Biologics Process Analytical Sciences and Technologies

Global Process Analytical Science

Application of Free Cysteine Footprinting by Differential Alkylation in BioProcess Development

September 29th, 2022

Roger Liu, Jacob Bongers, Tingwei Ren, Nela Zvereva, Chris Chumsae, Julia Ding

Biologics Development,
Bristol Myers Squibb

Bristol Myers Squibb™

BMS Biologics Development Network

PROCESS/ANALYTICAL DEVELOPMENT & CLINICAL MANUFACTURING

Devens, MA

- US Biologics COE
- Biologic Development
- MS&T, Quality AS&T
- Clinical Manufacturing
- Commercial Manufacturing

New Brunswick, NJ

US Biologic Development-Early Phase

Bioassay COE; GMP Testing

Summit, NJ

US Biologic Development-Early Phase

Clinical Manufacturing

Global Process Analytical Sciences in BMS

High-throughput Collaborative Team	Process Optimization Efficiency Improvements	Drive for Innovation and Scientific Excellence
Focus on: **Build High-Throughput Assays** **Deliver Quality Data**	**Focus on:** **Data Analytics Automation** **Procedure Automation**	**Focus on:** **Process Analytical Technologies** **Build Deep Process Knowledge**

• Standardize **High Throughput** Platform Assay procedure for mAb across network
• Standardize Assay Performance tracking and monitor across network
• Build **High-Throughput** assays for non-mAb molecules
	• **E2E Automation with Tecan Platform**
	  • Building Integrated components (centrifuge, thermocycler, plate washer, plate reader etc.) with Tecan platform to enable more assay automation
	• **Empower Data Process Automation**
	• **Spotfire** for Data Trending and Operation excellence
		• Establish technologies on:
		  • **Spectroscopy** (FT-IR, Raman)
		  • **Multi-attribute Chromatography** (Segflow/2D LC, Patrol)
		  • Delta-V feedback loop control
		  • Centralized PAT Data Integrator
		• Advance **PAT** into manufacturing space
		  • Raman for glucose/lactate
		  • Flow VPE for protein concentration at UF/DF step

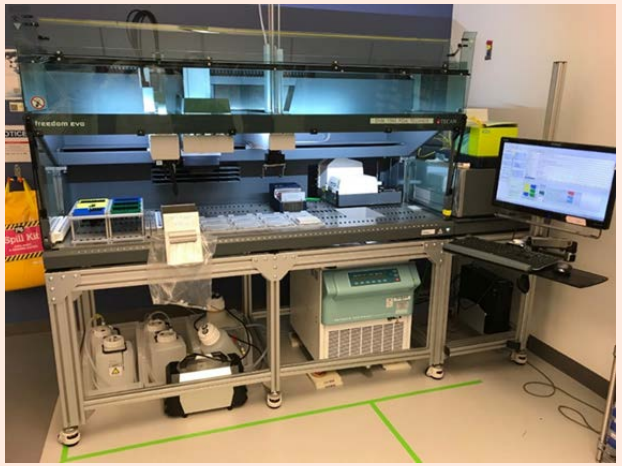
Our Operating Model

Platform Approach with High Throughput Instrumentation Capability




- Plate based analysis
- Multiplexing
- High-throughput Autosamplers

Automation to replace manual sample preparation and handling



Example Assays

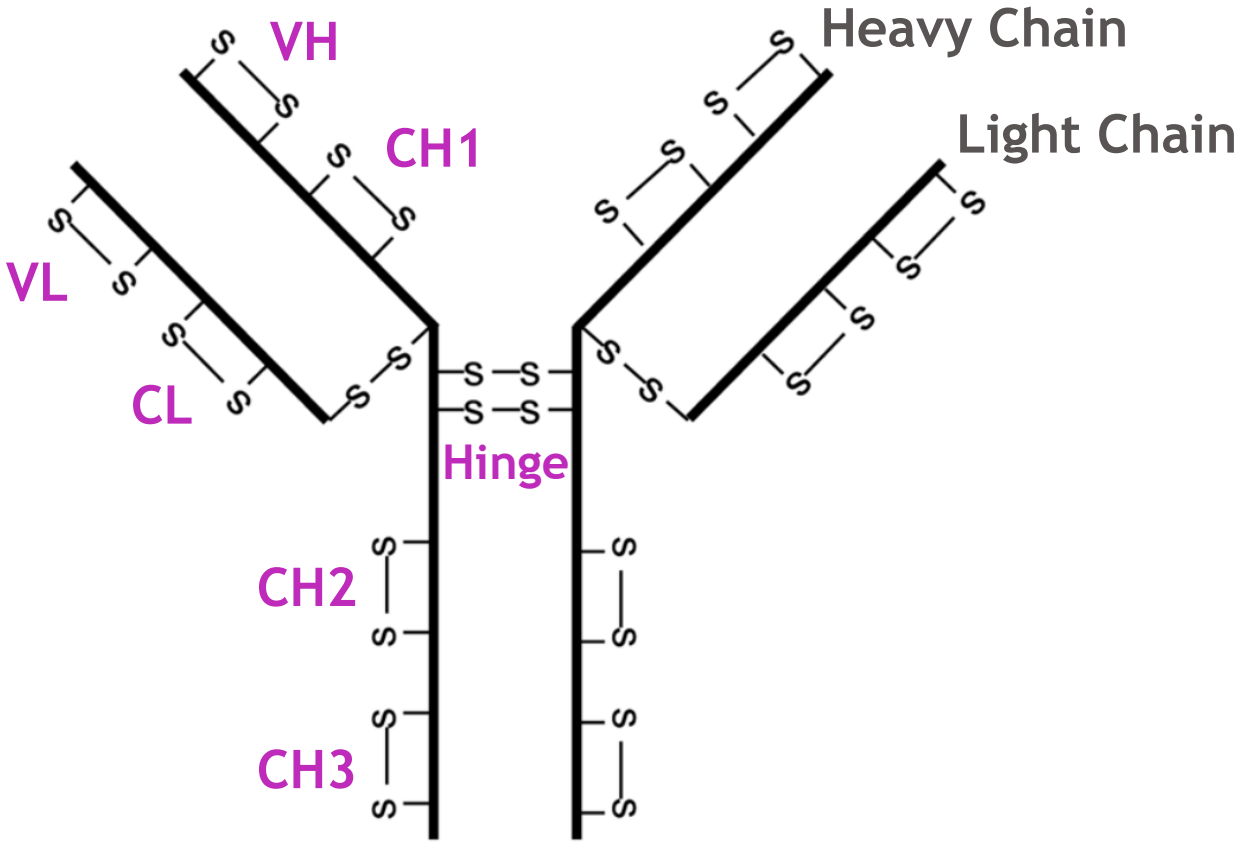
- Glycan Analysis
- CE-SDS
- Impurity Assays (HCP, residual DNA)

Real Time & Near-Real Time Analytics through PAT



- In-line Sensors - Spectroscopy
- On-line Analytics - Chromatography
- At-line Assays

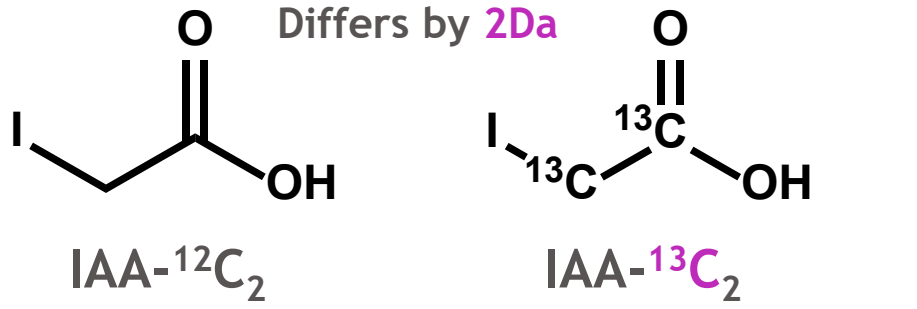
Disulfide Bond Network in IgG1 mAbs

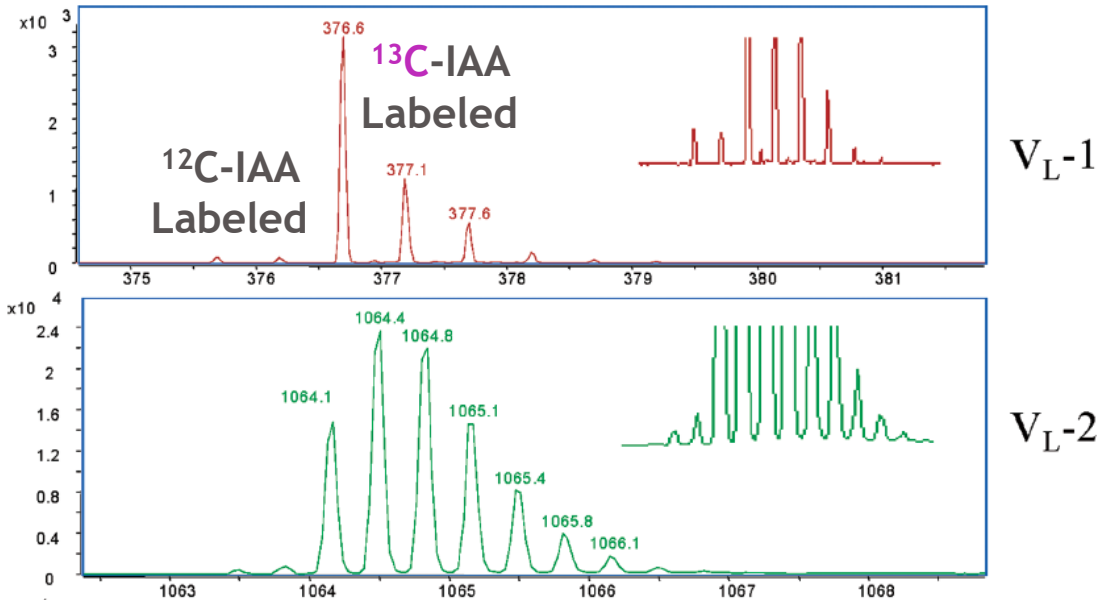


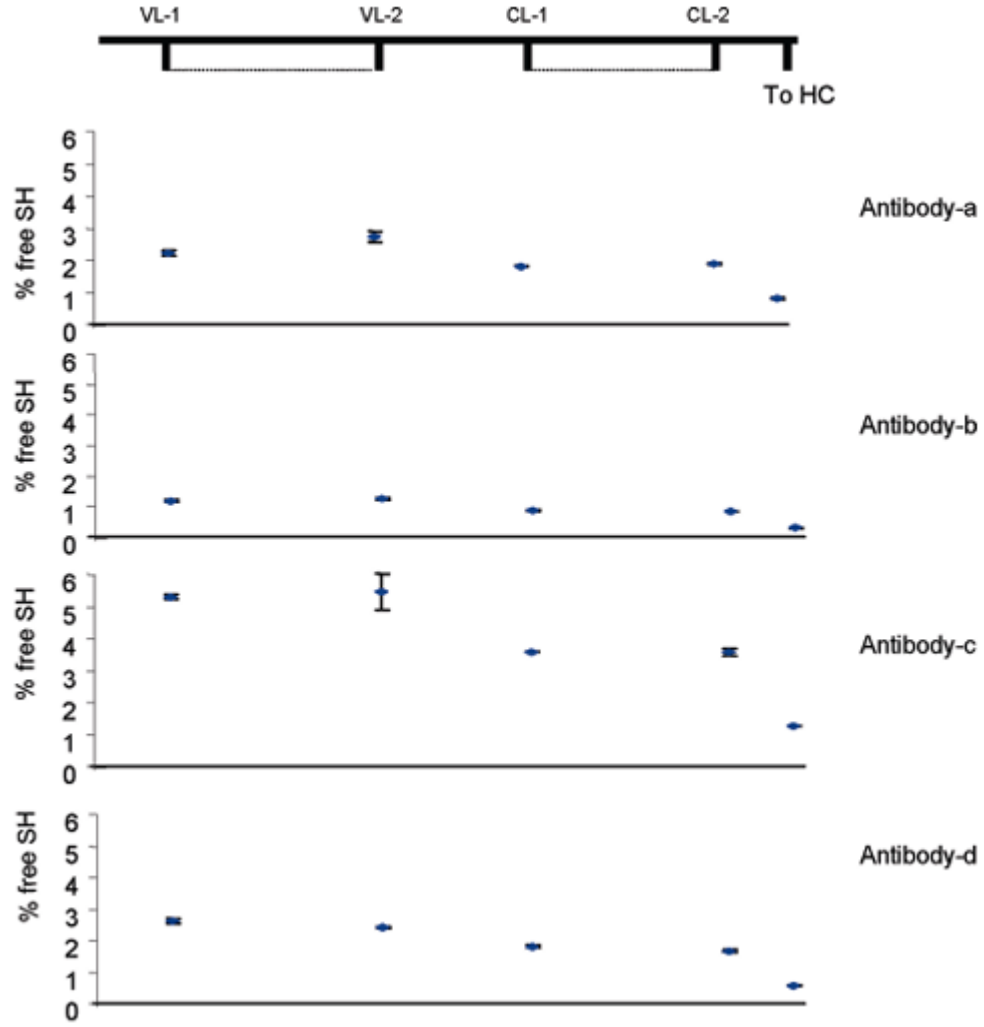
- All Cys residues in IgG are disulfide-bonded
- High free Cys content may lead to high LMW (unformed disulfide bond between LC and HC) and aggregation (inter-molecular disulfide linkages through free Cys residue)

Free Cysteine Footprinting by Differential Alkylation

- Cys Alkylation by Iodoacetic Acid (IAA)



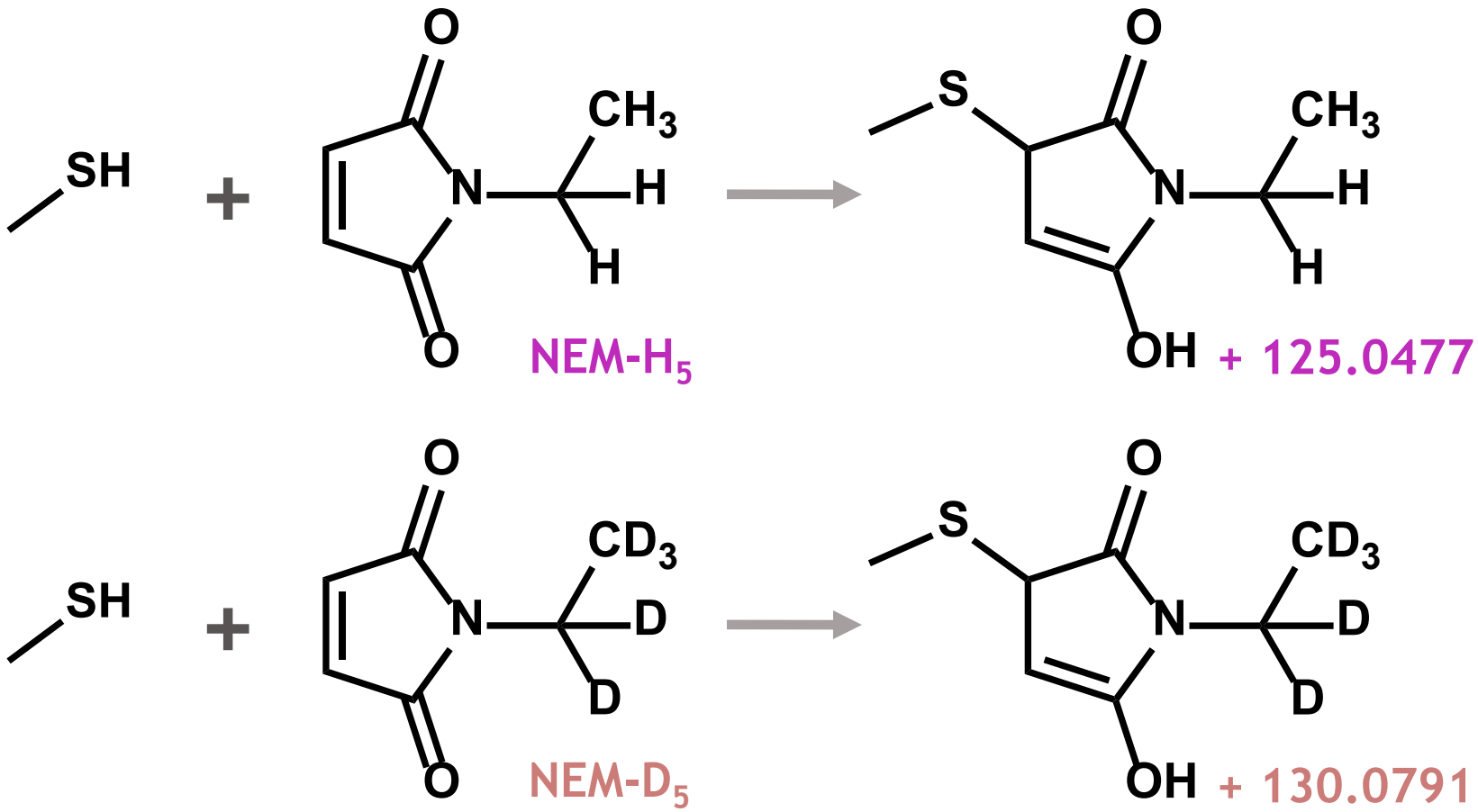




Xiang, T.; Chumsae, C.; Liu, H. *Anal. Chem.* **2009**, *81*, 8101-8108.

An Improved Workflow with New Reagent

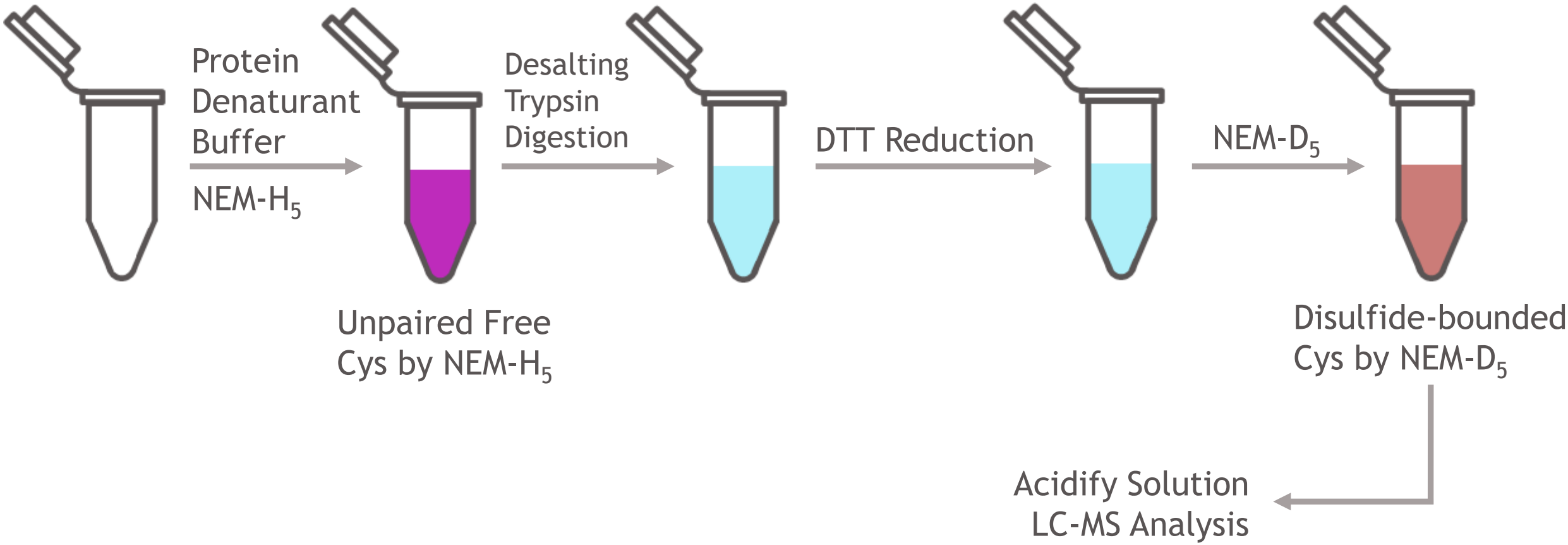
- Cys Alkylation by *N*-EthylMaleimide (NEM)



Differs by 5Da

Free Cysteine Footprinting by Differential Alkylation

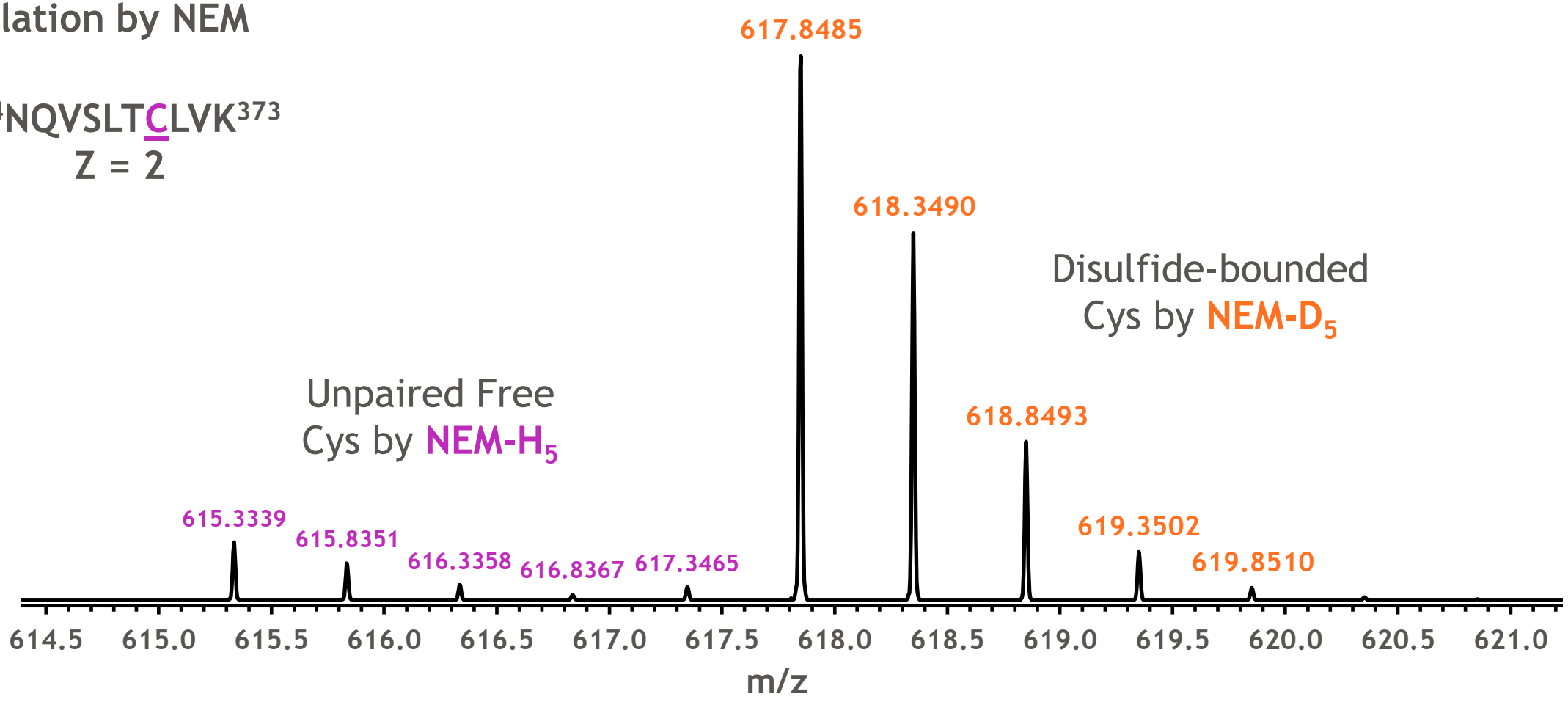
- **Experimental Workflow**



An Improved Workflow with New Reagent

- Alkylation by NEM

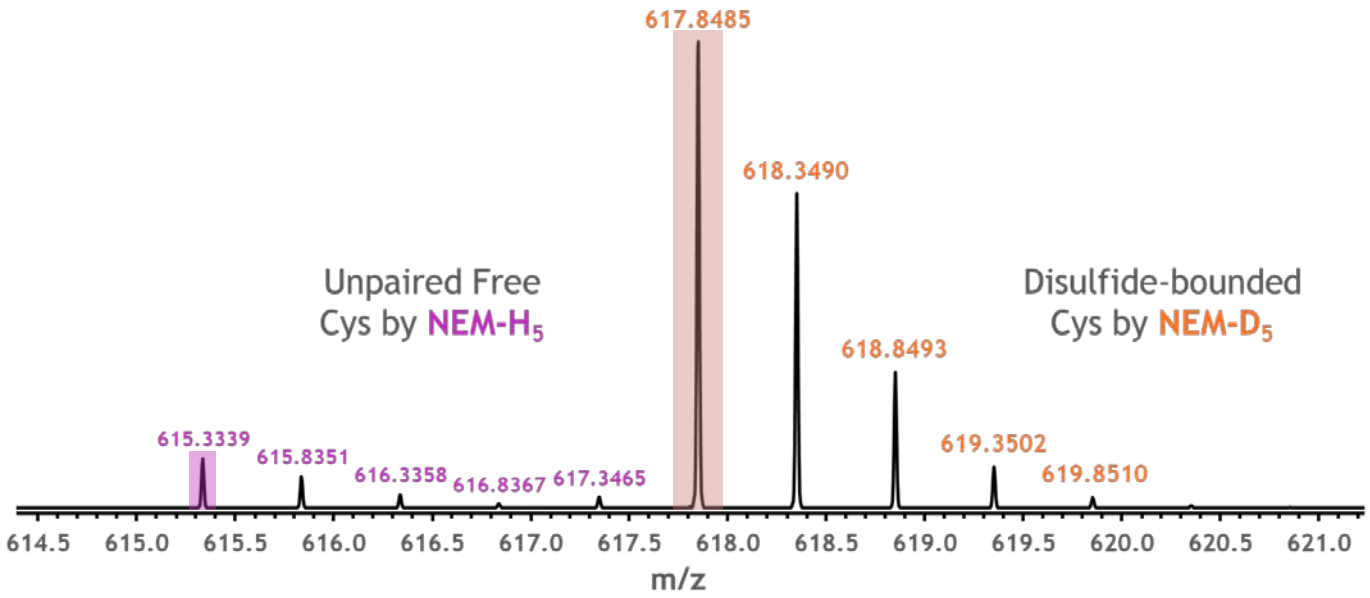
^{364}NQVSLTCLVK^{373}
Z = 2



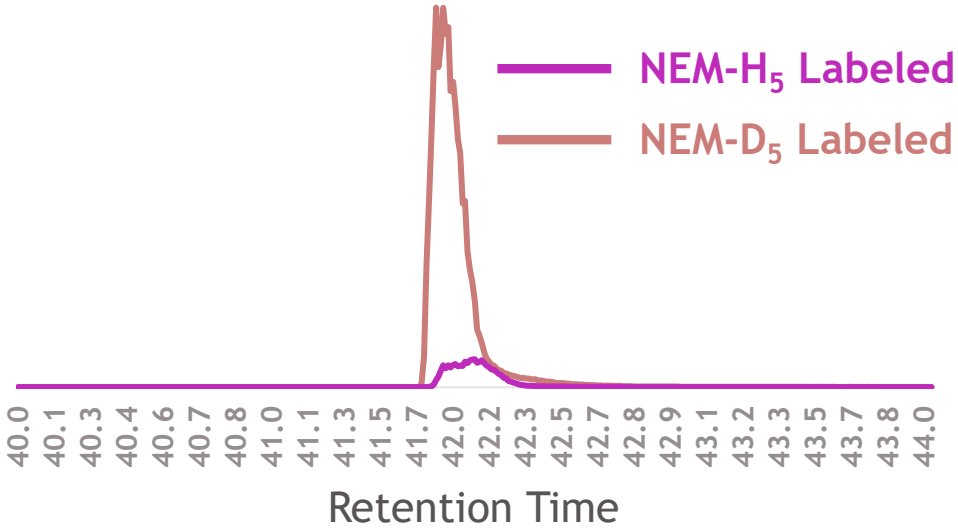
- A clear separation of isotopic cluster between the heavy and light-labeled species

Free Cysteine Footprinting by Differential Alkylation

- Data Processing



Extracted Ion Chromatogram (EIC)



$$Free\ Cys\ Fraction = \frac{EIC\ Area_{NEM-H_5-Labeled}}{EIC\ Area_{NEM-H_5-Labeled} + EIC\ Area_{NEM-D_5-Labeled} + EIC\ Area_{Non-Labeled}}$$

Instrumentation

• Mass Spectrometer

- Thermo Q-Exactive Plus
- Data dependent acquisition with top 10 precursors

• Chromatography

- Waters I-Class
- 200 µL/min
- ACQUITY Premier CSH C18 Column 1.7 µm, 2.1 x 150 mm
- HESI Source

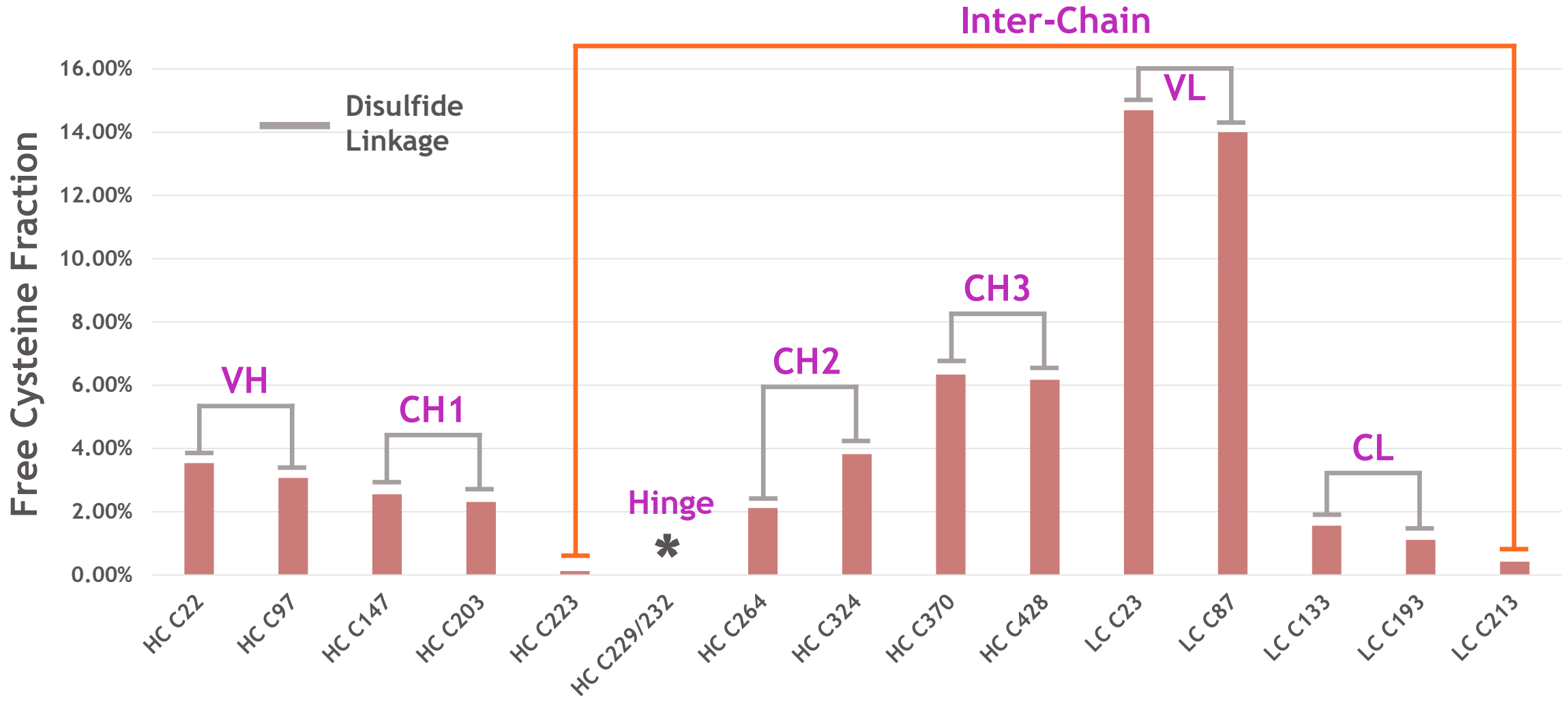
• Data Analysis

PROTEIN METRICS
Advancing Life Science with Data Science

- Customized reporting template that focus only on Cys & NEM modifications, for high-throughput data processing

		MS Id ←		1
		MS Alias name ←		NISTmAb_FreeCys (%)
Protein name ↑	Var. Pos. Protein ↑	Mod. Names ↑	Mod. AAs ↑	
NIST mAb HC	22	NEM:2H(5)/130.0791	C	96.5
		Nethylmaleimide/125.0477	C	3.54
	97	NEM:2H(5)/130.0791	C	96.9
		Nethylmaleimide/125.0477	C	3.07
	147	NEM:2H(5)/130.0791	C	97.4
		Nethylmaleimide/125.0477	C	2.55
	203	NEM:2H(5)/130.0791	C	97.7
		Nethylmaleimide/125.0477	C	2.31
	223	NEM:2H(5)/130.0791	C	99.9
		Nethylmaleimide/125.0477	C	0.121

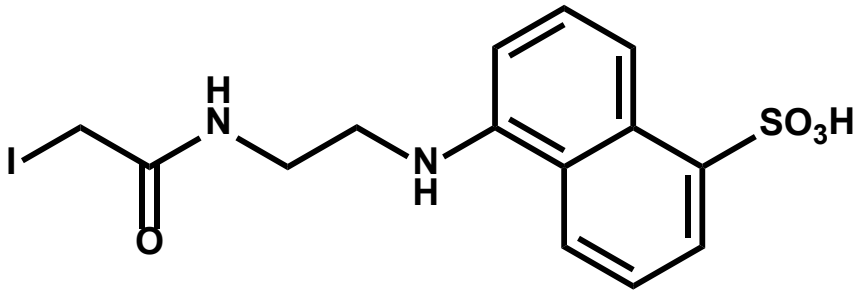
Proof of Concept – NIST mAb



- Free cysteine level at each cysteine site is quantified individually

Case Study 1 – In-Process Sample Support

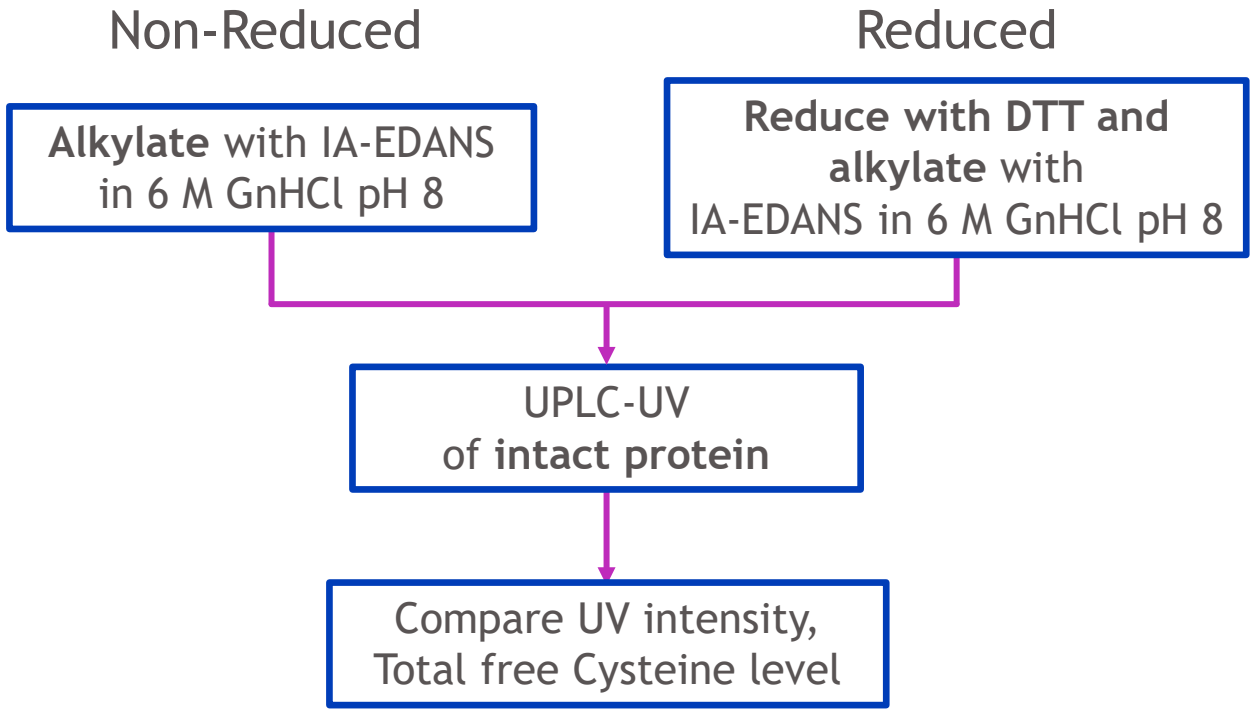
- Program A is a therapeutic protein
- Upstream developed a two-step process, for which two fragments of the target protein are expressed separately then assembled through disulfide bond
- Total free cysteine assay revealed high free thiol content for the precursor after step 1



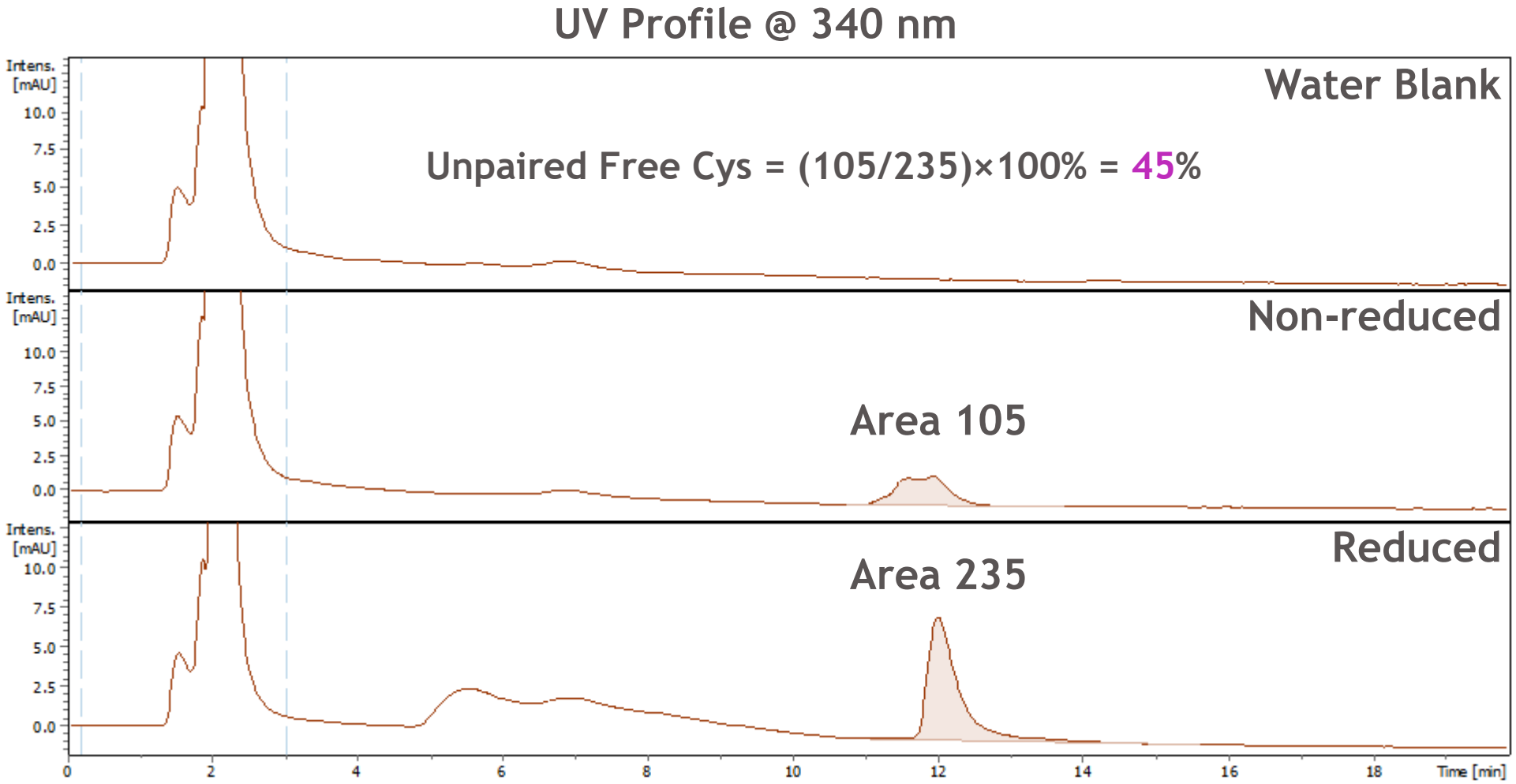
N-(Iodoacetyl)-*N*'-(5-sulfo-1-naphthyl)ethylenediamine

IA-EDANS

- Fluorescent/colorimetric alkylating reagent
- UV/Vis λ_{max} at 340 nm



Total Free Cysteine Assay on Precursor

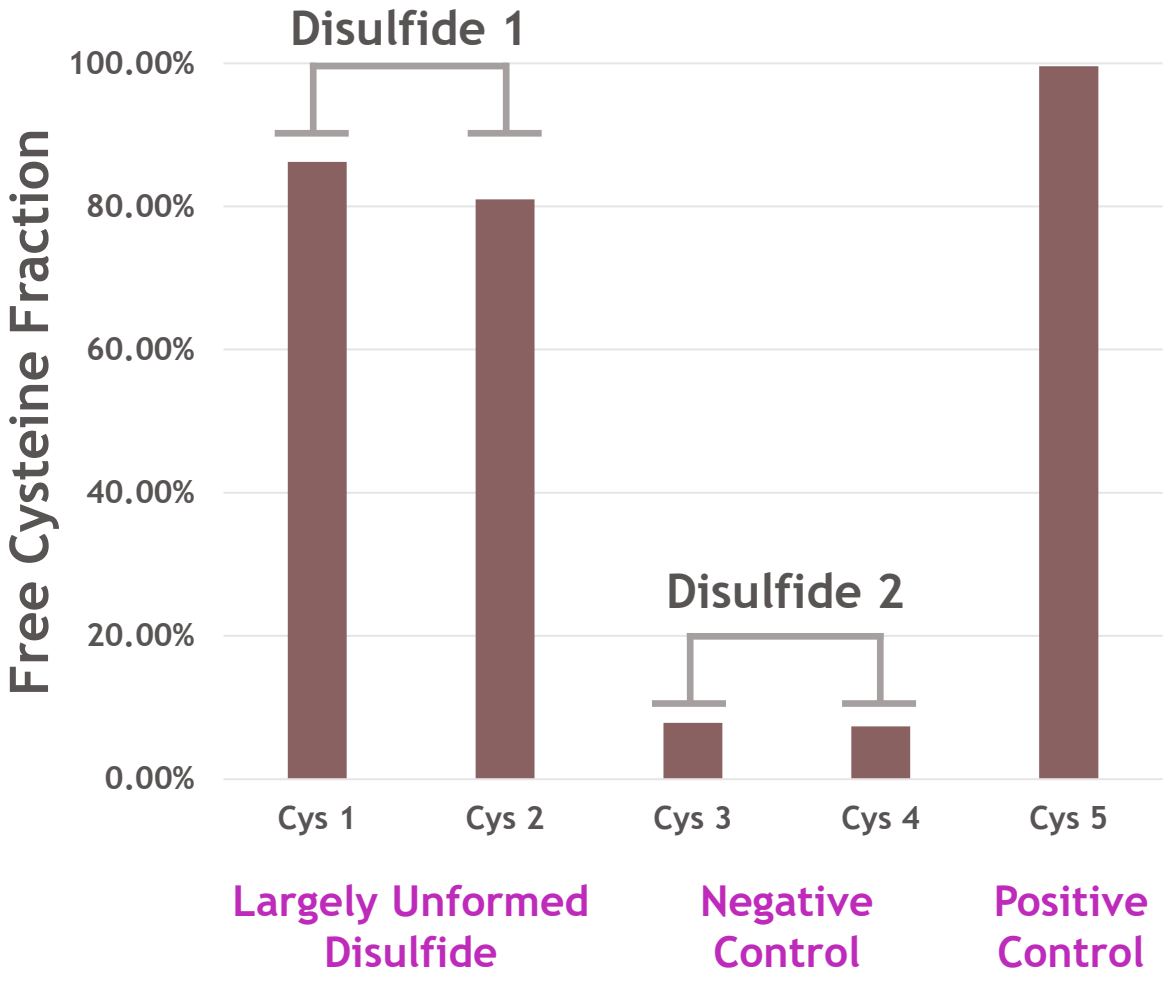


- Observed high free thiol content for precursor yet cannot localize the free thiol

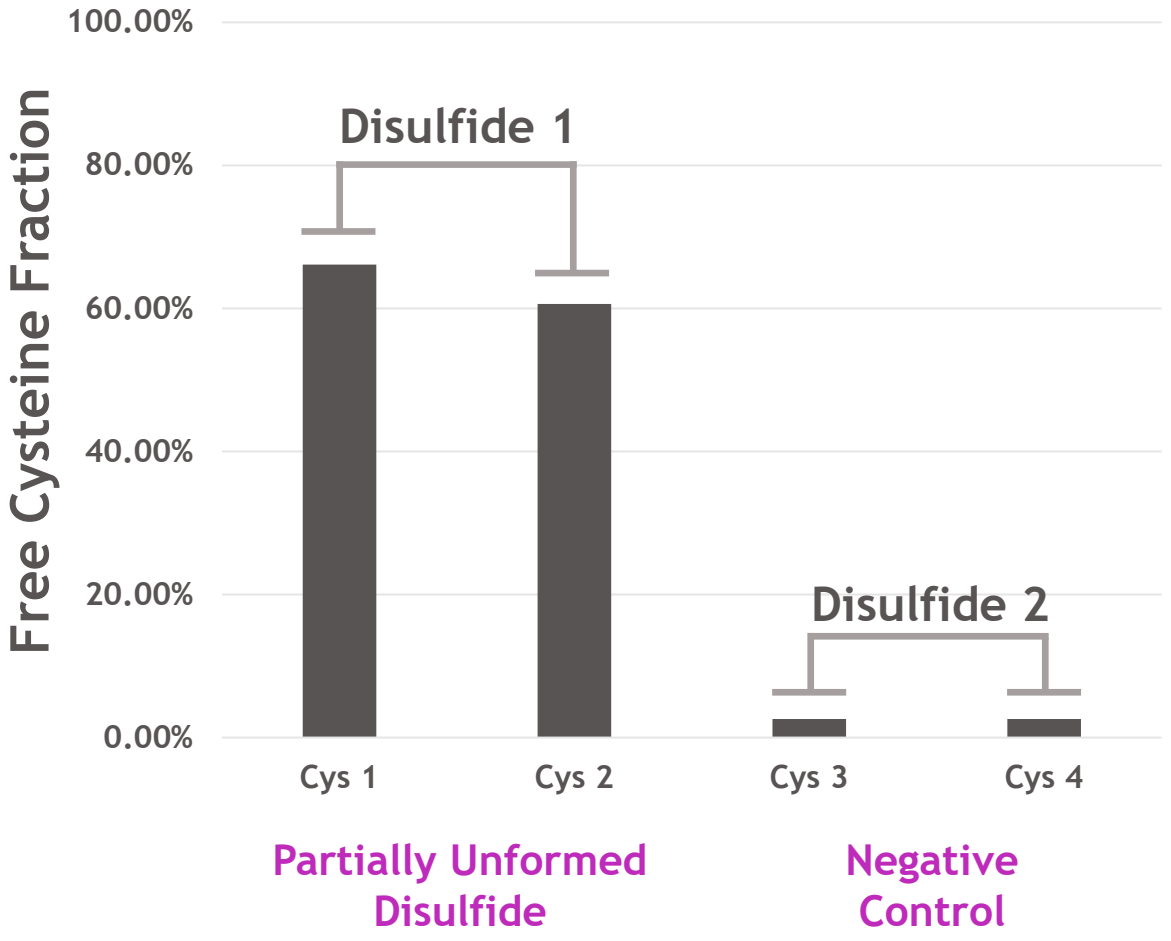
Jacob Bongers

Free Cysteine Footprinting with Differential Alkylation

- Precursor after Step 1



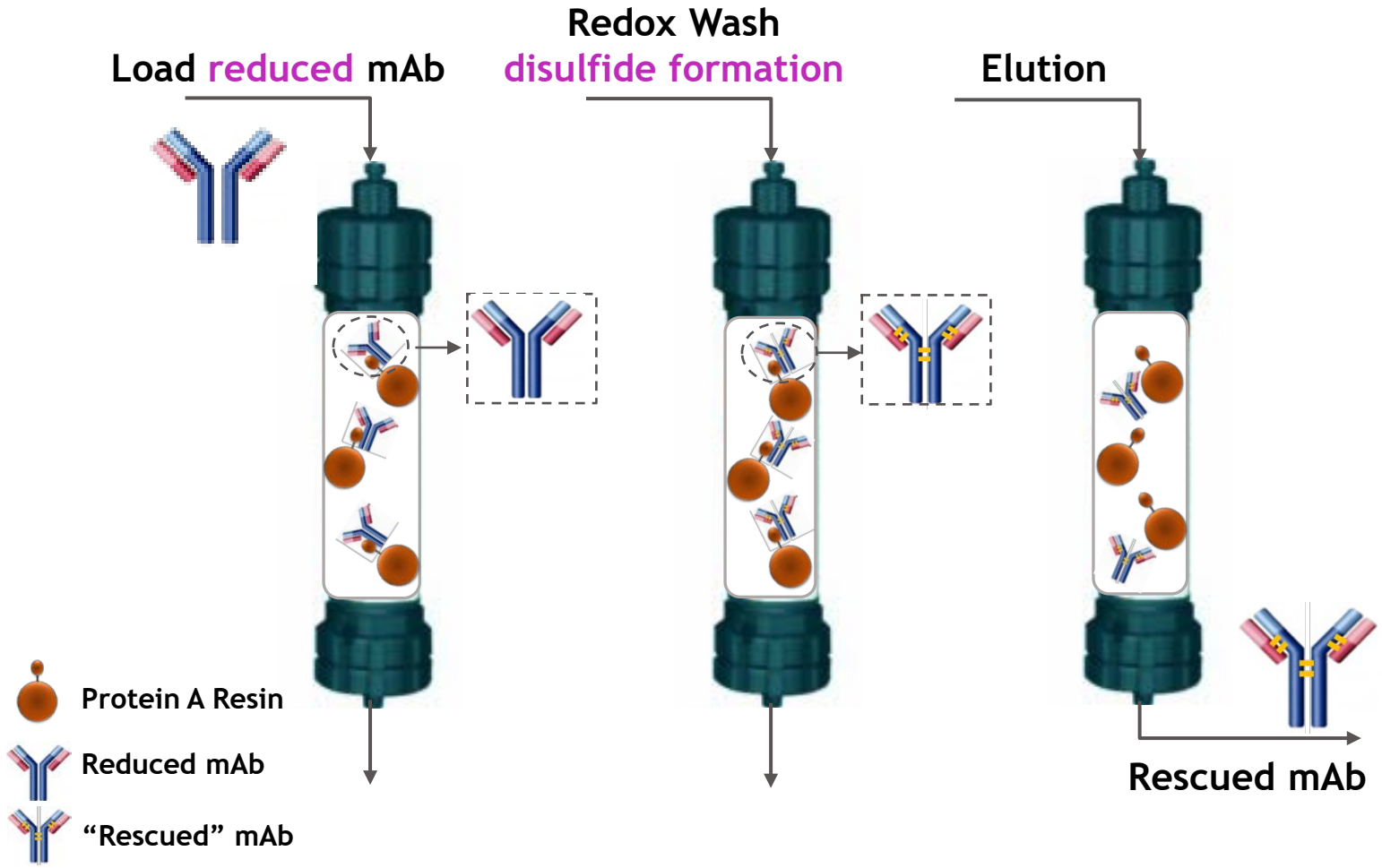
- Final Assembly



Case Study 1 – Conclusion

- Total free cysteine assay revealed high free thiol content for precursor
- Free cysteine footprinting by differential alkylation identifies the root cause of high free thiol content by locating a pair of unformed disulfide bond
- Localization of unformed disulfide bond enlightens process team to request further analytical evidence to better manage the risk
- This pair of unformed disulfide bond is buried inside the protein from an HOS perspective and have minimum impact on protein structural integrity and potency

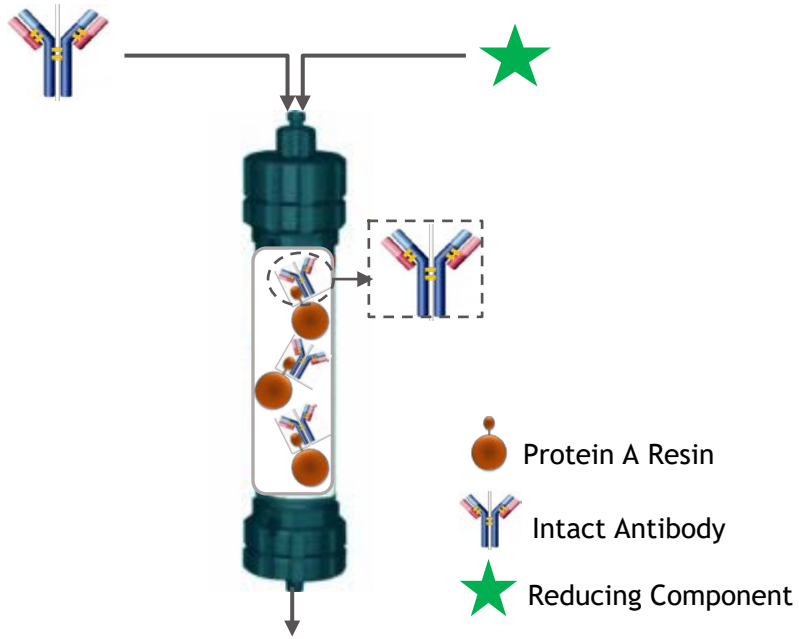
Case Study 2 – On-Column Disulfide Bond Rescue



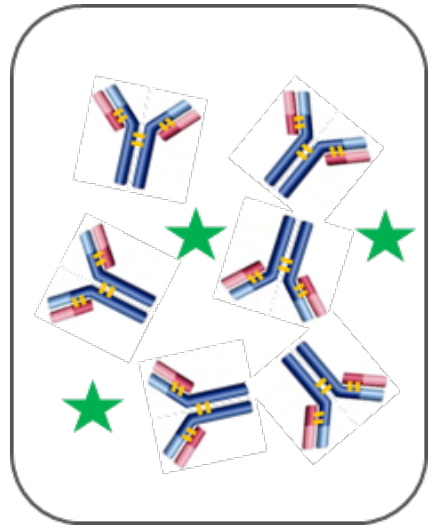
Tan, Z.; Ehamparanathan, V.; Ren, T.; Tang, P.; Hoffman, L.; Kuang, J.; Liu, P.; Huang, C.; Du, C.; Tao, L.; Chemmalil, L.; Lewandowski, A.; Ghose, S.; Li, Z. J.; Liu, S. *mAbs* **2020**, *12*, 1829333.

On-Column Disulfide Bond Reduction

- **On-column evaluation**
  - Load intact mAb on affinity column
  - Wash with reducing reagent for with various times
  - Elute for non-reduce purity analysis



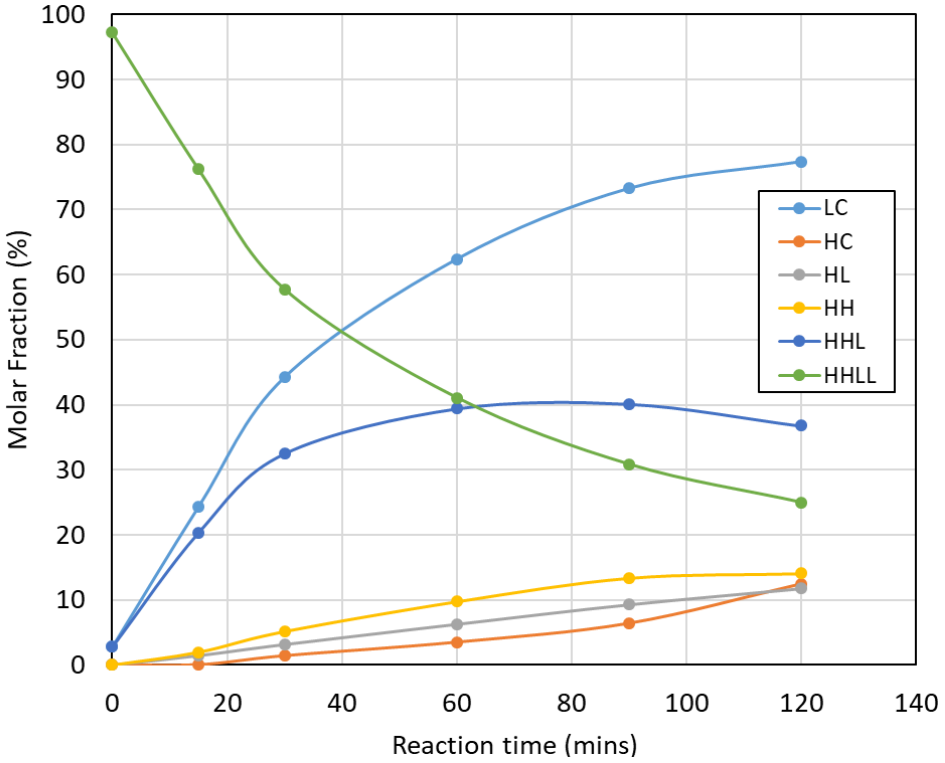
- **Free solution evaluation**
  - Mix intact mAb with reducing reagent in solution
  - Incubate for multiple time points and quench the reaction by adding iodoacetamide
  - Non-reduce purity analysis



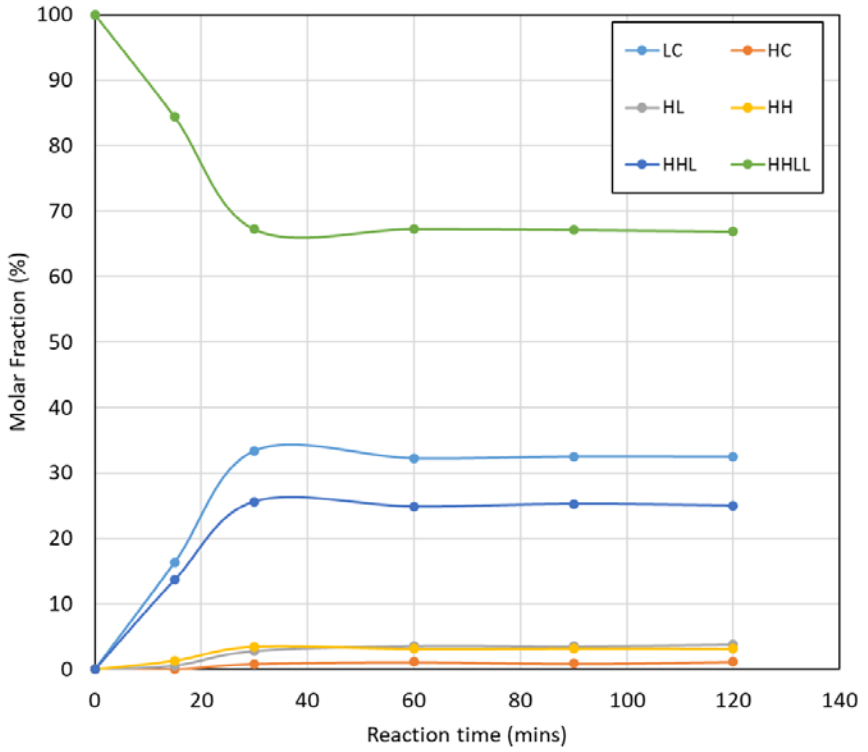
- **Build a kinetic model for mAb disulfide bond reduction**

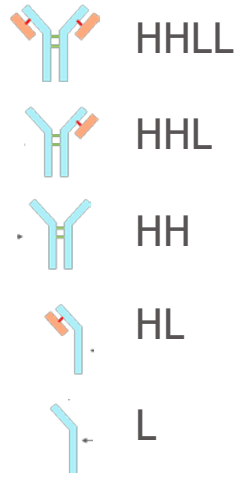
Caliper Analysis of On-Column Disulfide Bond Reduction

- On-column evaluation (5 mM Cys)



- Free solution evaluation (same Cys:mAb ratio)

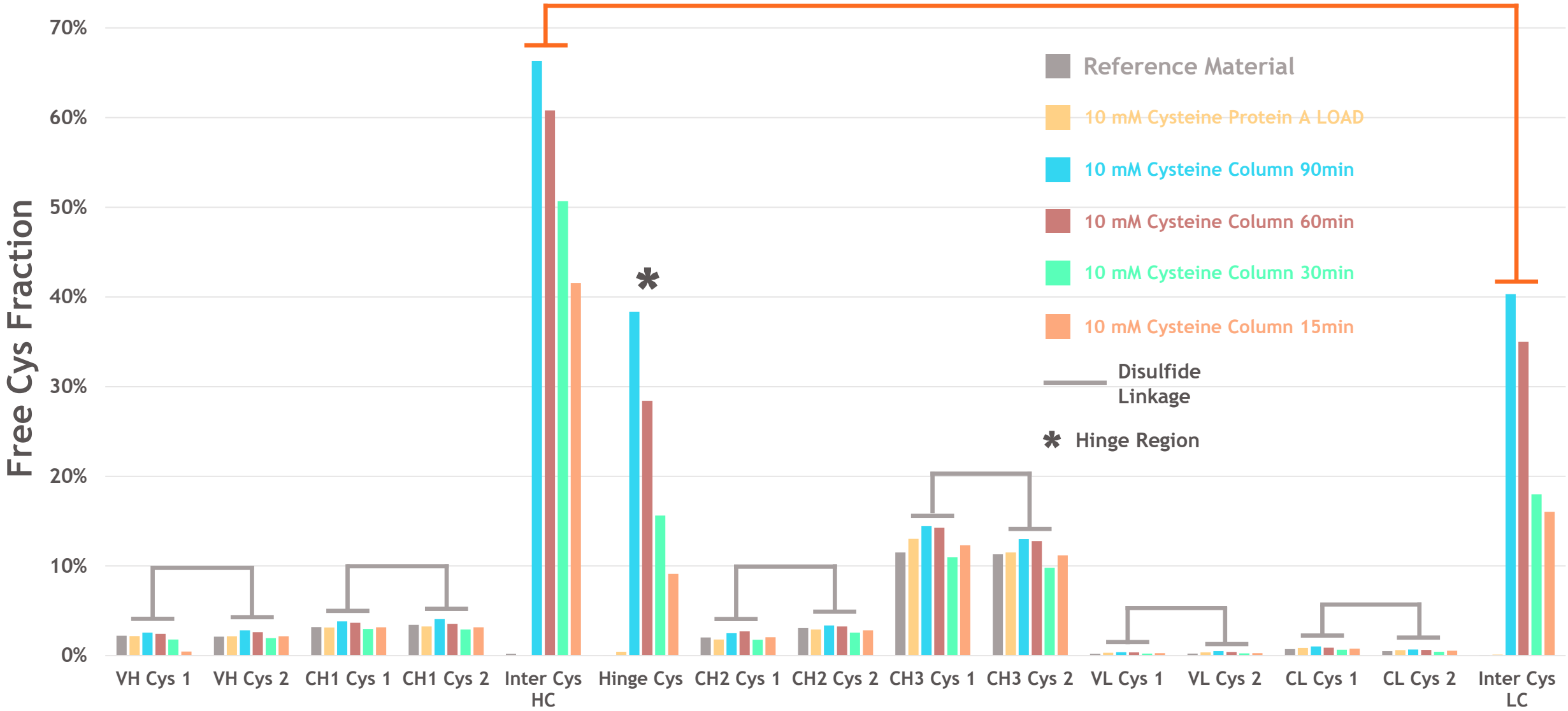




- On-column mAb reduction by Cys allows higher “resolution” at longer reducing time

Nela Zvereva & Tingwei Ren

Free Cys Footprinting for On-Column Disulfide Bond Reduction



Case Study 2 – Conclusion

- Process team developed an on-column mAb reduction strategy to model the disulfide bond reduction process
- An IgG1 was selected to demonstrate and validate this strategy
- Free Cys footprinting reveals that reduction happens exclusively on the solvent accessible inter-chain disulfide bonds
- Footprinting data allows the process team to focus only on inter-chain disulfide bonds when building kinetic models

An improved free cysteine footprinting method by differential alkylation, allowing stakeholders better manage risks in the process development space

Acknowledgement

- **Global Process Analytical Science**
  - Chun Shao
  - Emmanuel Cudjoe
  - Gloria Li
- **Global Upstream Process Development**
  - Yikun Huang
  - Dingzhou Zhao
  - Daniel Mendez
  - Steven Sowa
- **Global Downstream Process Development**
  - Yiran Wang
  - Magfur Alam
  - Daniel Cetnar
  - Steven Noyes
  - Mithun Saha
  - Ujjwal Bhaskar
  - Wai Keen Chung
  - George Tan
- **Analytical Development and Attribute Science**
  - Li Tao
  - Anthony Leone
- **Biologics Development**
  - Gargi Maheshwari
- **Protein Metrics**
  - Janet Tam
  - James Moore

Thank you

Bristol Myers Squibb™