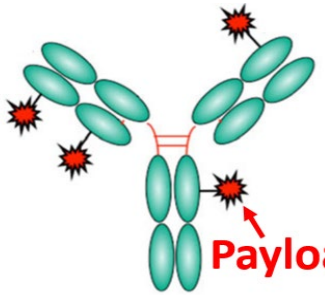
05-June-2024

Kasie Fang, Hermes Licea-Perez, Timothy Sikorski

gsk.com

72th ASMS Workshop: LC-MS Bioanalysis of Drug Conjugate

Challenges for Payload Bioanalysis



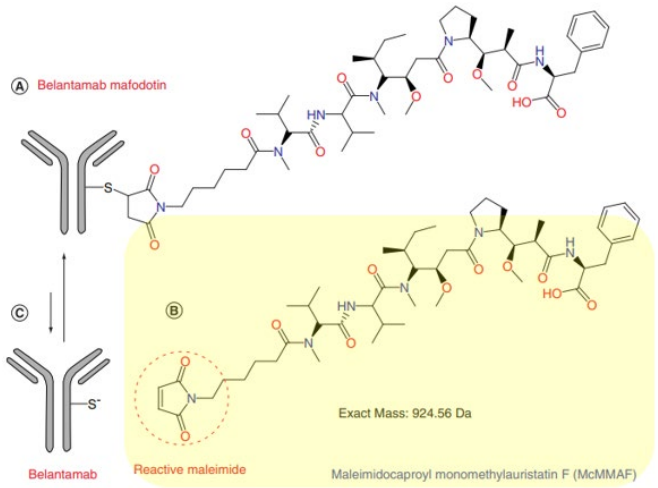
- What is the analyte of interest and sample composition?
- Sensitivity and selectivity requirements
- Analyte stability in the presence of ADC
- Interference and ADC purification
- Matrix effect and non-specific binding

Chemistry →

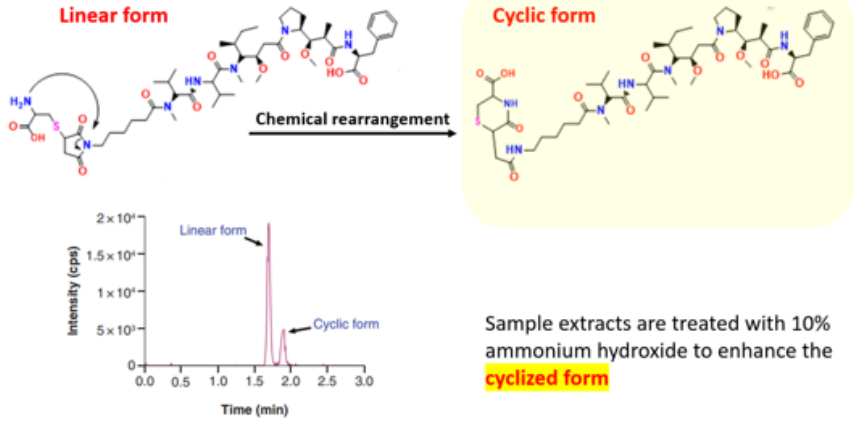
Conjugation	Lysine, cysteine, site-specific engineered cystine
Linker	▪ Uncleavable (thioether, maleimidocaproyl group)
	▪ Chemical cleavage (hydrazone bond, disulfide bond)
	▪ Enzyme cleavage (glucuronide bond, peptide bond)
Payload	Tubulin inhibitors (e.g., Auristatins) , DNA damaging agents (e.g., Exatecans), and immunomodulators (e.g., TLR agonists, STING agonists)

Case I: Prevent Deconjugation in Sample Preparation

ADC and Linker-payload

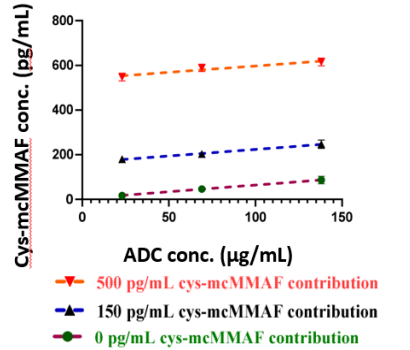


Cys-mcMMAF

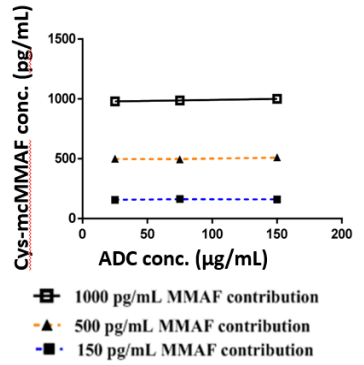


Sample extracts are treated with 10% ammonium hydroxide to enhance the **cyclized form**

SPE ⇨ Acidic PPT



	0 pg/mL cys-mcMMAF	150 pg/mL cys-mcMMAF	500 pg/mL cys-mcMMAF
Slope	0.6061 ± 0.06489	0.5870 ± 0.07936	0.5765 ± 0.1111
Y-intercept	3.783 ± 5.845	165.1 ± 7.147	540.0 ± 10.00



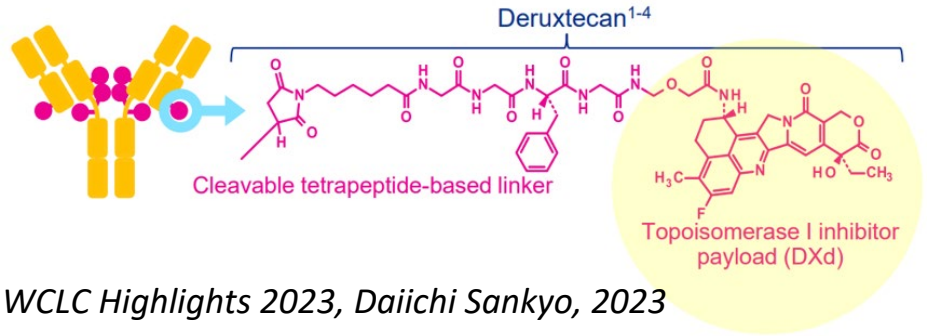
	150 pg/mL cys-mcMMAF	500 pg/mL cys-mcMMAF	1000 pg/mL cys-mcMMAF
Slope	0.01607 ± 0.02794	0.09777 ± 0.04855	0.1639 ± 0.09234
Y-intercept	157.8 ± 2.735	493.2 ± 4.753	975.2 ± 9.040

Analyte	**Cys-mcMMAF (50 pg/mL)**
Conjugation/linker	Cysteine conjugation; Uncleavable linker-payload "mcMMAF" (maleimidocaproyl-monomethyl auristatin F) could react with free cystine to form Cys-mcMMAF
Structure alert	Linear vs. cyclic through isomerization reaction
Major Interference	Deconjugated payload during SPE
Sample extraction	**Acidic protein precipitation (PPT) to remove ADC earlier in sample preparation**
Stability control	Low temperature; Incubation at basic condition to complete cyclization

A Mayer, H Licea-Perez, et.al. Bioanalysis (2021) 13(18)

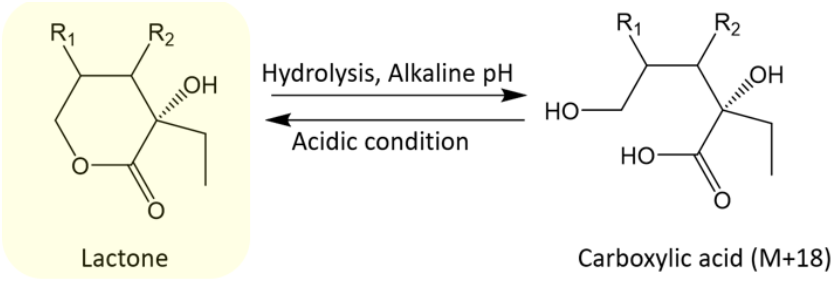
Case II: ADC Purification to Remove Impurity

ADC, Linker and Payload

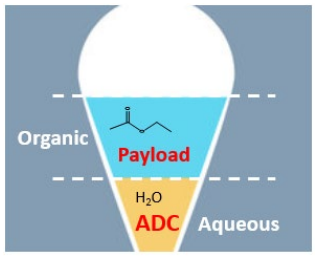


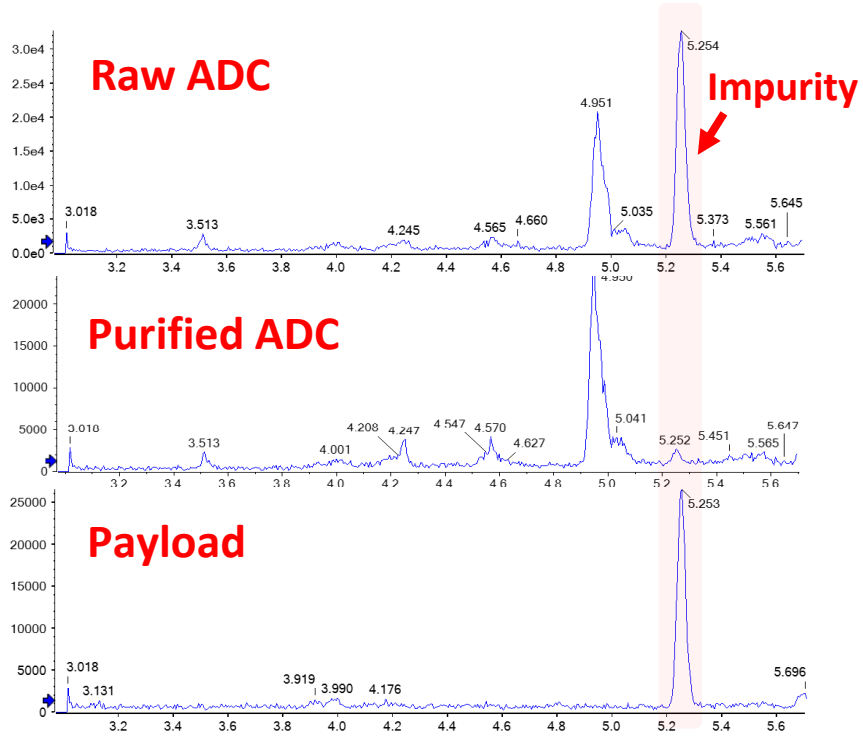
WCLC Highlights 2023, Daiichi Sankyo, 2023

Payload



Analyte	Deruxtecan derivative (50 pg/mL)
Conjugation/Linker	Cysteine-conjugation; Cleavable tetra-peptide linker
Structure alert	Lactone vs. carboxylic acid form
Interference	Payload impurity from ADC reference standard
ADC purification for stability test	**2xLLE (ethyl acetate) to remove interference**
Sample extraction	Protein precipitation + SPE (MAX)
Stability control	Low temp; Acidic pH for reconstitution in the last step





Conclusions

- Understand ADC conjugation, linker and payload chemistry is the key to determine analyte of interest and method development strategy
- Design sample extraction proactively to prevent ADC deconjugation during process
- Evaluate analyte stability in the presence of purified ADC after impurity removal

Acknowledgement

Hermes Licea-Perez

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Minjoo Jung

Sharon Boram

Christopher A Evans
(Now at Adaptimmune)

Clara Andonian

Kristen E Pannullo

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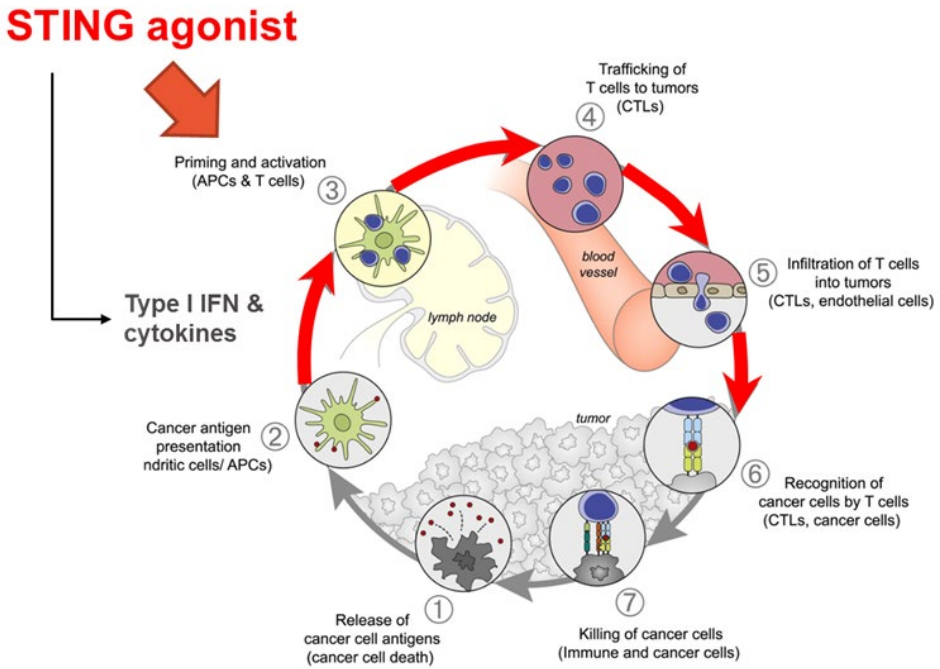
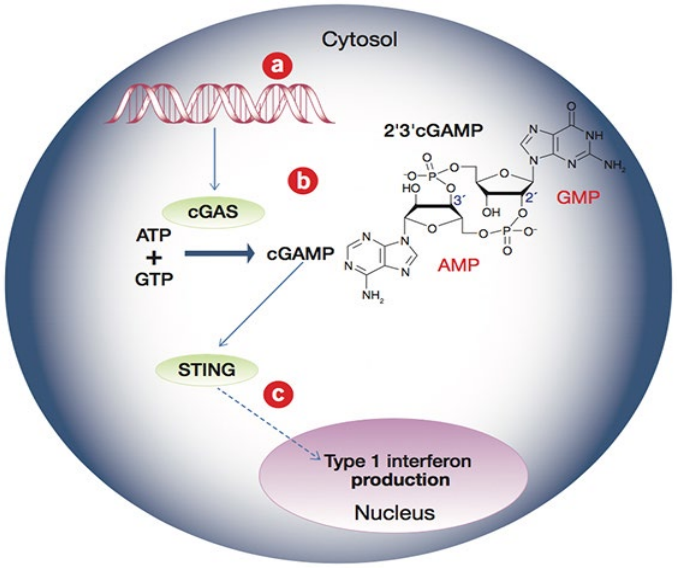
A 2-in-1, two-cycle immunoaffinity enrichment LC/MS assay strategy for bioanalysis of an iADC

Yipei Zhang, Mark Qian, Linlin Dong

DMPK&M, Takeda Development Center America, Inc.

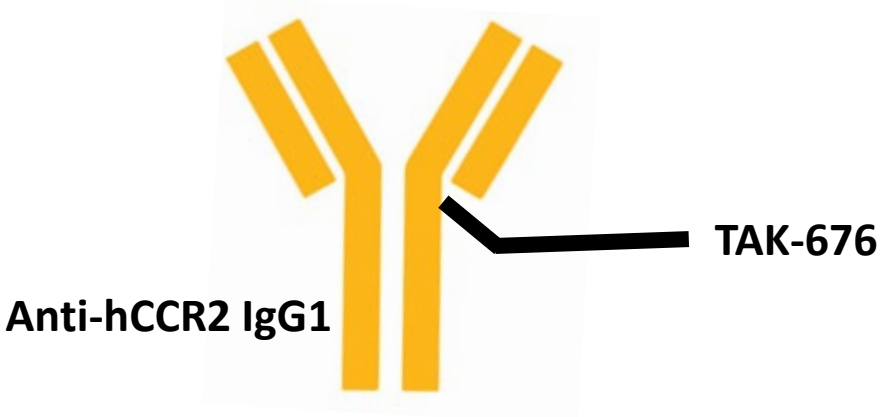
STimulator of INterferon Genes Catalyzes Cancer Immunity Cycle





Cytosolic DNA induces type-I interferons

Chen et al. Immunity. 2013 Jul 25;39(1):1-10



TAK-500, an immune-cell directed antibody drug conjugate (iADC) for targeted delivery of the STING agonist, TAK-676, to tumors

BA Strategy for iADC

- ➢ PK/TK assay support (≥3 analytes)
  - Total antibody
  - Conjugated payload or antibody
  - Free or deconjugated payload + metabolites

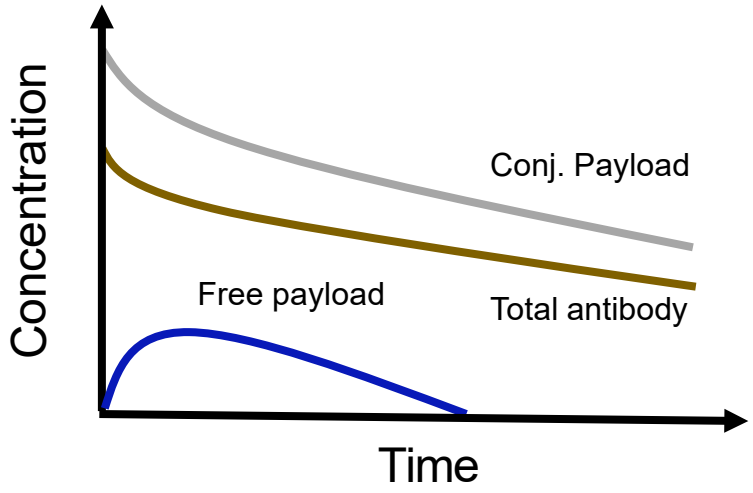


Table 1. The advantages and challenges of the ELISA-based immunoassay platform and hybrid ligand binding LC-MS (liquid chromatography-mass spectrometry) platform.

Assay	Advantage	Challenges
LBA	Sensitive quantitative analysis for large molecules	DAR insensitive; does not provide measurement of the DAR or the overall drug load
	Low equipment cost	Typically not sensitive to biotransformation
	High throughput	Specificity and selectivity is determined only by the capture and detection antibodies
	Easy to implement	Lack of structural/sequence information of the ADCs
		Potential cross-reactivity between antibodies in a multiplexed immunoassay
		Limited multiplexing capability
		Time-consuming to develop highly selective and specific antibodies
Hybrid LBA-LC-MS	Sensitive quantitative analysis for complex biotherapeutics such as ADCs	Relatively higher equipment cost compared to LBA assays
	DAR sensitive–able to measure DAR/drug load	Complexity of instrument operation and data interpretation
	Specificity and selectivity achieved using antibody capture, chromatographic separation and characteristic fragmentation of surrogate peptides	Lower throughput due to additional steps such as proteolytic digestion and chromatographic separation requiring samples to be injected one at a time
	Able to provide ADC analyte structure information	Relatively low sensitivity for intact ADC analysis
	Can be sensitive to biotransformation	Reliance on surrogate analytes for quantification
	Could be highly multiplexed; many analytes can be analyzed at a time in a single LC-MS analysis	

LBA, ligand-binding assays; DAR, drug-antibody ratio; ADCs, Antibody-drug conjugates; LBA-LC-MS, LBA-liquid chromatography coupled with mass spectrometry.

Antibodies **2018**, *7*(4), 41; https://doi.org/10.3390/antib7040041

2-in-1 iADC Assay to Quantify Total Antibody and Conjugated Payload

❑ **Papain digestion conditions**

Previously: 37°C for 3 hr at pH 6.5

Now: 37°C for 1 hr at pH 6.5

❑ **Trypsin/Lys-C digestion (with Rapigest®) to Rapid Digestion**

Previously: 37°C for 1 hr at pH 8 (microwave assisted)

Now: 70°C for 1 hr at pH 8

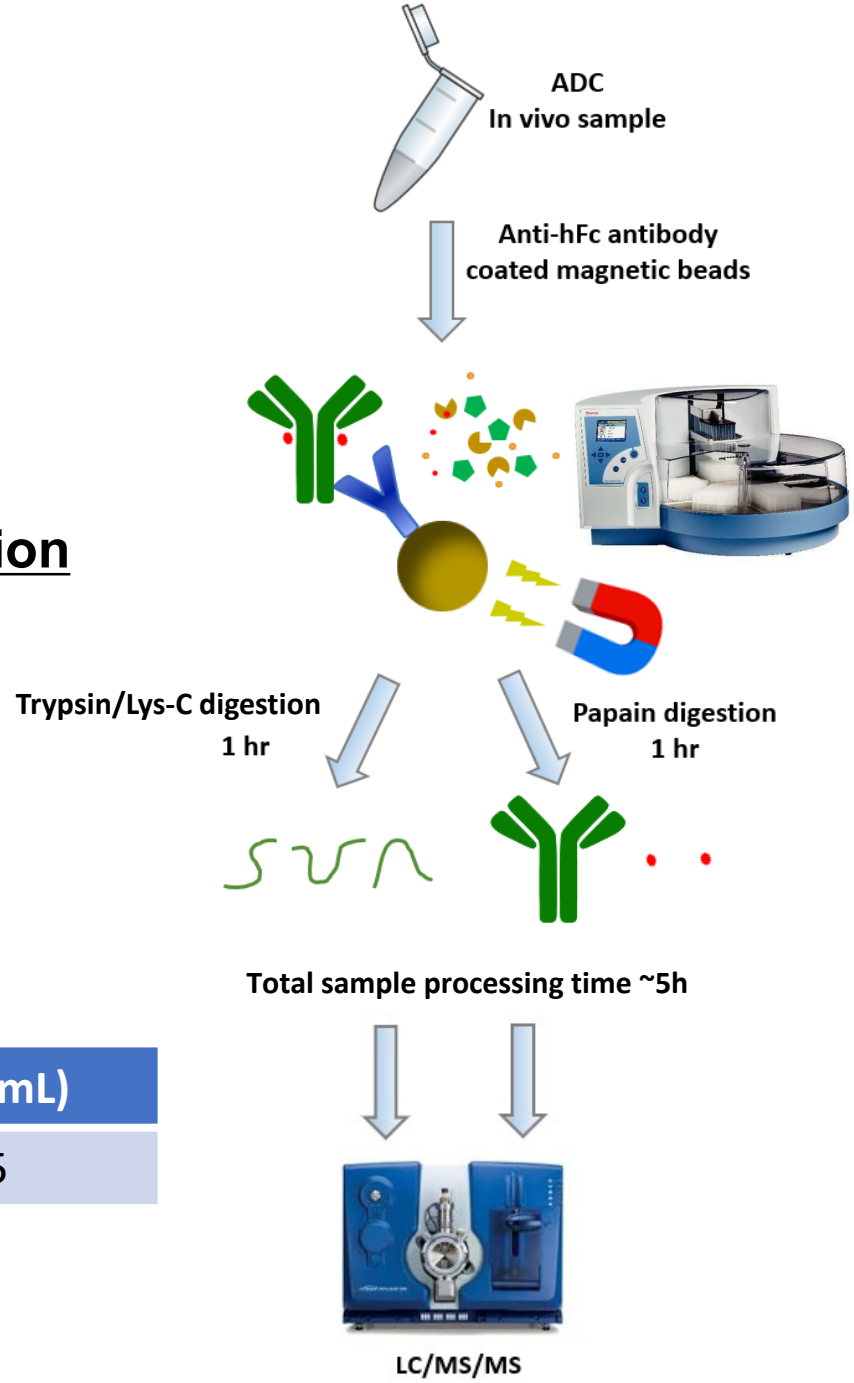
❑ **New parallel digestion workflow saves time**

4 hr → 1 hr

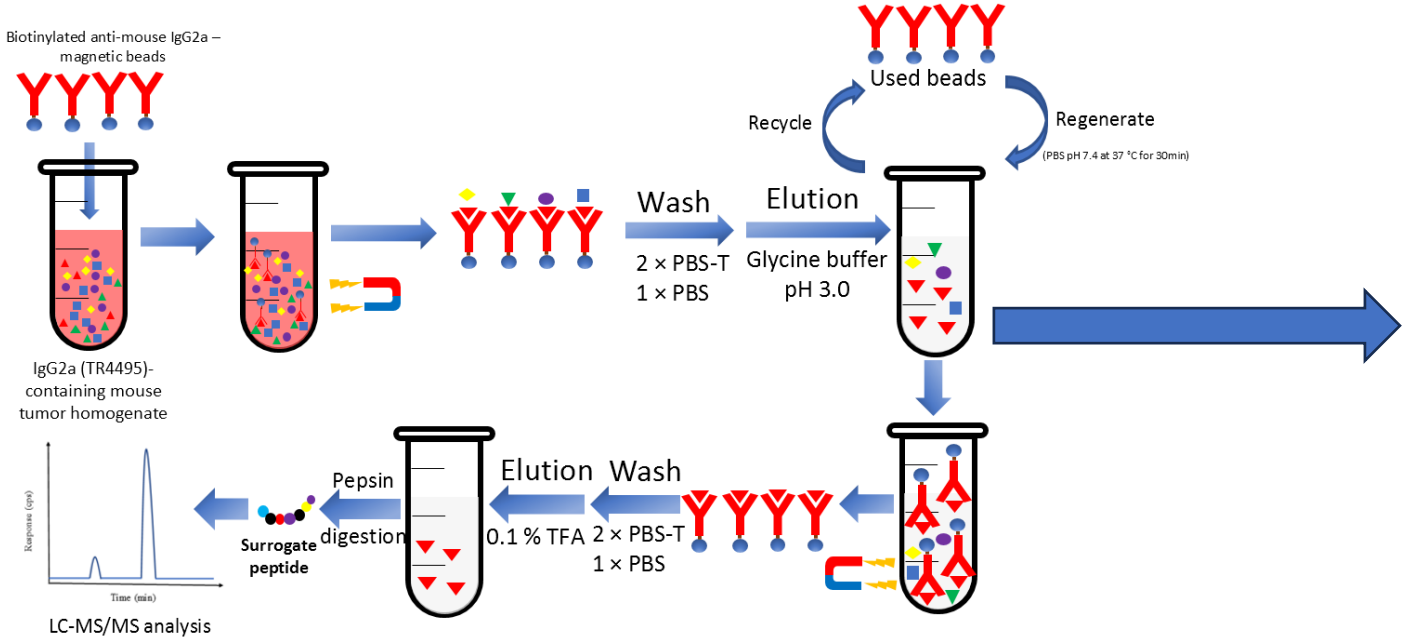
❑ **Assay sensitivity:**

Analyte	Sample volume (µL)	LLOQ (ng/mL)
Total antibody/ Conjugated TAK-676	50	30/0.55

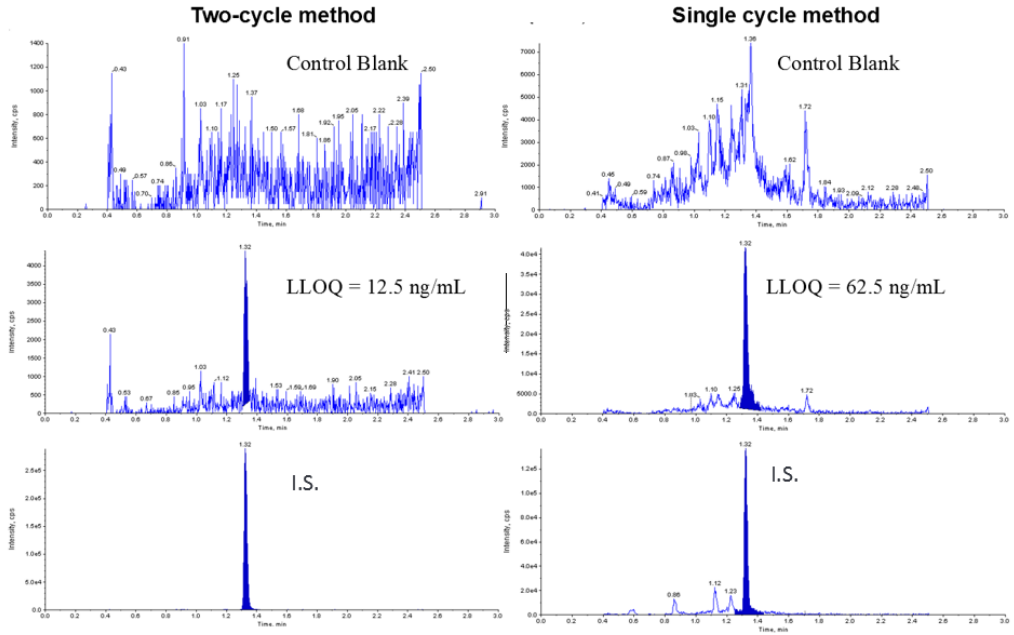
*Linear range based on antibody concentration: 30-10000 ng/mL



A Novel Two-Cycle Immunoaffinity Enrichment Method to Enhance Assay Sensitivity of STING ADC in tissues



Total antibody sensitivity for TAK-500 in mouse tumor samples increased fivefold by using this two-cycle immunoaffinity enrichment method



Chromatograms of surrogate peptide for TAK-500 total antibody

Conclusion

- A 2-in-1 hybrid LC/MS assay has been developed for TAK-500, enabling the measurement of both total antibody and conjugated payload within a single immunoaffinity enrichment
- A novel two-cycle immunoaffinity enrichment method was utilized for analyzing tissue samples, resulting in a 5x enhancement in sensitivity for total antibody measurement

David Zuluaga, MS (Resolian)

Senior scientist and team lead of method development at Resolian, with an M.S. in biochemistry from the National Autonomous University of Mexico, focused on the quantitation of large molecules such as oligonucleotides, ADCs, and biomarkers within a regulated framework. Previously worked at Eurofins in the protein characterization group. With over 8 years of experience in the bioanalysis field.

Min Meng, Ph.D. (Resolian)

Dr. Min Meng, the COO and President APAC in Resolian Bioanalytics, has more than 25 years of working experience in the US and China CRO industries. She has served as Founder/CEO for a Chinese bioanalytical CRO, Chongqing Denali Medpharma, and the Laboratory and Technical Director of Covance/Labcorp, Salt Lake City. She has extensive bioanalytical experience in various drug modalities, developed and supervised over thousands of bioanalytical methods using LC-MS/MS, LC-HRMS and published many academic articles and book chapters. Dr. Meng is the editorial member of Journal of Bioanalysis and has been very active in the bioanalytical community for regulatory insights and interpretation, promoting best practice and training hundreds of young bioanalytical scientists. Dr. Meng received her bachelor and MS degrees from Beijing Normal University and Ph.D. from the University of Maryland School of Pharmacy.

Translational Challenges in ADC Bioanalysis: Preclinical Insights to Clinical Implementation

David Zuluaga, MS.

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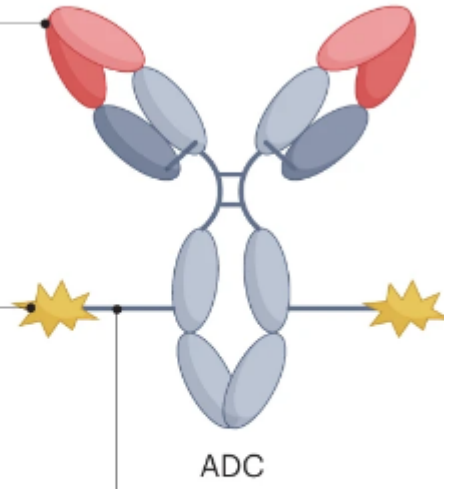
ADC Characteristics

Antibody (human IgG1 in general)
- High tumour specificity
- Long circulation life
- Rapid internalization
- With or w/o immune activation
- Minimal immunogenicity

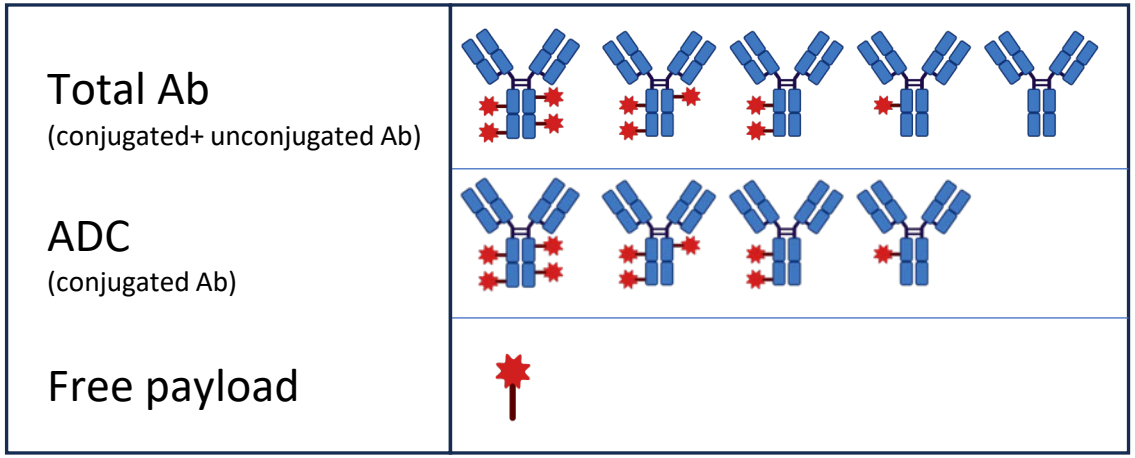
Payload
- Highly toxic compound
- Various mechanisms of action (such as microtubule inhibition and direct DNA damage)
- Bystander effect if hydrophobic
- Optimal DAR

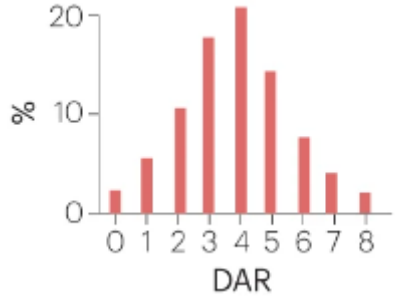
Linker and conjugation chemistries
- Links the monoclonal antibody and the payload
- Homogeneity
- Non-cleavable or cleavable
- Affects physicochemical properties, stability in circulation and potency



Tsuchikama, K., Anami, Y., Ha, S.Y.Y. *et al.* Exploring the next generation of antibody–drug conjugates. *Nat Rev Clin Oncol* **21**, 203–223 (2024).

Matrix Characterization





ADC Approved by FDA until 2021

Name	FDA approval year	ADC assay format	Total antibody assay format	Unconjugated warhead assay format
Tisotumab vedotin-tftv	2021	LBA	LBA	LC-MS/MS
Loncastuximab tesirine-lpyl	2021	LBA	LBA	LC-MS/MS
Sacituzumab govitecan-hziy	2020	Derived[b]	LBA	LC-MS/MS
Belantamab mafodotin-blmf	2020	LBA	LBA	LC-MS/MS
Fam-trastuzumab deruxtecan-nxki	2019	LBA	LBA	LC-MS/MS
Polatuzumab vedotin-piiq	2019	Hybrid LBA LC-MS/MS[c]	N/A	LC-MS/MS
Enfortumab vedotin-ejfv	2019	LBA	LBA	LC-MS/MS
Inotuzumab ozogamicin	2017	LC-MS/MS[c]	N/A	LC-MS/MS
Trastuzumab emtansine	2013	LBA	LBA	LC-MS/MS

Mu R, Yuan J, Huang Y, et al. Bioanalytical Methods and Strategic Perspectives Addressing the Rising Complexity of Novel Bioconjugates and Delivery Routes for Biotherapeutics. BioDrugs. 2022;36(2):181-196. doi:10.1007/s40259-022-00518-w

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ADC Preclinical Strategy by LC-MS

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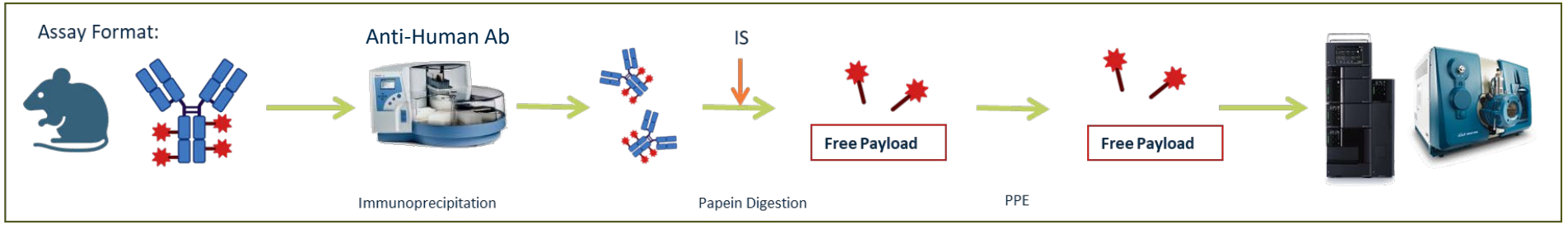
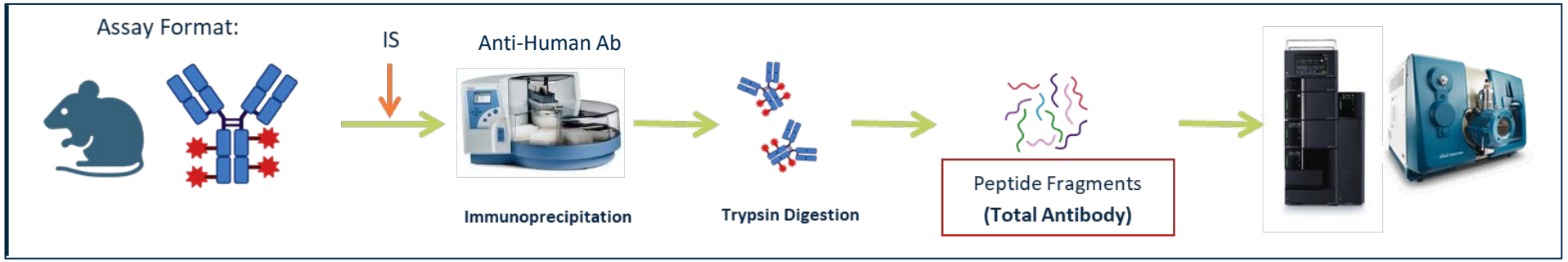
Free payload

ADC
(conjugated Ab)



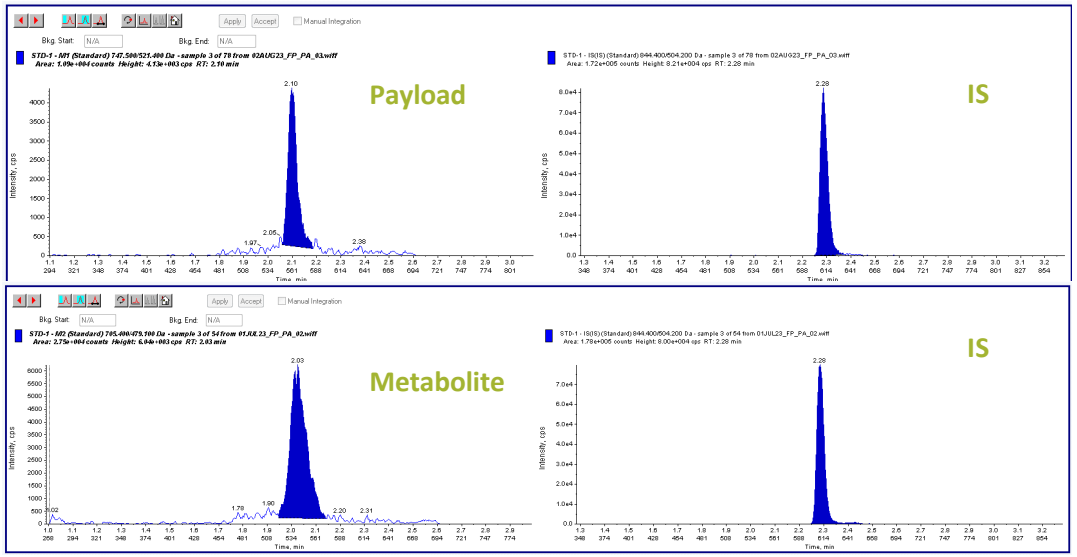
Total Ab
(conjugated+ unconjugated Ab)



*Short development time and applicable to a wide number of ADCs.

Case Study ---ADC by LCMS

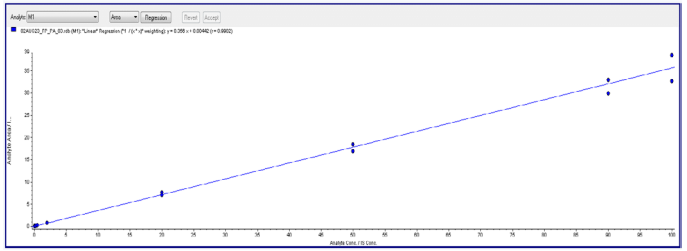
Unbound Payload and Metabolite Assay



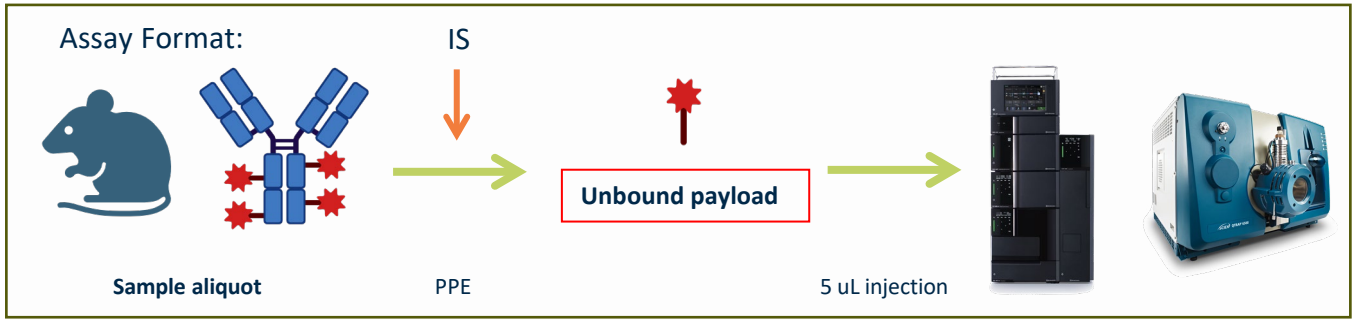
LLOQ Chromatograms

Concentration (ng/mL)				
Batch ID	**LLOQ-QC**	**LQC**	**MQC**	**HQC**
	0.100	**0.300**	**100**	**800**
n	18	18	18	18
Mean	0.100	0.300	104	844
SD	0.01	0.02	0.29	3.19
CV(%)	10.1	6.2	2.8	3.8
Accuracy(%)	104.8	100.1	104.2	105.5

	Concentration (ng/mL)							
Batch ID	**0.100**	**0.200**	**0.500**	**2.00**	**20.0**	**50.0**	**90.0**	**100**
n	5	6	6	6	6	6	6	6
Mean	0.100	0.200	0.480	2.02	20.8	51.1	91.0	95.8
SD	0.01	0.03	0.05	0.13	0.76	2.25	4.34	8.17
CV (%)	5.8	12.6	10.1	6.5	3.6	4.4	4.8	8.5
Accuracy(%)	101.3	99.5	95.5	100.9	103.9	102.2	101.1	95.8



Calibration curve: Range: 0.1 - 100 ng/mL



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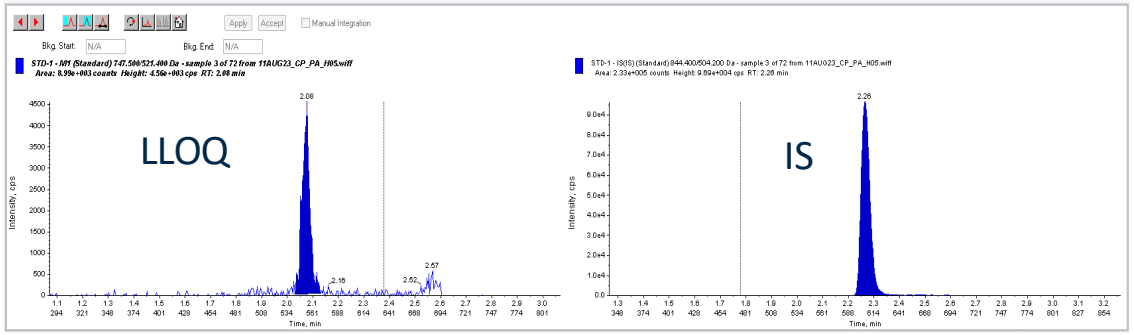
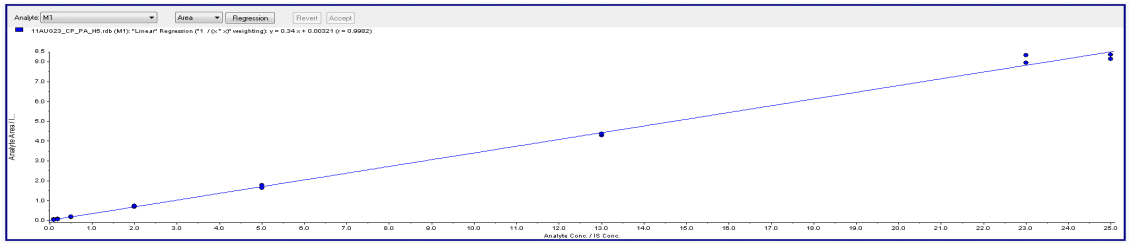
Case Study ---ADC by LCMS

➢ Conjugated Payload Assay





Calibration curve: Range: 0.100 – 25.0 µg/mL

Concentration (ug/mL)				
Batch ID	**LLOQ-QC**	**LQC**	**MQC**	**HQC**
	0.100	**0.300**	**2.50**	**20.0**
n	18	18	18	18
Mean	0.100	0.330	2.70	20.7
SD	0.01	0.02	0.18	1.22
CV(%)	8.6	7.2	6.6	5.9
Accuracy(%)	103.1	109.2	107.9	103.3

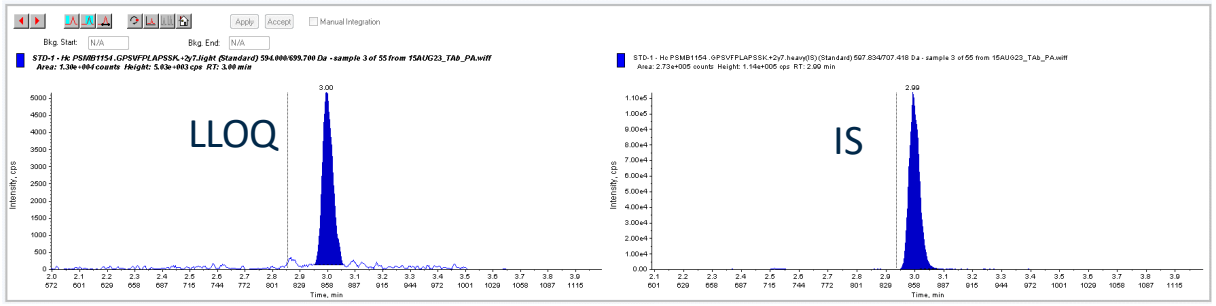
	Concentration (ug/mL)							
Batch ID	**0.100**	**0.200**	**0.500**	**2.00**	**5.00**	**13.0**	**23.0**	**25.0**
n	6	6	6	6	6	6	6	6
Mean	0.100	0.200	0.510	2.04	5.10	12.6	23.5	23.8
SD	0.004	0.013	0.025	0.054	0.201	0.393	0.834	0.578
CV (%)	3.8	6.4	4.9	2.6	3.9	3.1	3.6	2.4
Accuracy(%)	99.5	100.1	101.8	102.2	102.0	97.2	102.0	95.3

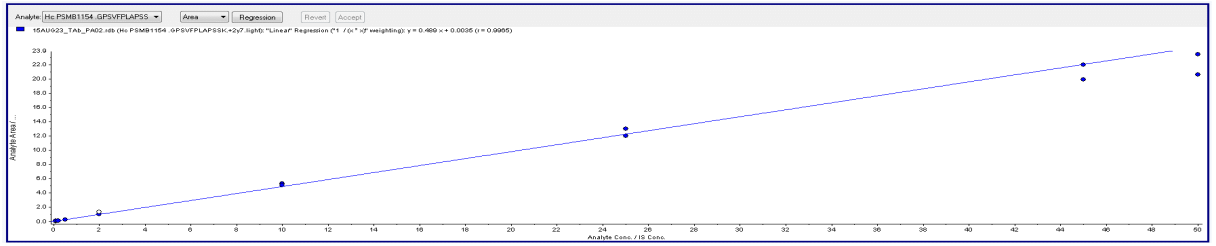
Assay Format:

25 uL Aliquot | Immunoprecipitation | Papein Digestion | PPE | 5 uL Injection

Case Study ---ADC by LCMS

➢ Total Antibody Assay





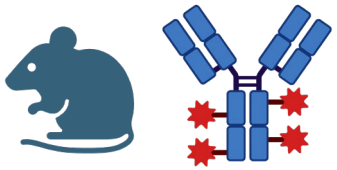
Calibration curve: Range: 0.100 – 50.0 ug/mL

Batch ID	Concentration (µg/mL)			
	LLOQ-QC	LQC	MQC	HQC
	0.100	**0.300**	**5.00**	**40.0**
n	17	18	18	18
Mean	0.103	0.323	4.95	40.7
SD	0.01	0.03	0.50	3.98
CV(%)	10.8	9.5	10.1	9.8
Accuracy(%)	102.7	107.5	99.0	101.7

	Concentration (µg/mL)							
Batch ID	**0.100**	**0.200**	**0.500**	**2.00**	**10.0**	**25.0**	**45.0**	**50.0**
n	6	6	6	5	6	6	6	5
Mean	0.0992	0.204	0.493	2.03	10.5	25.2	43.7	47.2
SD	0.01	0.01	0.05	0.16	0.62	1.35	3.16	3.16
CV (%)	5.9	5.7	9.1	7.9	5.8	5.4	7.2	6.7
Accuracy(%)	99.2	102.2	98.6	101.6	105.4	100.9	97.1	94.4

Signature Peptide Selection

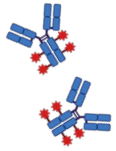
Assay Format:



25 uL Aliquot

IS

Immunoprecipitation

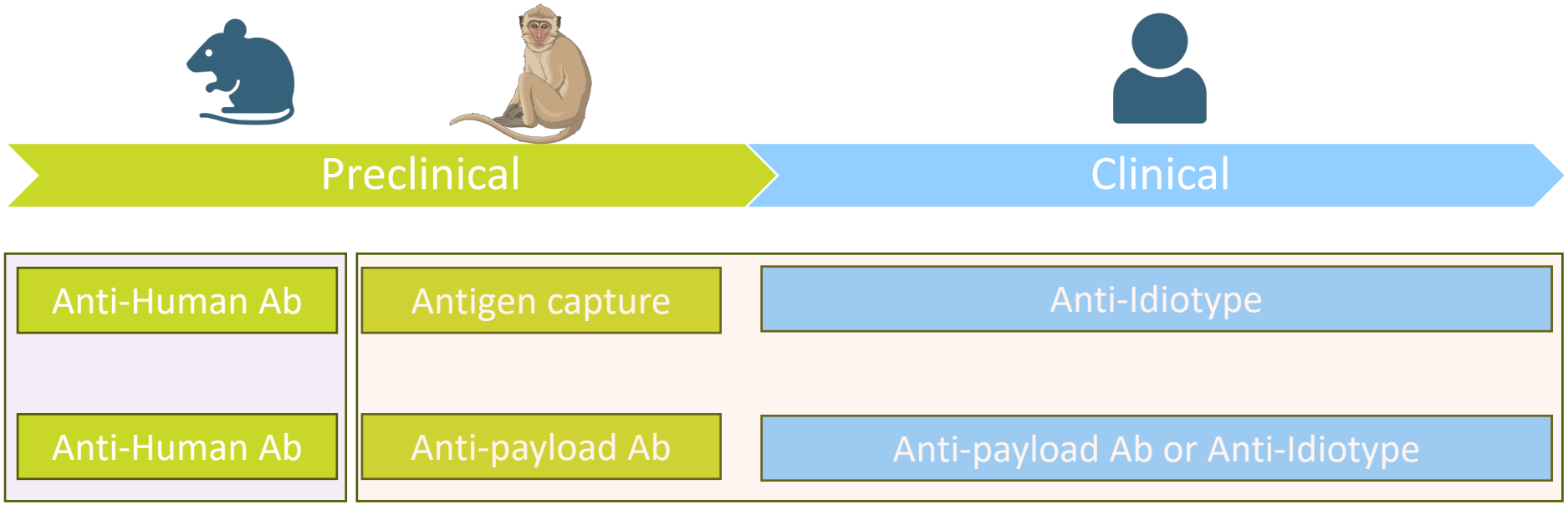


Trypsin Digestion

Peptide Fragments
(Total Antibody)

5 uL Injection

ADC Capturing Reagent Development by LCMS



Preclinical

- Capturing strategy can uses anti-human generic Ab.
- Can be develop in short time frame.
- Metabolites need to be evaluated, if significant it need to be quantified.
- Starting methods are in place and can be tailored to a particular ADC.
- No soluble target expected.

Clinical

- Capturing strategy uses anti-idiotype Ab and anti-payload Ab.
- Long timeframe for reagent creation.
- Metabolites need to be evaluated in free payload assay and conjugated payload.
- Need to evaluated the impact of soluble target in quantitation.
- Sensitivity need to be increased.

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Thank you !

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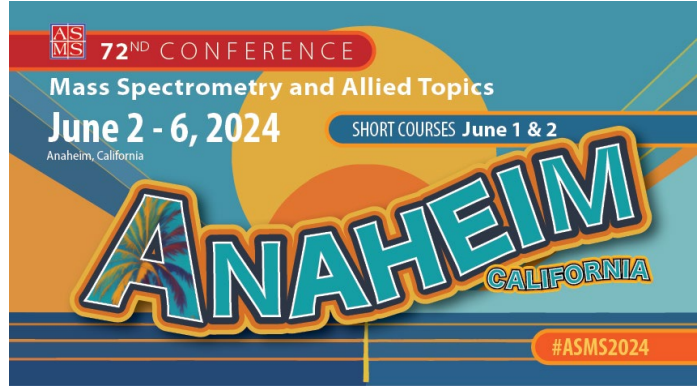

Yang Xu, Ph.D. (Merck)

Scientific Sr. Director and Lead of the Critical Reagent Group in Regulated Bioanalytics in PDMB at Merck, supporting both biologics and vaccine projects. Yang obtained her B.S. in Pharmaceutical Sciences from Beijing Medical University, M.S in Analytical Chemistry from Chinese Academy of Sciences, Ph.D. in Biochemistry from Wesleyan University, followed by postdoc fellowship in Molecular Biophysics and Biochemistry (MB&B) at Yale University. Since joined Merck in 2001, Yang developed key capabilities in Reg. BA, including microsampling implementation in GLP and clinical studies, immunoaffinity purification (IP) coupled with LC/MS for peptide/protein quantification, while her work using the hybrid IP-LC/MS/MS clinical PK assay enabled biosimilar approval of LusdunaTM NexvueTM. Outside of Merck, Yang serves as the co-chair for Critical Reagent Working Group in AAPS BA Community. She has over 50 publications in peer-reviewed journals and over 20 invited oral presentations at scientific meetings.

Considerations for critical reagents used in ADC PK/ADA/NAb assays

- Target capture – pros and cons?
- Antibody to mAb of ADC
  - LBA
    - blocker vs. non-blocker for PK vs. ADA PC vs. Nab PC?
    - pAb vs. mAb as ADA/NAb PC
    - DAR sensitive?
  - LCMS – sensitivity, linearity, amount vs. cost?
- Antibody to payload – stability of payload (e.g. PNU)?
- Internal standard to LCMS assay
  - SIL payload
  - SIL peptide
  - SIL ADC - Pros and cons?

Q&A1

Choice of matrix and stabilization conditions

- Continuity from Pre-clinical to Clinical – **plasma or serum**
- Stability Assessments
- Determine which component is unstable (mAb, linker, payload)
  - Whole blood Stability – collection procedures
  - Sample handling/processing temperature
  - Storage conditions
  - Freeze/thaw

Q&A1

Payload assays

1. What is the best practice to evaluate the **payload stability** for the ADCs with cleavable or uncleavable linkers?
2. What would be a practical solution in clinical setting when the payload stability results are not acceptable?

Q&A T1

Platform Selection

- Which platform do you prefer to use for ADC bioanalytical support: **LC/MS or LBA**?
- What are the pros and cons of the LC/MS and LBA platforms?

Q&A2

2. Sample Preparation using Immuno Capture

- *Choice of capture reagent: Anti-ID or Target Protein?*
- Assessment of Recovery
- Assessment of Digestion efficiency
- *Choice of Internal standard*
- *(SIL-ADC or SIL-surrogate peptides and SIL-payload)*
- How to test presence of DAR bias without reference materials of different DARs?

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Q&A2

Payload assays

What is the best strategy to improve payload assay selectivity and sensitivity?

Q&A T2

1. What are the challenges for bioanalysis of ADC for **tissue samples**

- **Stability** in tissue homogenates
- Sensitivity of the assay for tissue samples

Q&A3

3. Conjugated Payload Measurement by enzymatic release of payload

- Standard curve concentrations of ADC or equivalent payload concentration?
- Concentrations of unknown reported as ADC or Conjugated Payload?

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Q&A3

ADC reference standard

1. What is the best strategy to purify ADC reference standard for the stability evaluation?
2. What is the best practice to quantify the purified ADC concentration?

Q&A T3

- Is the LC/MS based assay still the best for DAR determination?