Advanced Charge Heterogeneity Profiling of a Model ADC via icIEF Fractionation and Mass Spectrometry

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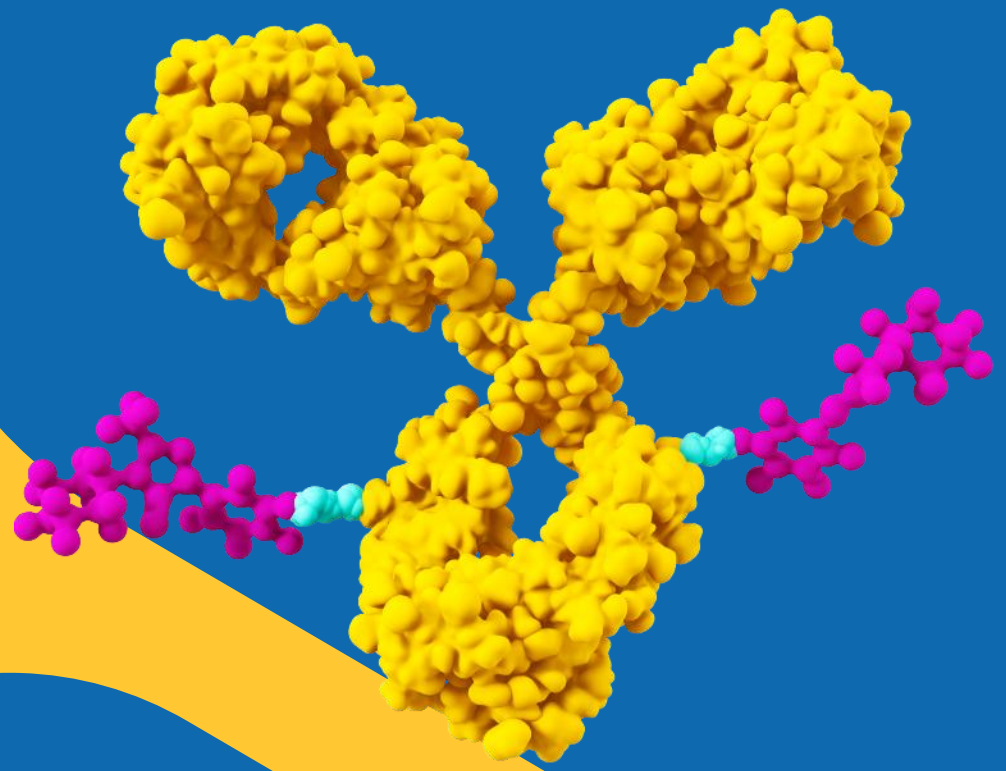
Process and Analytical Development (PAD) | Life Science | Life Science Services

September 10th, 2025



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IN SALES (2023)

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COUNTRIES

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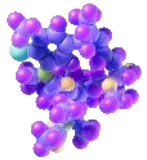
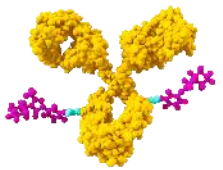


Small Molecules

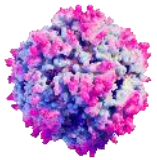
APIs, HPAPIs, Advanced int., aPEGs



ADC

Antibody-Drug and Bioconjugates

mRNA & LNP Formulation



Viral Vectors

Gene and Cell Therapies

Contract Testing Services

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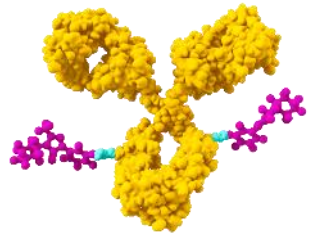
The PAD Experience
St. Louis Bio-Conjugation Capabilities



- Over 100 different constructs and **>1000 batches** in development
- Batch Scale **- 1mg to 750g** in development
- Transferred over 70 products to manufacturing – supporting over **70 INDs**
- Experience in 3 validated programs and 5 ongoing
- Significant experience in chromatographic purification development
- Broad conjugation chemistry history
  - Cysteine – stochastic and full reductive
  - Lysine conjugations
  - Site directed conjugation chemistries including:

    Engineered cysteines
    Enzyme-assisted
    Non-natural amino acids
    Engineered tags
    Metal-free click
    Glycan remodel platform

- Various Payloads and Linker chemistries:
  Cytotoxic, Immunotoxins, Oligonucleotides, Dyes, Antibiotics, Chelators
- Single-Use Reactor in PAD since 2017

01

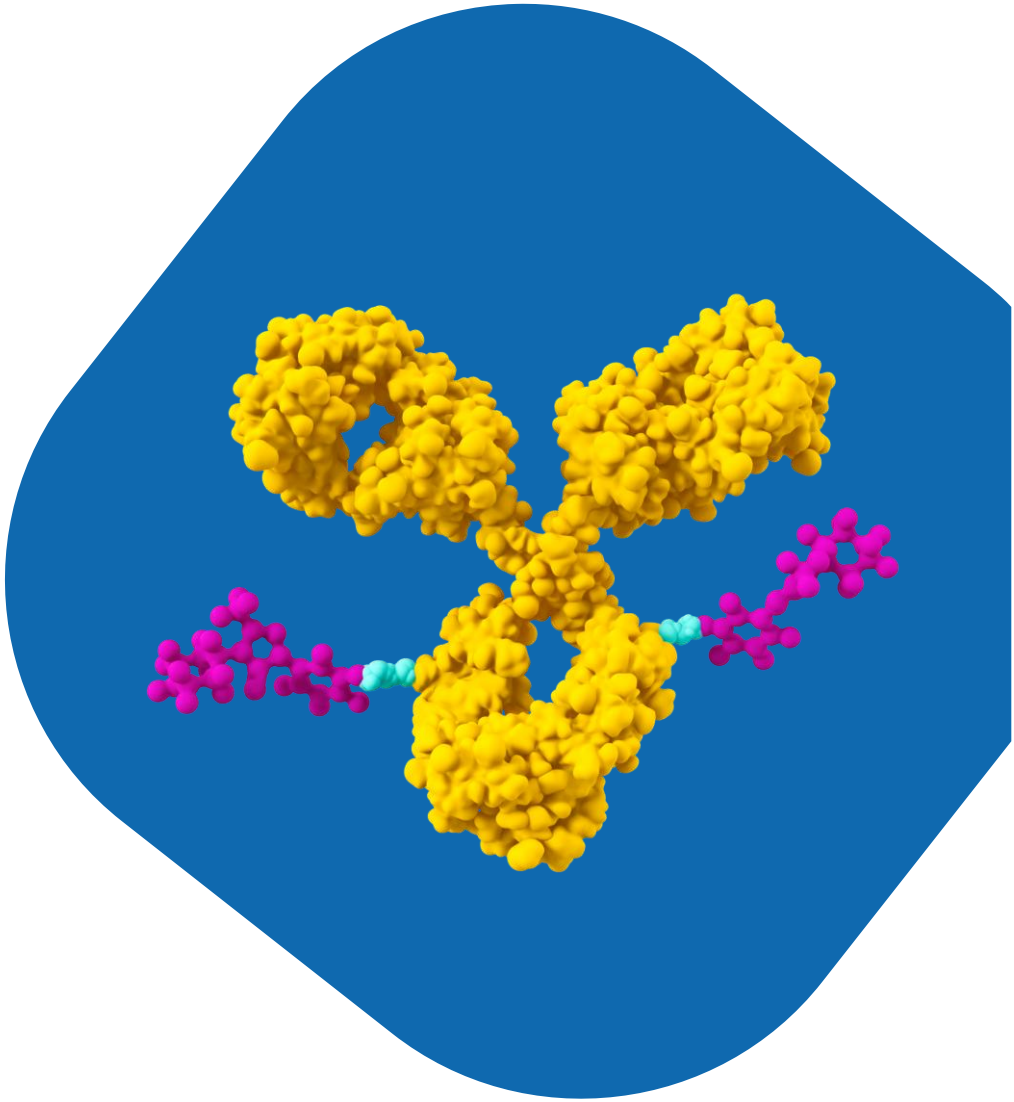
Background and Introduction

Background and Introduction
Antibody-Drug Conjugates (ADCs)

- An **Antibody-Drug Conjugate (ADC)** is a targeted cancer therapy that combines an antibody specific to cancer cells with a potent cytotoxic drug to selectively destroy those cells.

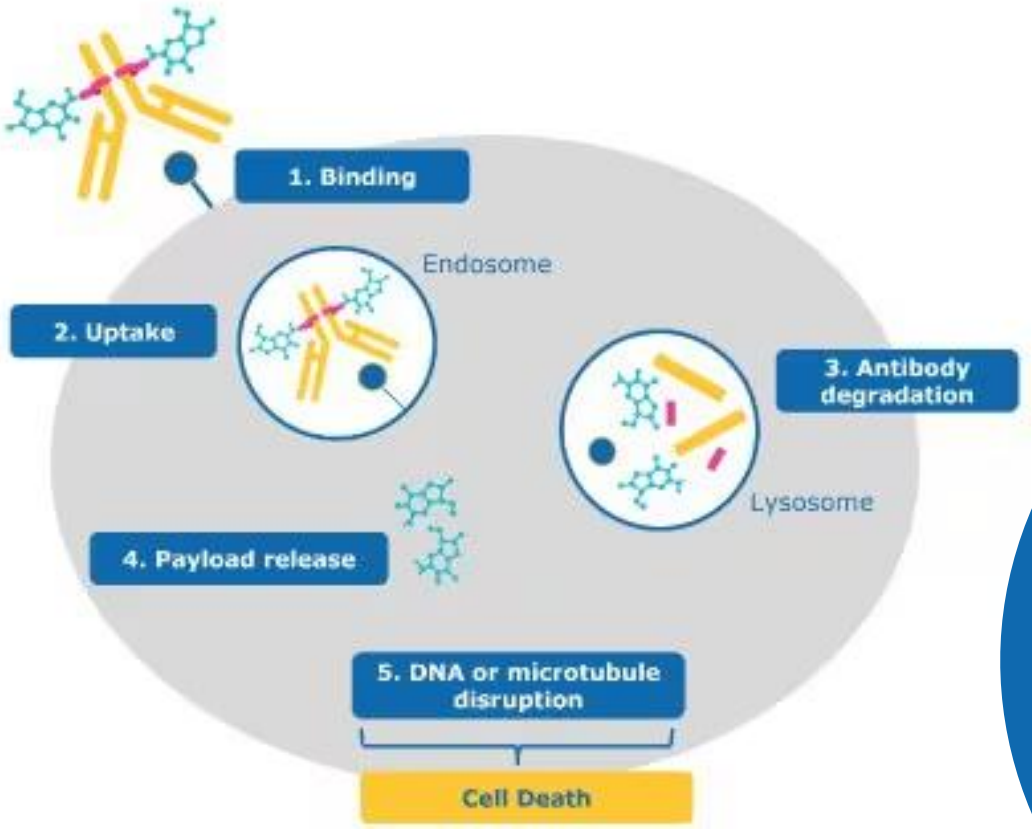
Three components of ADC:

1. An **antibody** targeting a specific receptor, unique to cancer cells.
2. A **linker** ensuring the payload remains attached until reaching the cancer cell.
3. A **payload** that is a highly potent anti-cancer agent.

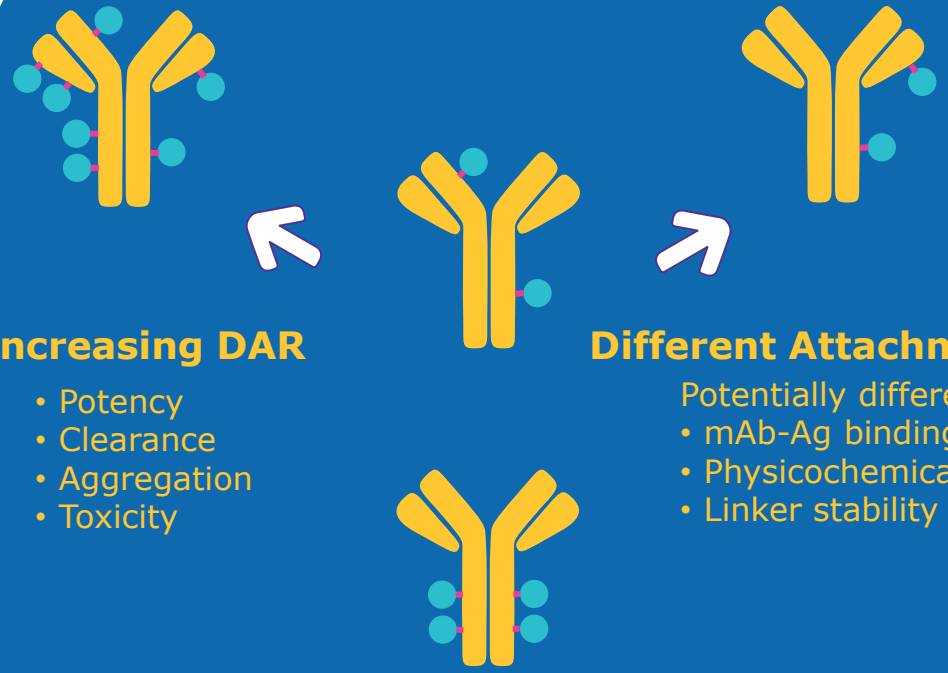


Background and Introduction
Mechanism of Action and Drug Attachment in ADCs

Mechanism of Action



Effects of DAR and Attachment Site



Increasing DAR

- Potency
- Clearance
- Aggregation
- Toxicity

Different Attachment Sites

Potentially difference in:

- mAb-Ag binding
- Physicochemical properties
- Linker stability

Equal DAR same Attachment sites → Optimal efficacy Optimal safety

9 Walsh, S. J., Bargh, J. D., Dannheim, F. M., Hanby, A. R., Seki, H., Counsell, A. J., ... & Spring, D. R. (2021). Site-selective modification strategies in antibody–drug conjugates. Chemical Society Reviews, 50(2), 1305-1353.

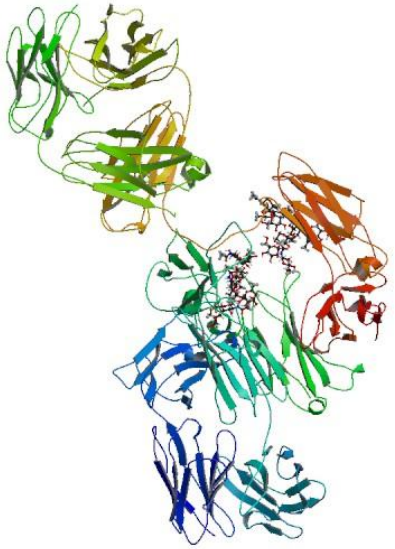
Model ADC Library
Cetuximab with MMAE through Cysteine Conjugation

Design and Characterization

of Model ADC utilizing Cetuximab and MMAE through Cysteine conjugation chemistry

Cetuximab

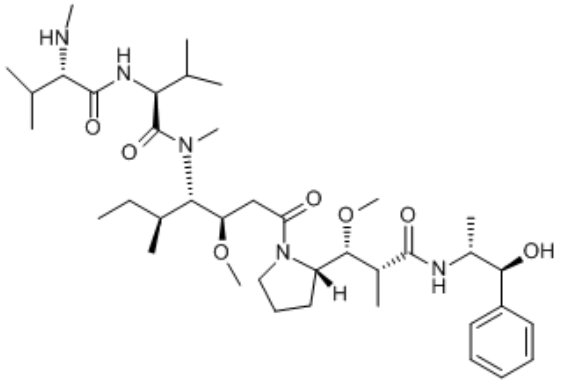
Cetuximab is an epidermal growth factor receptor (EGFR) inhibitor medication used for the treatment of metastatic colorectal cancer and head and neck cancer.



Cysteine Conjugation

Monomethyl auristatin E (MMAE) is a very potent antimitotic agent that inhibits cell division by blocking the polymerization of tubulin.

The family of auristatins are synthetic analogues of the antineoplastic natural product Dolastatin 10, ultrapotent cytotoxic microtubule inhibitors.



Hsu, H., **Zhang, X**., Richards, D., Davis, K., Stockel, J., Ramsay, J., Hernandez, E., & McDermott, L. (2025). Comparative evaluation of structure activity between stochastic and site-specific cetuximab (CmAb) - MMAE conjugates. World ADC London 2025.

Background and Introduction
Characterization of Antibody-Drug Conjugates (ADCs)

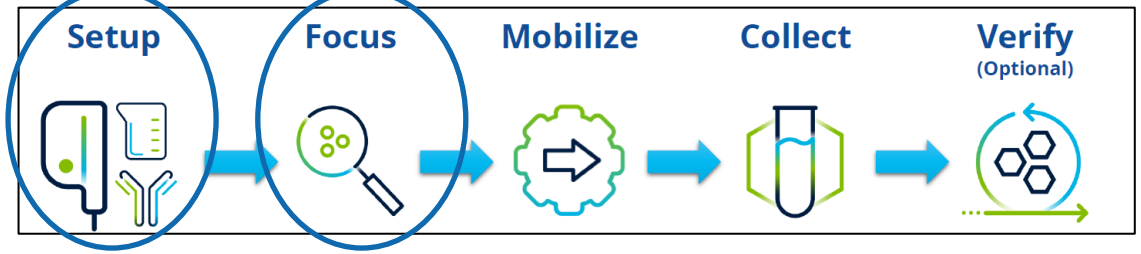
Aggregation	Size-Exclusion Chromatography (**SEC**): Detects and quantifies aggregates.
Free Drug	High-Performance Liquid Chromatography (**HPLC**): Quantifies the amount of free drug present. Liquid Chromatography-Mass Spectrometry (**LC-MS**): Detects and quantifies free drug.
Drug-to-Antibody Ratio (DAR)	Hydrophobic Interaction Chromatography (**HIC**): Separates ADC species based on hydrophobicity differences.
Purity	Size-Exclusion Chromatography (**SEC**): Separates based on molecular size, helps assess purity and aggregates. Capillary Electrophoresis (**CE**): Separates molecules based on charge or size.
Identity and Stability	Ion Exchange Chromatography (**IEX**): Separates based on charge differences. Imaged Capillary Isoelectric Focusing (**icIEF**): Separates proteins based on their isoelectric point.
Antigen Binding	Enzyme-Linked Immunosorbent Assay (**ELISA**) and Surface Plasmon Resonance (**SPR**): Quantifies the binding activity of the ADC to its target antigen.

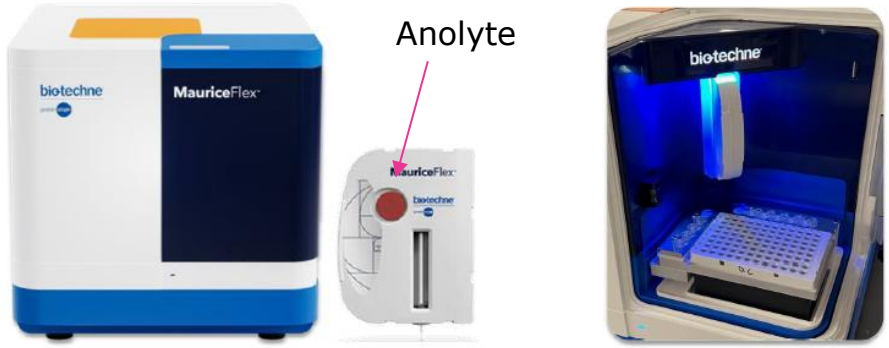
Cahuzac, H., & Devel, L. (2020). Analytical Methods for the Detection and Quantification of ADCs in Biological Matrices. Pharmaceuticals (Basel, Switzerland), 13(12), 462.
Wakankar, A., Chen, Y., Gokarn, Y., & Jacobson, F. S. (2011). Analytical methods for physicochemical characterization of antibody drug conjugates. mAbs, 3(2), 161–172.

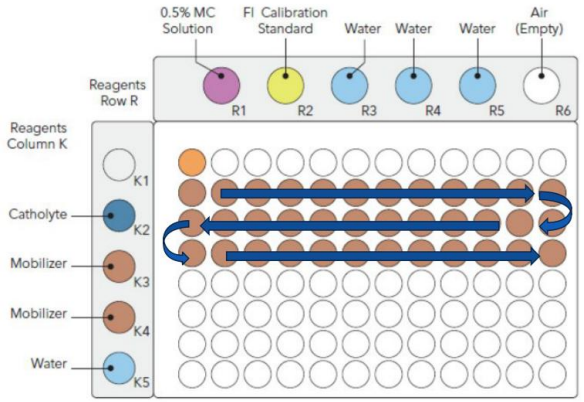
02

Workflow - icIEF Fractionation and Mass Spectrometry

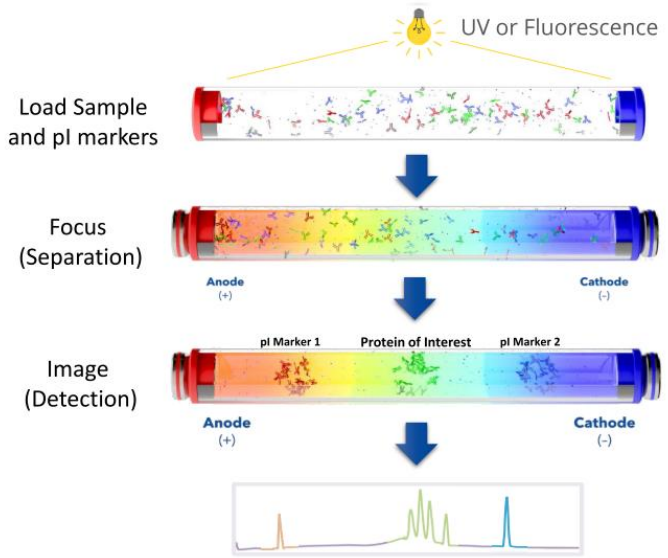
icIEF Fractionation – MauriceFlex Workflow
Instrument Setup and Sample Focusing Step







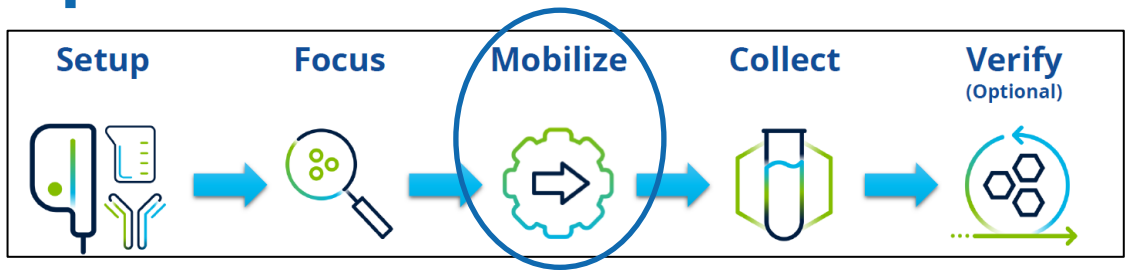
*Mobilizer solution: 5mM Ammonium Acetate



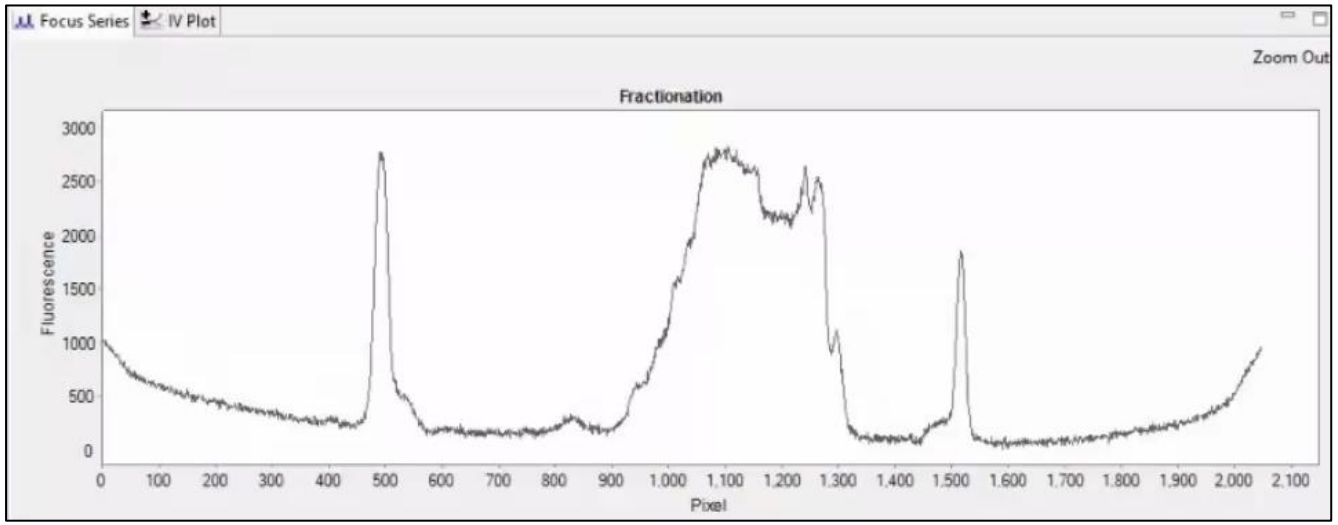
Convert Maurice/iCE3 icIEF focusing methods to MauriceFlex icIEF Fractionation Focusing methods:

- Concentrate stock sample to ~ 30 mg/mL to allow target concentration of working solution to 3 ~ 5 mg/mL.
- Incorporate Arginine and Glycerol to preserve the pI marker.

icIEF Fractionation – MauriceFlex Workflow
Mobilization Step



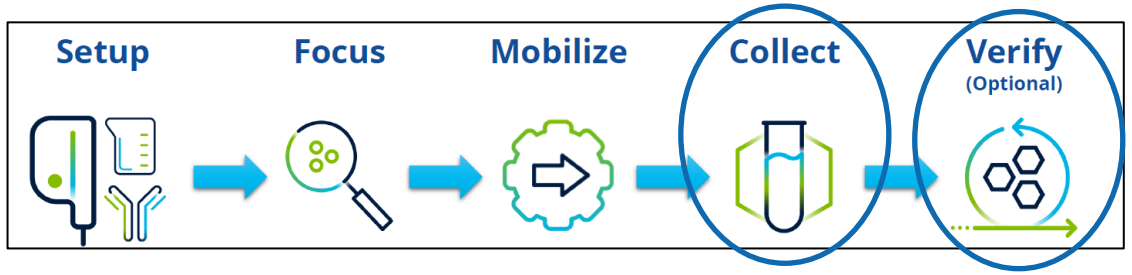
Video - Visual Representation of the Mobilization Process

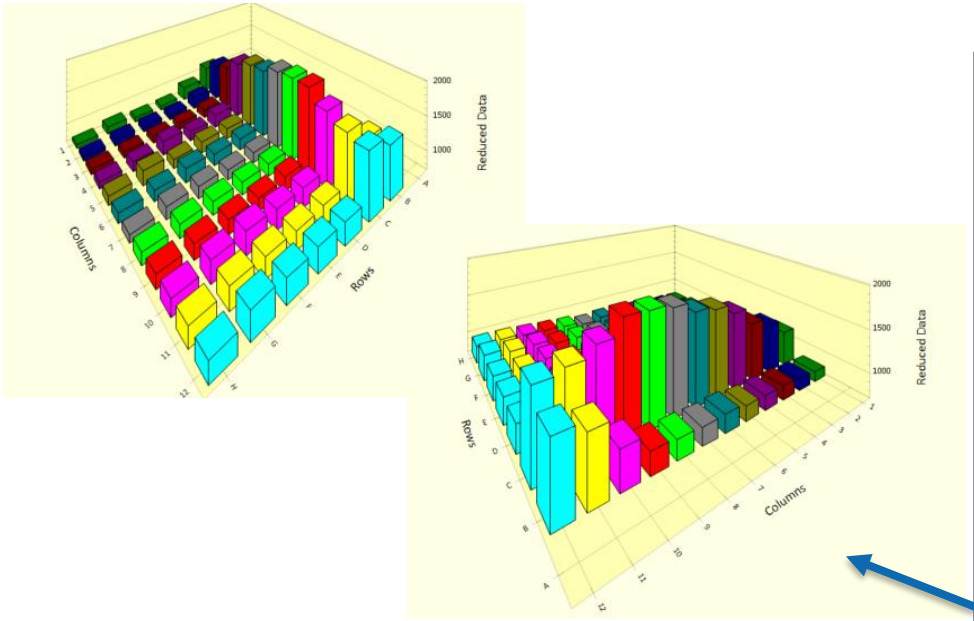


Mobilization	Refocus	Fractions
30.0 min 1500 Volts	5.0 min 2500 Volts	20.0 sec 1500 Volts

- Only charged molecules, like protein charge variants, are mobilized and collected
- Method additives like methylcellulose and urea are not mobilized

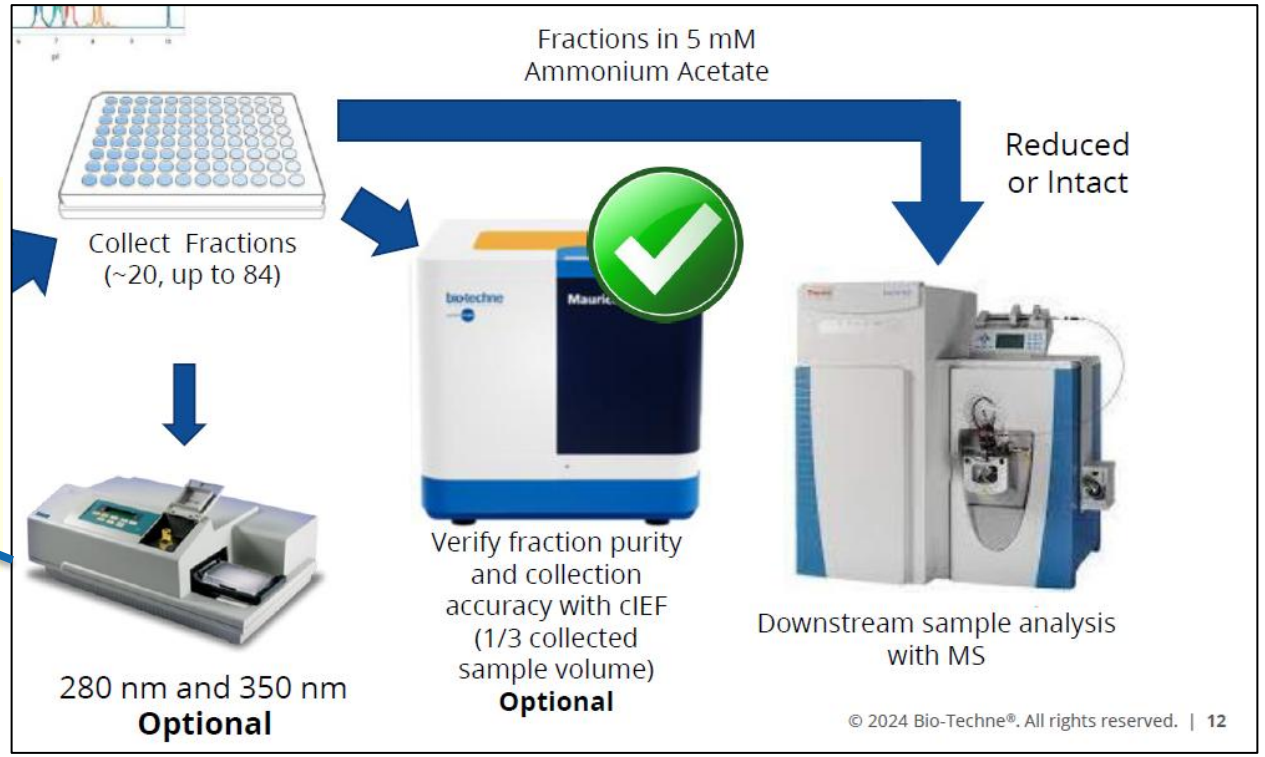
icIEF Fractionation – MauriceFlex Workflow





3D Graph of Fractions from Plate Reader

Two views showing signal intensity across the plate, helping to visualize where each fraction is located and confirming successful collection.



Mass Spectrometry (MS) Analysis of icIEF Fractions Workflow

Collect Fraction

No additional treatment needed

Fc Glycan Removal Incubation with PNGaseF

LC-MS Analysis

Agilent 6545XT AdvanceBio LC/Q-TOF

Waters BioResolve RP mAb Polyphenyl Column

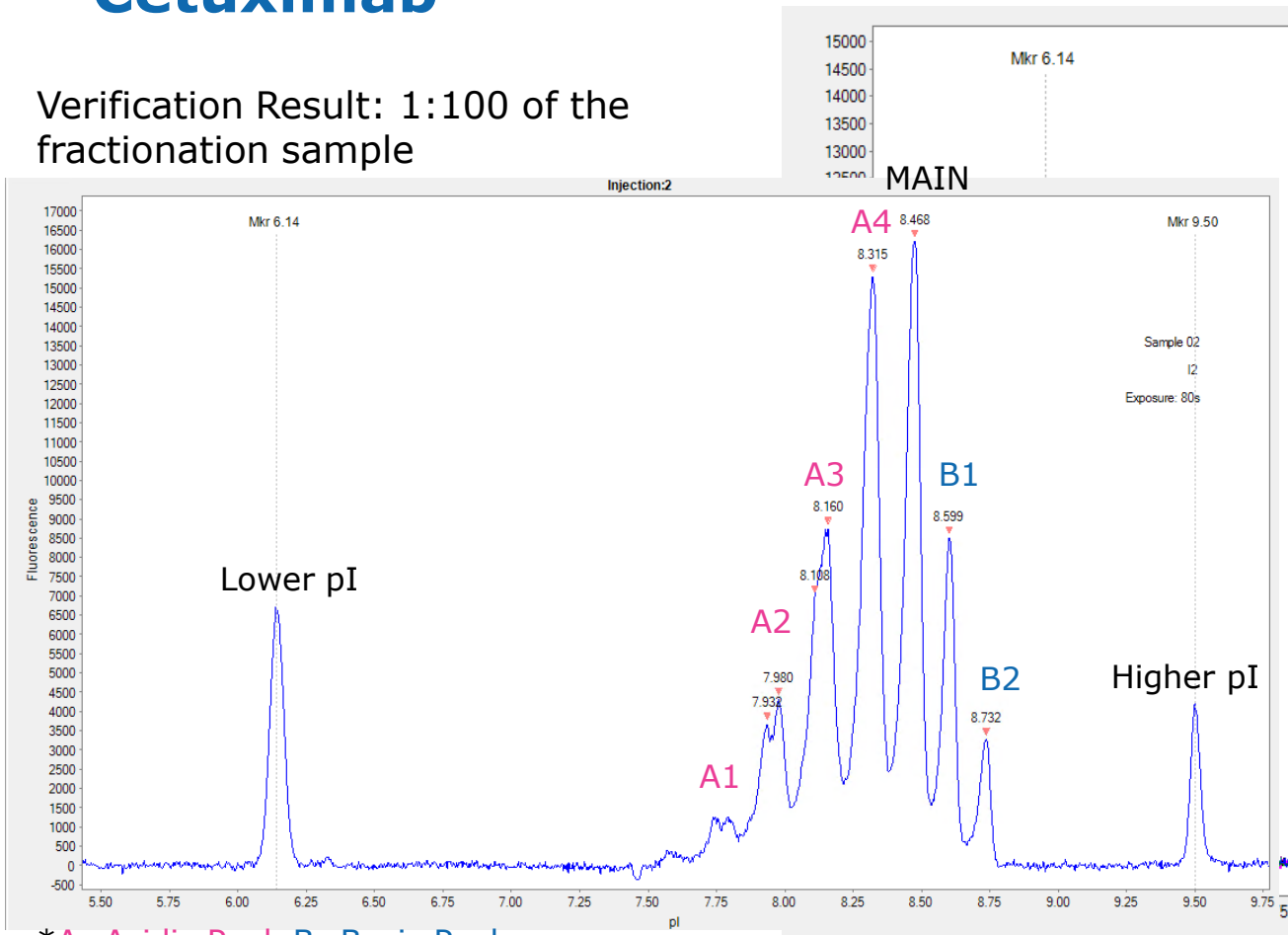
- Fractions were collected in 5 mM ammonium acetate, a buffer compatible with direct MS analysis without further treatment.
- Approximately 1 µg of protein from each fraction was subjected to LC-MS analysis under **Non-Denaturing Conditions**.

03

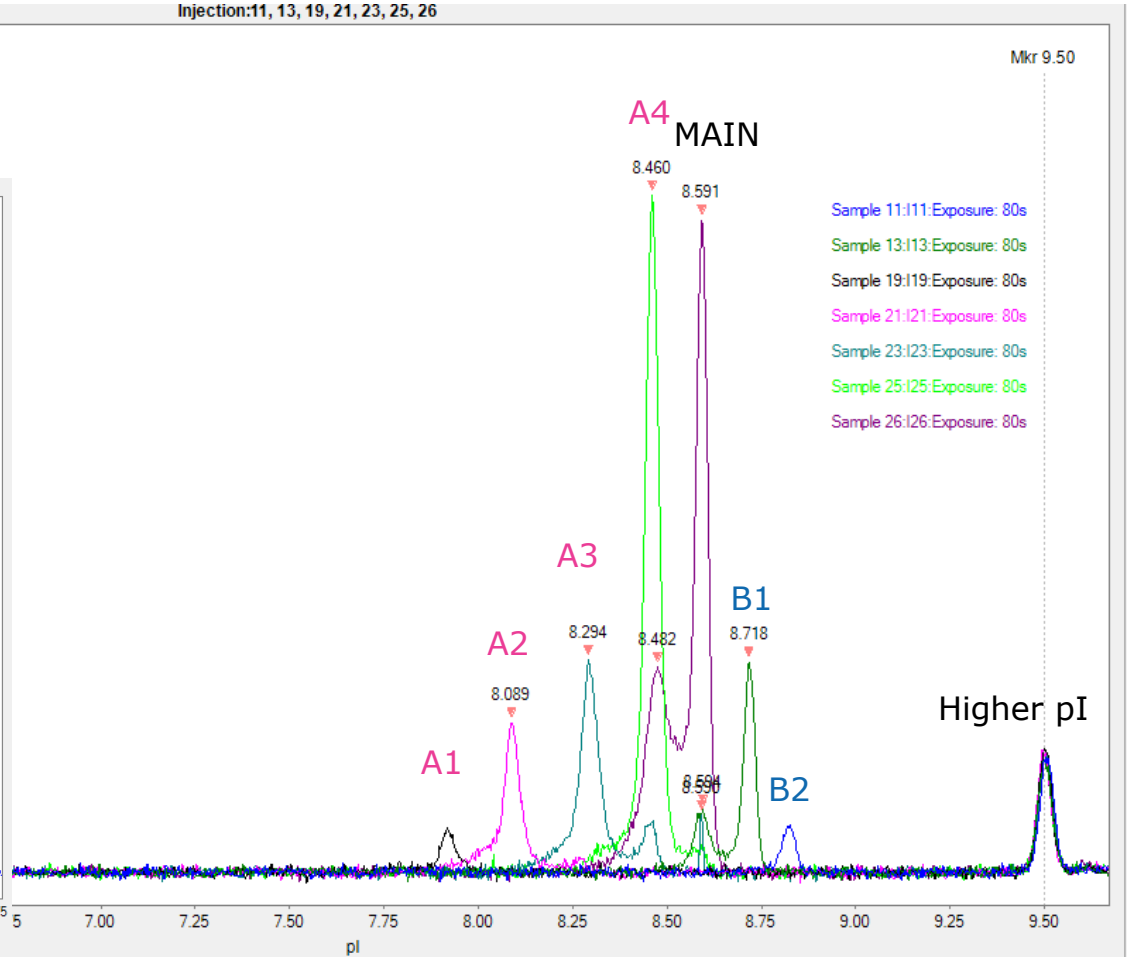
Model Study: Cetuximab & ADC

icIEF Fractionation – Results
Cetuximab

Verification Result: 1:100 of the fractionation sample



Overlay of the Fractions from Verification Run



*A: Acidic Peak B: Basic Peak

- **icIEF fractionation successfully separated the charge variants of cetuximab**, providing sufficient resolution of each fraction to support LC-MS analysis and facilitate deeper insight into its charge heterogeneity.

icIEF Fractionation – Results
Cysteine Conjugated ADC

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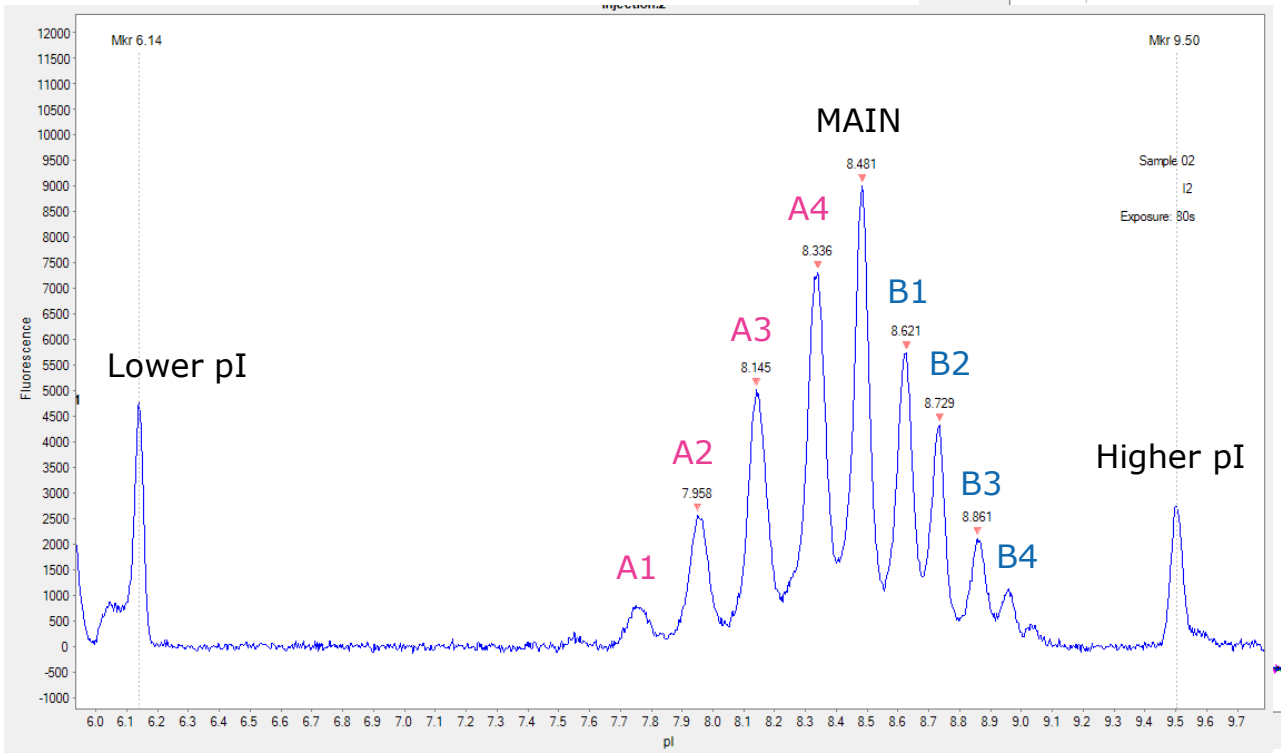
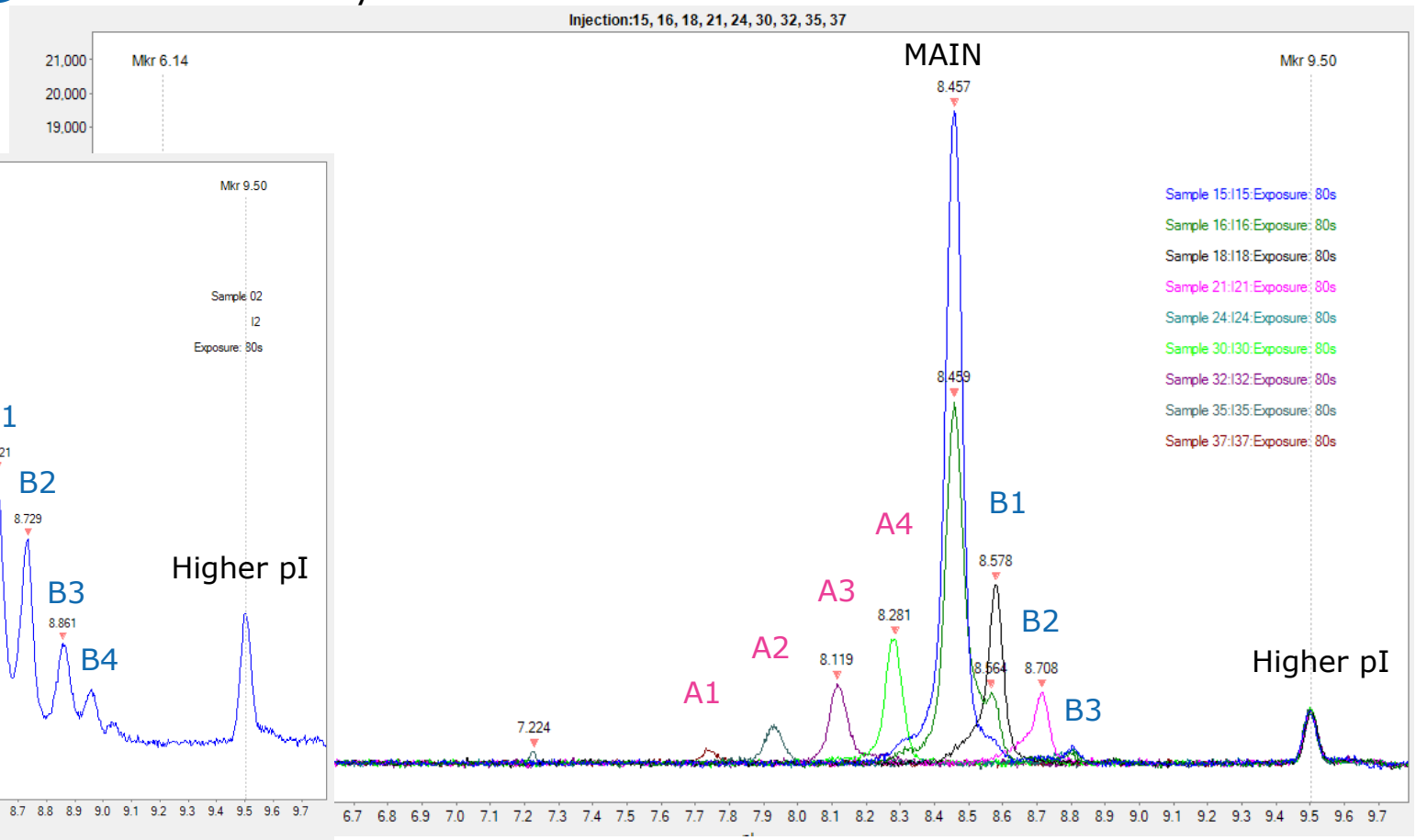
Verification Result: 1:100 of the fractionation sample



*A: Acidic Peak B: Basic Peak

Overlay of the fractions from Verification Run



- **icIEF fractionation successfully separated the charge variants of the cysteine-conjugated ADC**, providing sufficient resolution for LC-MS analysis.
- **The ADC displayed a charge profile highly comparable to the unconjugated mAb**, with an increased number of basic peaks.

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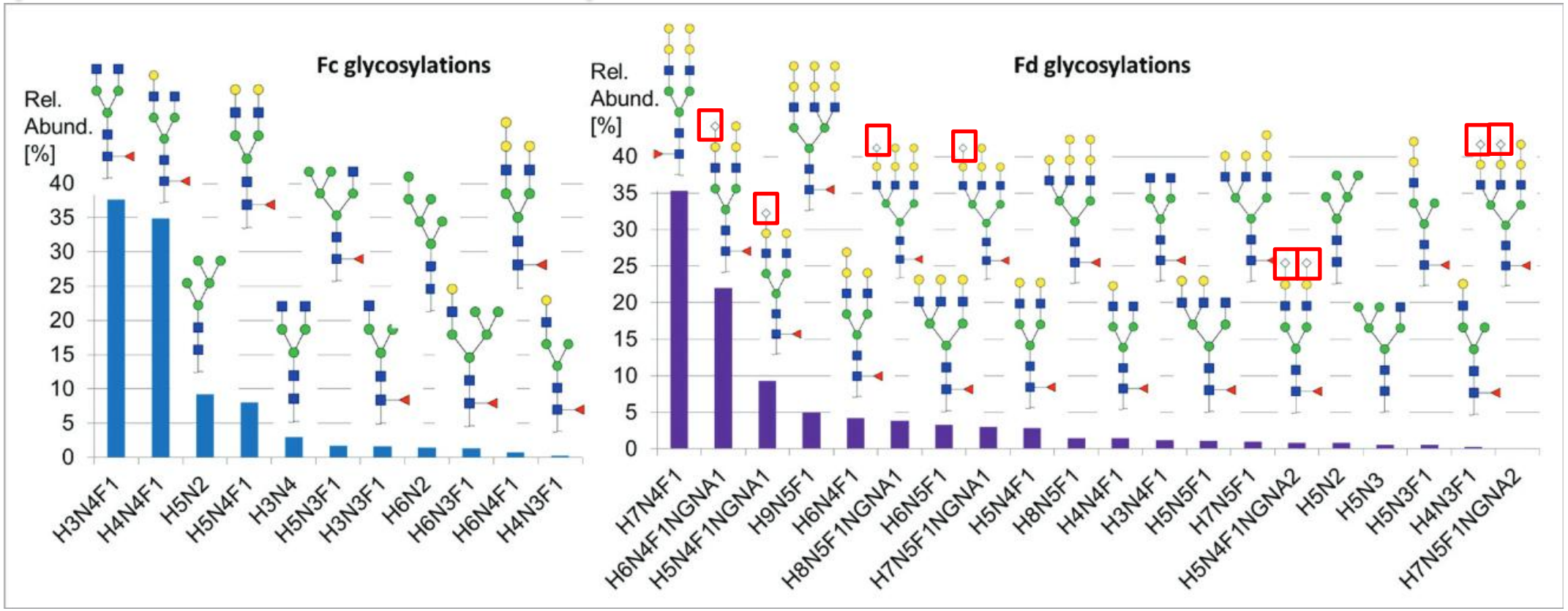
Mass Spectrometry Analysis of icIEF Fractions
Glycosylation Sites on Cetuximab

Cetuximab contains two different glycosylation sites: Fc and Fd (HC Fab)

Neutral
(no contribution to charge variation)

Some contain acidic sugars
(contribute to charge variation)



Ayoub, D., Jabs, W., Resemann, A., Evers, W., Evans, C., Main, L., … Beck, A. (2013). Correct primary structure assessment and extensive glyco-profiling of cetuximab by a combination of intact, middle-up, middle-down and bottom-up ESI and MALDI mass spectrometry techniques. *mAbs*, *5*(5), 699–710. https://doi.org/10.4161/mabs.25423

Mass Spectrometry Analysis of icIEF Fractions
Selective Removal of Fc Glycans Simplifies Charge Variant Analysis of Cetuximab

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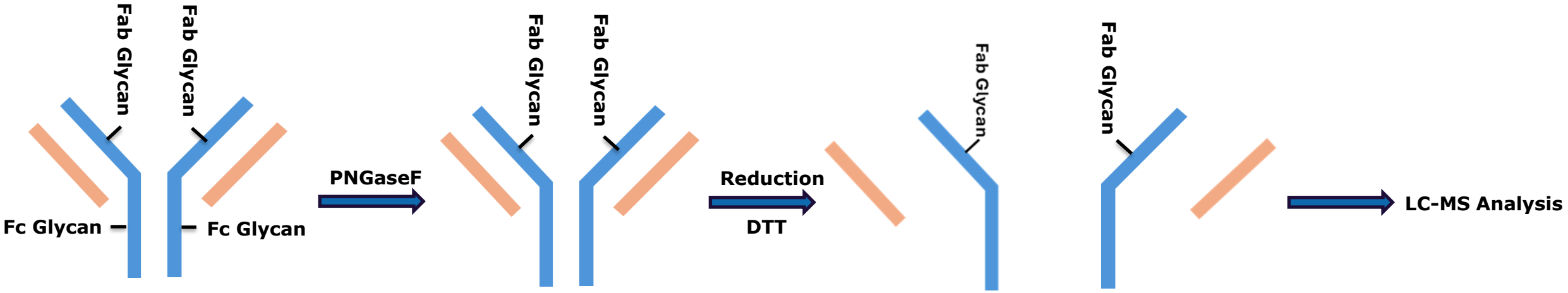
PNGaseF Only Cleaves Fc Glycans in Cetuximab under Non-Denaturing Conditions[1]:

Decreases structural heterogeneity by removing neutral Fc glycans (not relevant to charge variation)

Analysis simplified by reducing to subunits and retaining glycans which drive variation in charge:



[1]Janin-Bussat, MC. et al. (2013). Cetuximab Fab and Fc N-Glycan Fast Characterization Using IdeS Digestion and Liquid Chromatography Coupled to Electrospray Ionization Mass Spectrometry. In: Beck, A. (eds) Glycosylation Engineering of Biopharmaceuticals. Methods in Molecular Biology, vol 988. Humana Press, Totowa, NJ. https://doi.org/10.1007/978-1-62703-327-5_7

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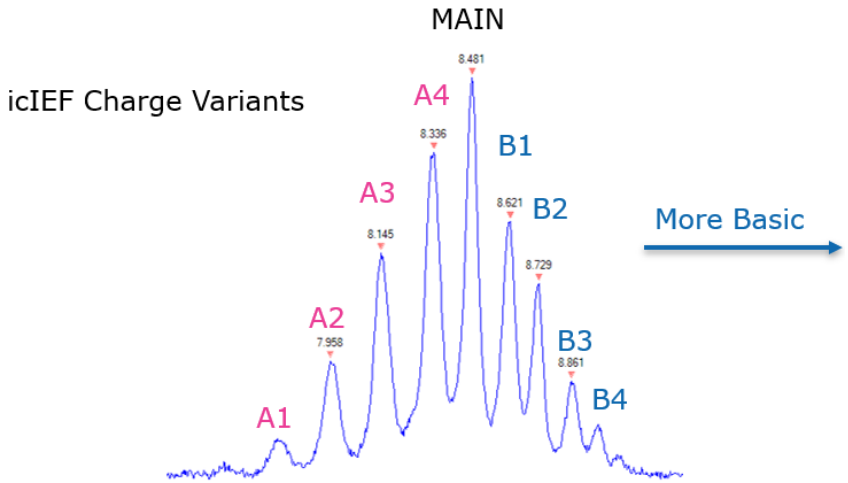
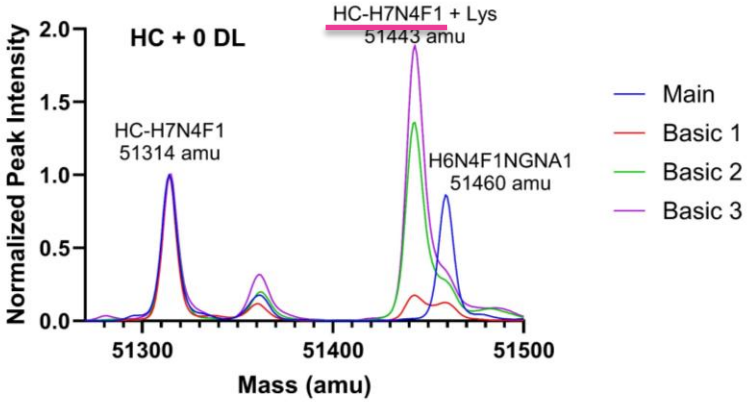
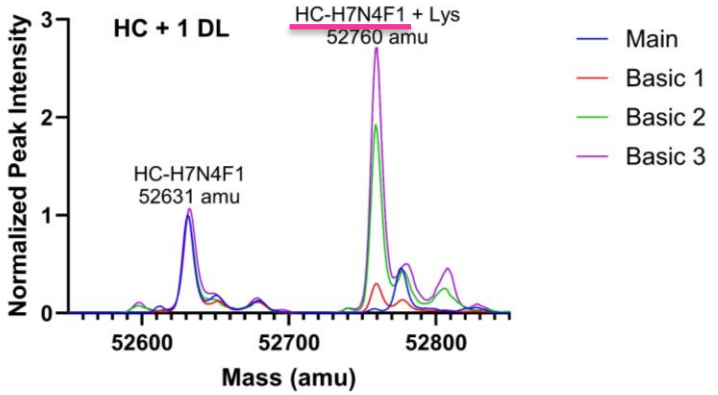
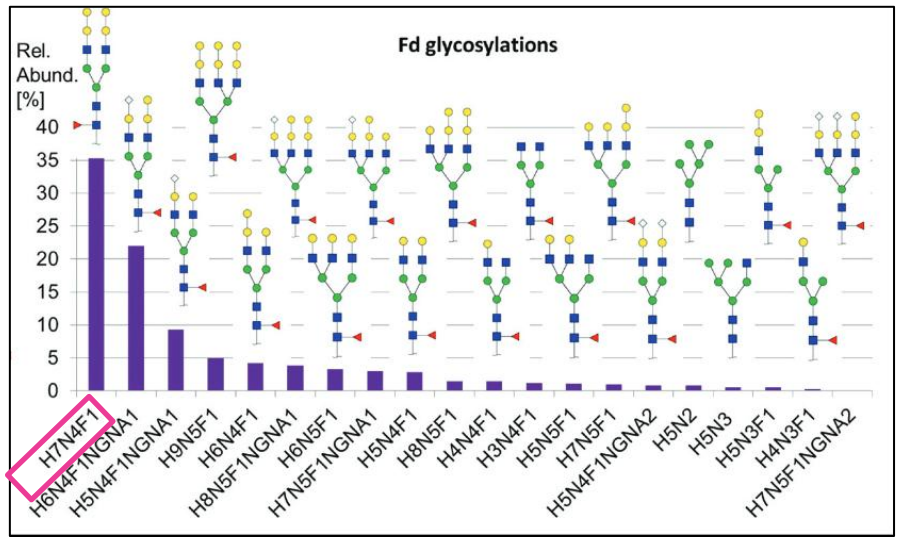
Mass Spectrometry Analysis of icIEF Fractions
Model Cysteine Conjugated ADC Results

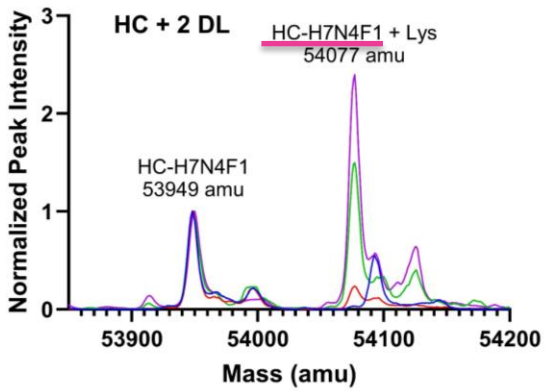
Main → Basic Fractions: Increase in Basicity Driven by Increase in HC C-Terminal Lys Abundance

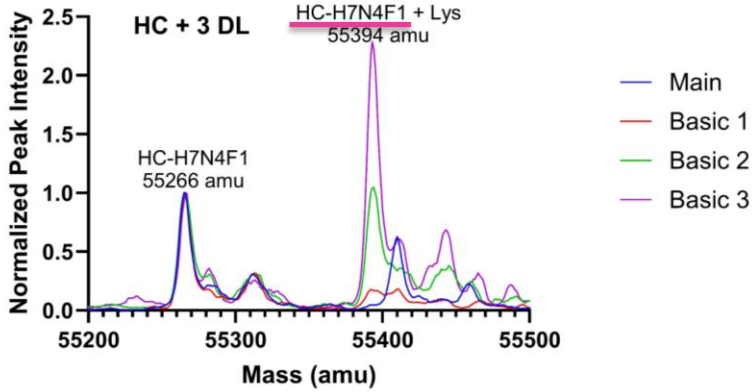












Mass Spectrometry Analysis of icIEF Fractions
Model Cysteine Conjugated ADC Results

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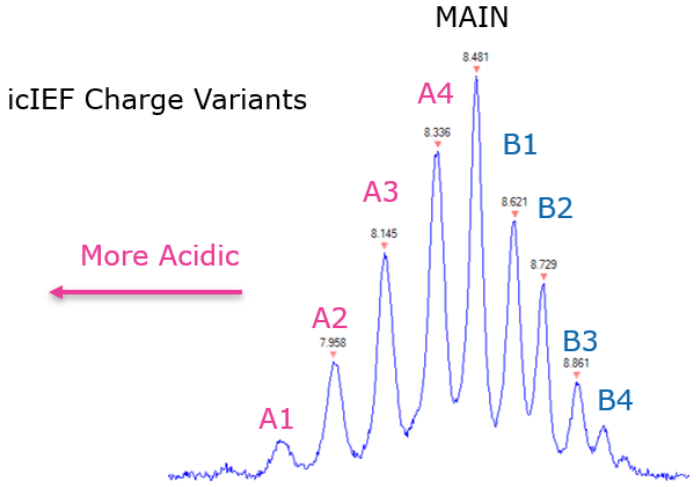
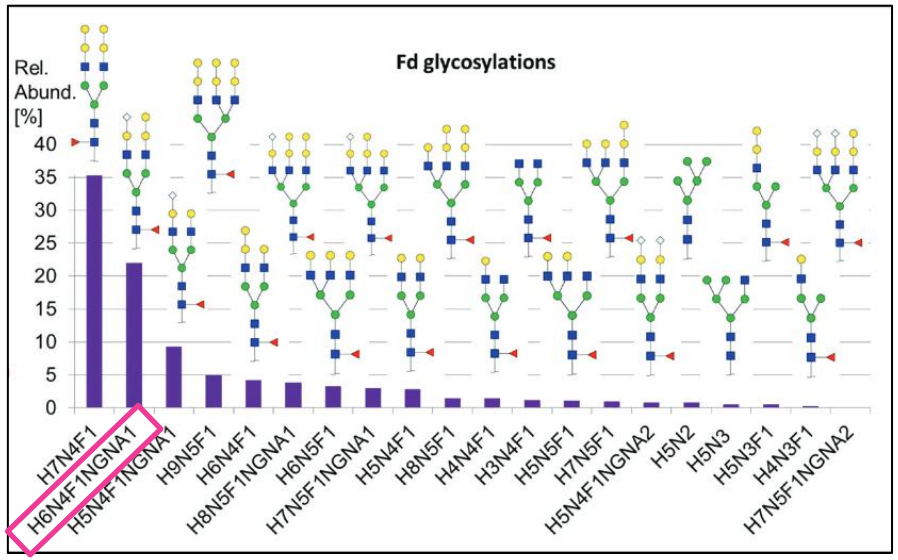
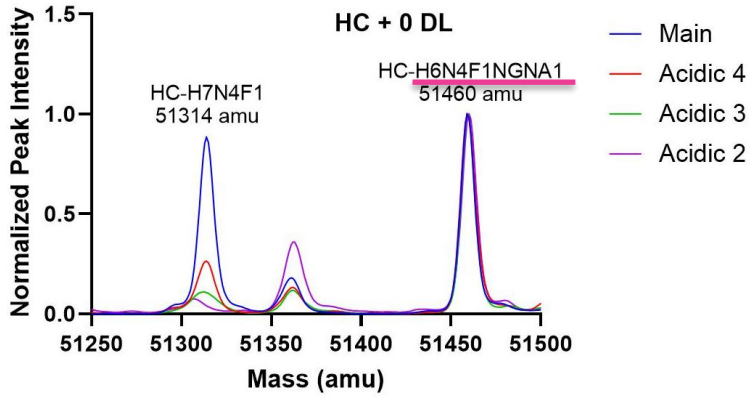
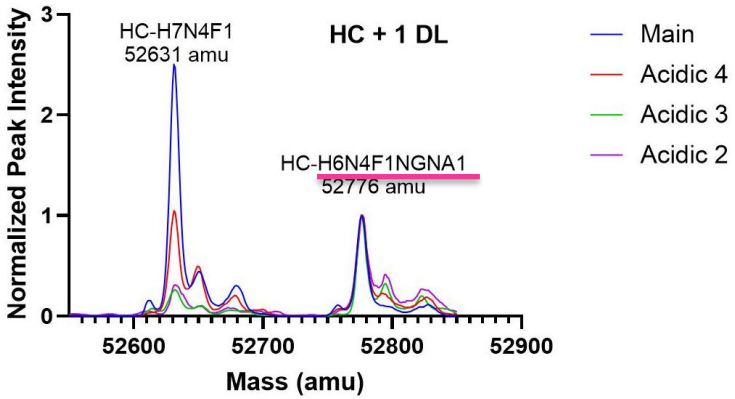
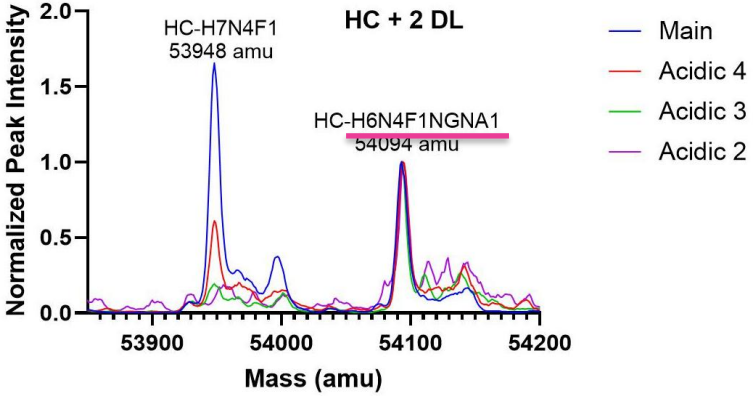
Main → Acidic Fractions: Increase in Acidity Driven by Increase in H6N4F1NGNA1 Fab Glycan Abundance

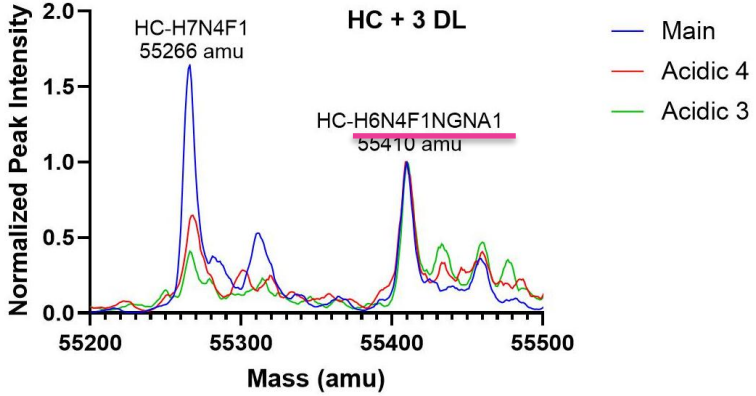












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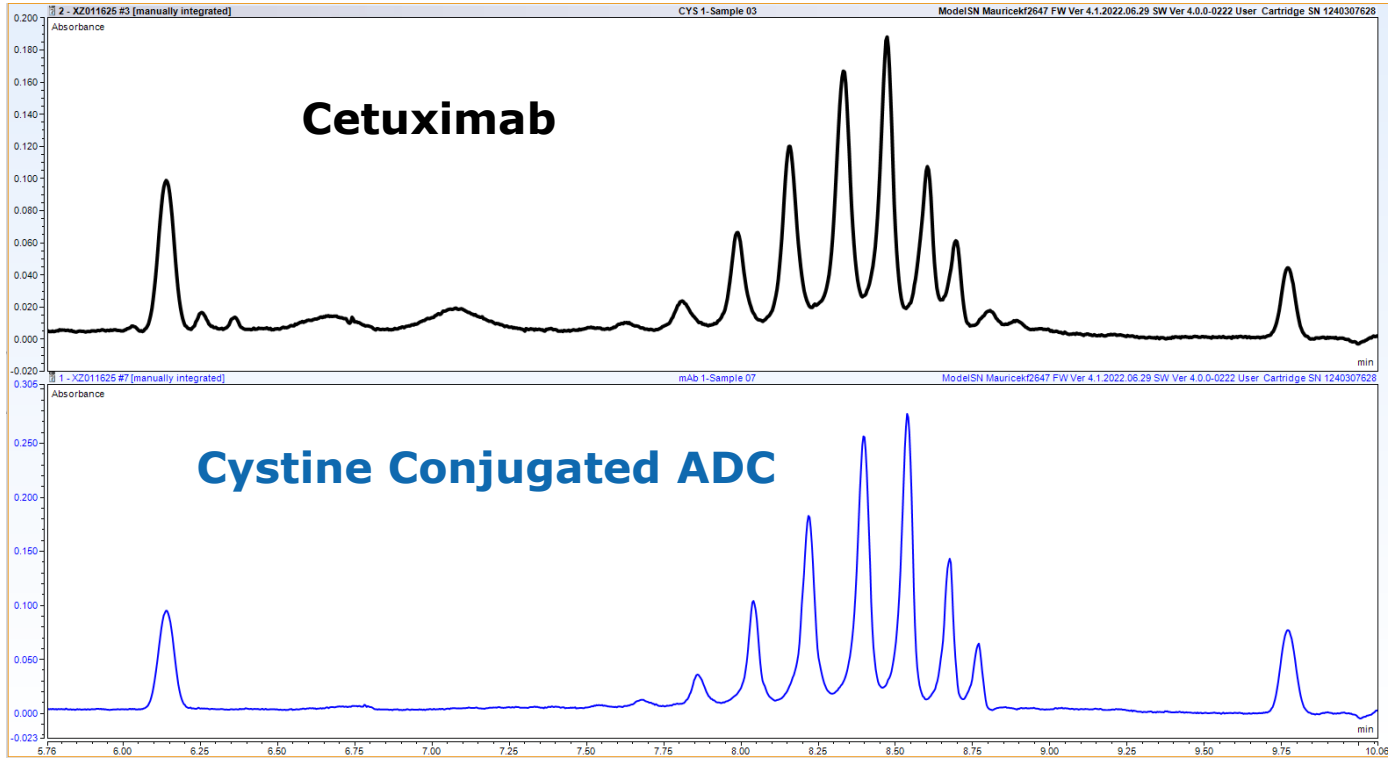
04 Discussion

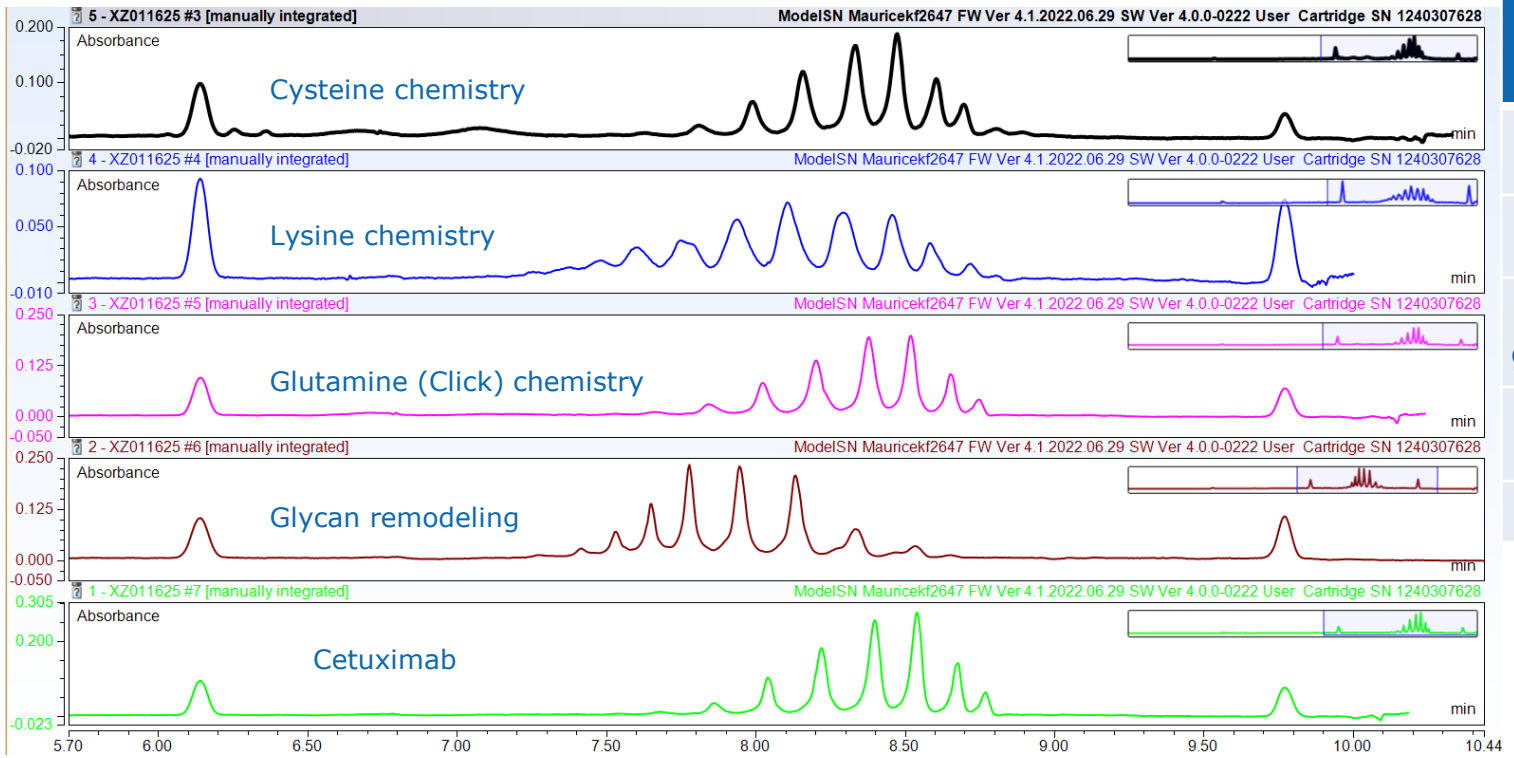
Charge Profile Comparison Between Cetuximab and Its Cysteine-Conjugated ADC
Insights from icIEF and LC-MS Analysis



Sample Name	Peak Numbers	Main Peak pI	%Acidic Species	%Main Peak	%Basic Species
Cetuximab	11	8.5	59.7	24.4	15.8
Cystine Conjugated ADC	10	8.5	53.8	23.3	22.9

- LC-MS data confirm that cetuximab and its cysteine-conjugated ADC exhibit comparable charge profiles and species distribution.
- Drug loading (DL) does not significantly impact the charge distribution in this case.
- Charge heterogeneity is primarily driven by:
  - **Glycan structures**
  - **C-terminal lysine variants**
- These charge-contributing features are present in both the native mAb and the ADC.
- icIEF combined with LC-MS provides deeper insight into the molecular basis of charge variation.

Influence of Conjugation Chemistry on mAb-to-ADC Charge Profile Changes



	Peak Numbers	Main Peak pI	% Acidic Species	% Main Peak	% Basic Species
Cysteine	10	8.5	53.8	23.3	22.9
Lysine	11	8.1	42.9	18.2	38.9
Trans-glutaminase	11	8.4	37.4	25.6	37.1
Glycan remodeling	10	7.9	41.9	23.0	35.1
CmAb	11	8.5	59.7	24.4	15.8

The icIEF profiles provide valuable insights into the charge heterogeneity of ADC conjugates. Like molecular fingerprints, these profiles reveal **unique attributes essential for establishing ADC identity**. Distinct conjugation strategies, such as lysine-based conjugation and click chemistry using charged drug-linkers, which introduce varying degrees of charge profile shifts between the mAb and its corresponding ADC. These shifts are reflected in the number of peaks, the main peak pI, and the distribution of acidic, main, and basic species.

- Coupling icIEF with MS enables identification of molecular species associated with each charge variant
- Provides insight into how drug load, linker charge, or modifications such as deamidation contribute to charge heterogeneity
- Enhances understanding of structure and function relationships
- Supports identity testing and comparability assessments for ADCs

Applications of icIEF-MS
Enabling Deeper Insight into ADC Charge Heterogeneity

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- **Characterization of process modifications:**
  - Differences in ADC charge profile (e.g. different lots of chemicals)
- **Identification and characterization of Charge Variants:**
  - Stability assessment and forced degradation studies
  - Drug load distribution (e.g. Certain ADCs with Cysteine Conjugation)

Specific LC-MS Methods for Supporting icIEF Fractionation Applications

Peptide Mapping	**Released Glycan LCMS**	**Native MS**
Relative quantitation of post-translational modifications	More detailed analysis of glycoforms	Characterization by intact mass

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Summary
Take Home Messages

- **Charge heterogeneity impacts ADC stability, efficacy, and safety.** Monitoring charge variants ensures batch-to-batch consistency and supports regulatory compliance.
- **icIEF supports stability and forced degradation studies.** Detects subtle charge shifts resulting from oxidation, deamidation, and linker instability.
- **MauriceFlex provides a simplified and efficient solution for icIEF fractionation.** Enables seamless collection of charge variants that are directly compatible with LC-MS, requiring no additional sample treatment. This supports high-resolution charge profiling and in-depth analysis of degradation pathways and drug load distribution in ADCs.
- **icIEF-MS analysis shows comparable charge profiles between cetuximab and its cysteine-conjugated ADC**, indicating in this specific case, the charge heterogeneity mainly driven by glycan structures and C-terminal lysine variants present in both molecules.
- **MauriceFlex icIEF fractionation enables deeper structural characterization.** Facilitates downstream analyses such as intact mass, peptide mapping, and LC-MS for precise charge variant identification.

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THANK YOU ALL!

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Questions?