Current Role of MS in Cell Line Development of Biotherapeutics

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Therapeutics Development & Supply

WCBP
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Johnson&Johnson

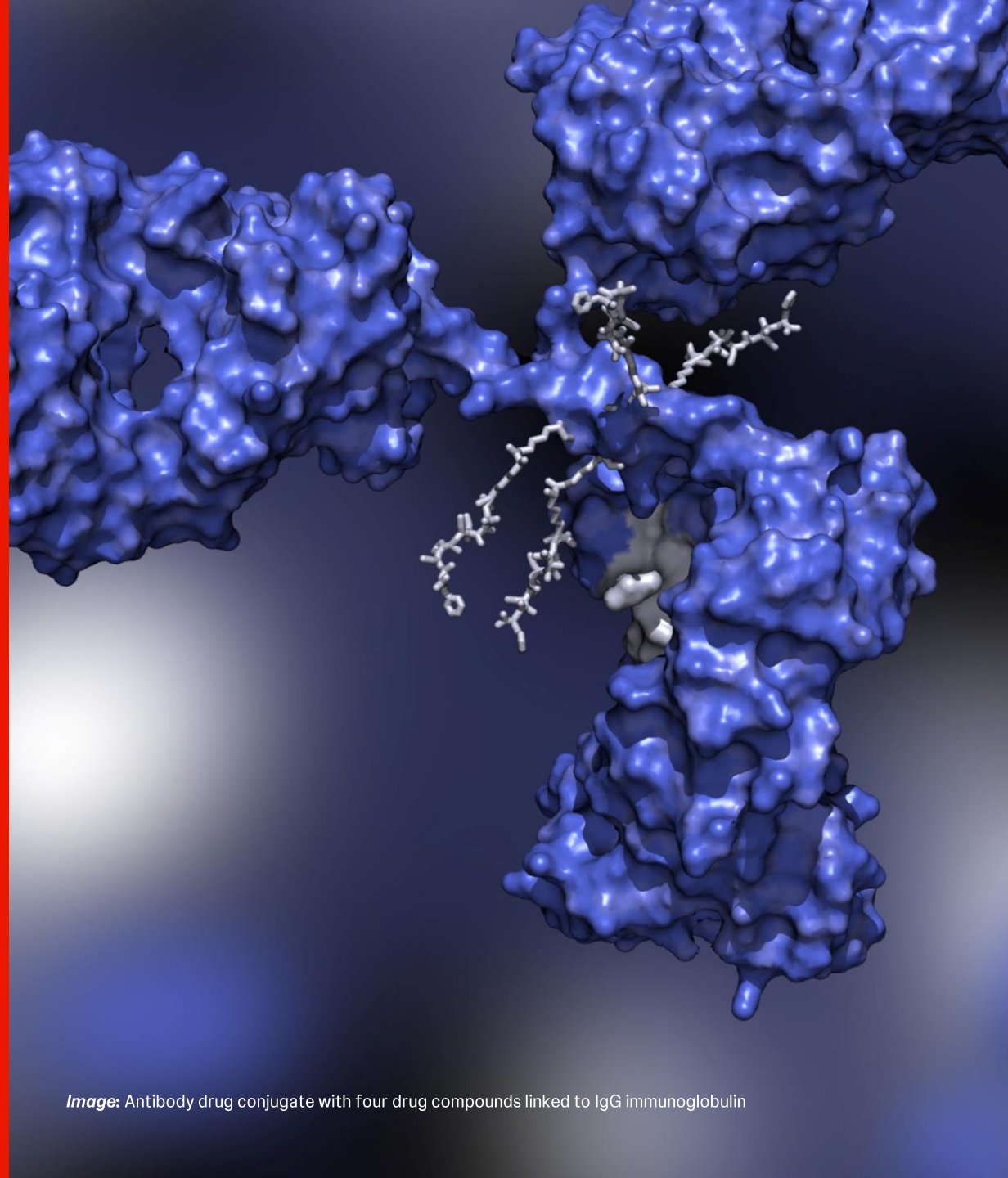
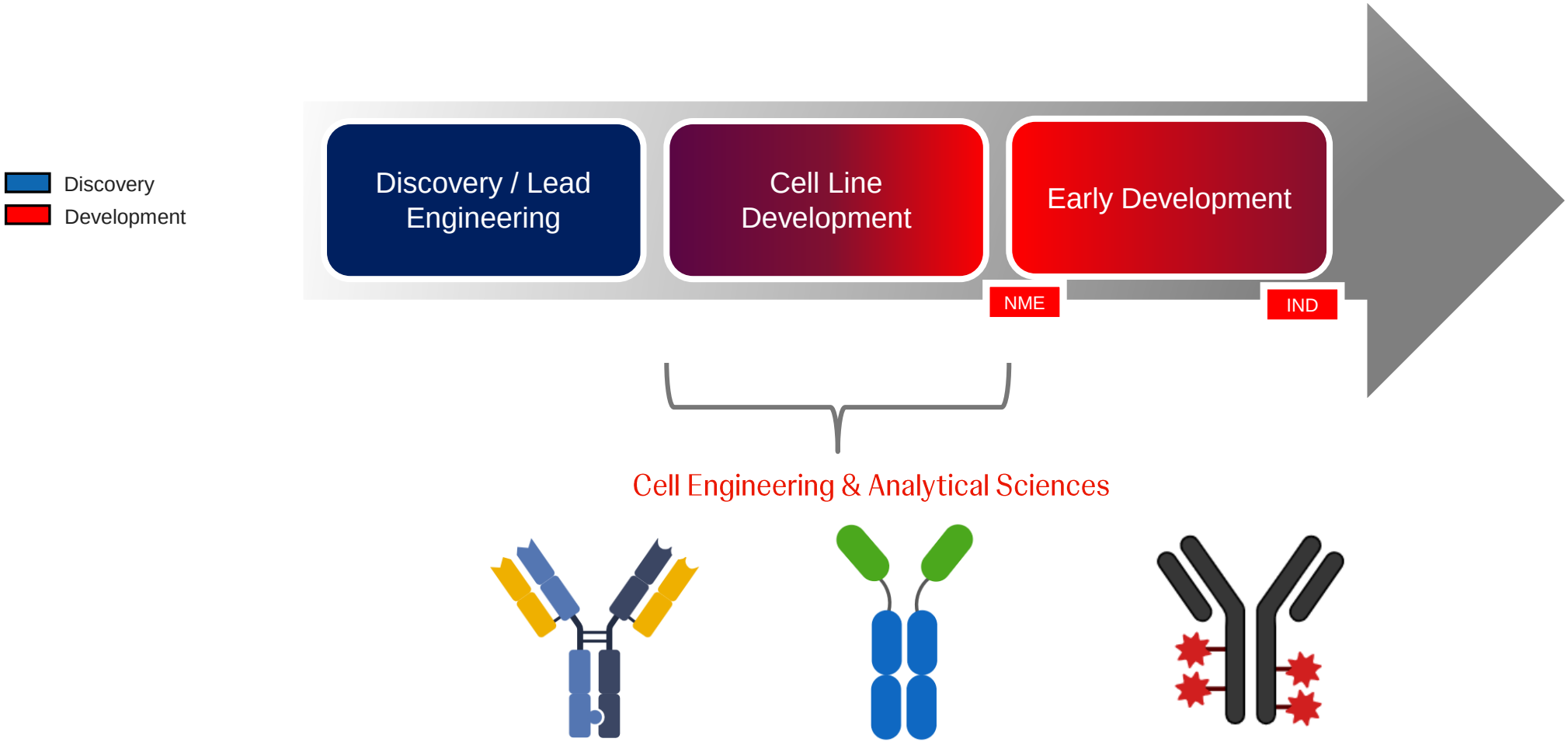


Image: Antibody drug conjugate with four drug compounds linked to IgG immunoglobulin

Outline - MS Platform for Biotherapeutic Characterization

- Cell Engineering – Analytical Sciences
- Analytical inputs to guide Cell Line Development
- Product quality attributes essential in selecting high quality cell lines
- HRAM mass spec platform built for biotherapeutic characterization
- Multi-specific Aggregate and Chain Mis-pairing analysis QE-UHMR
- Determination of LOD by Intact mAb spiking experiments
- Importance of avoiding sequence variants
- LOD, LOQ testing of SV and PTM by peptide map
- Disulfide map with ETD on Eclipse
- Role of Automation in Analytical Sciences
- Future Directions

Cell Engineering & Analytical Sciences

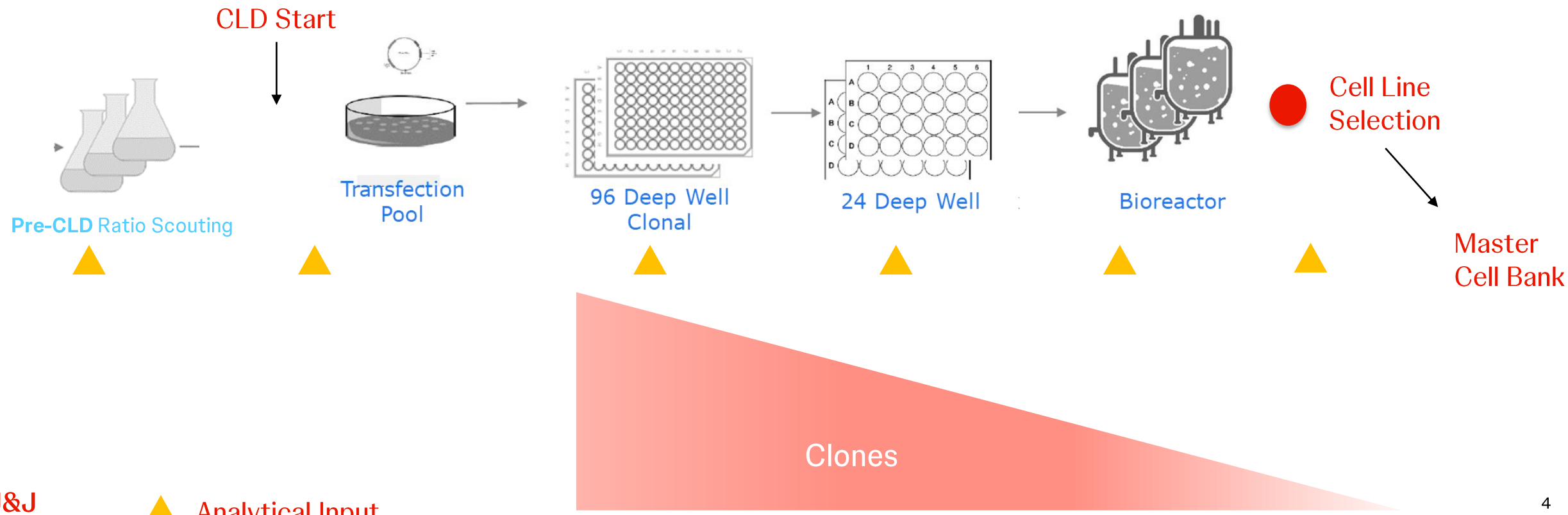


Analytical Inputs Guide Cell Line Development

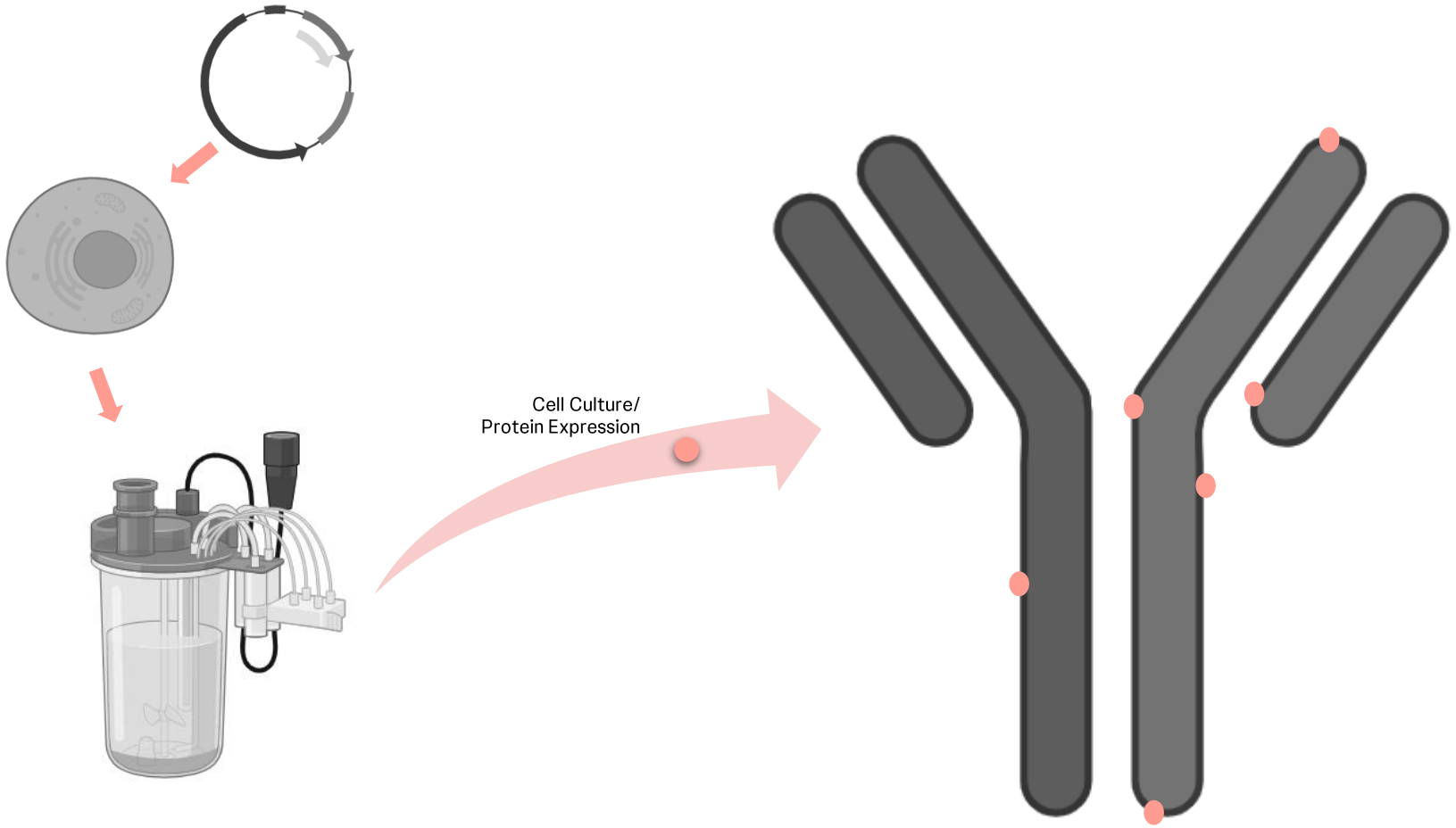
Frequent Analytical Touchpoints Along CLD Process

- Selection from different transfection pools
- Screen large numbers of 96DW plate clones
- Early look at product quality stable cell line (BRX)
- Selection of Final Mfg Clone

Integrated, Real-time Analytical Feedback: Highest Quality Clones move Forward



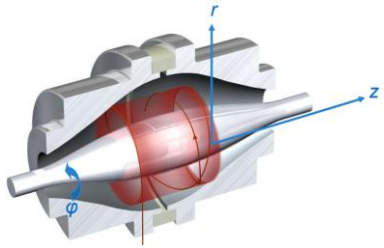
Product quality attributes essential to selecting high quality cell line



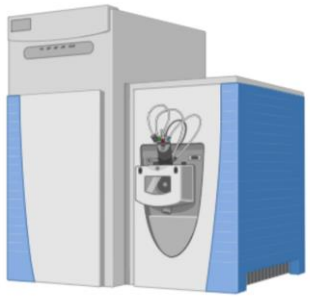
Product Quality Attributes

- Correct MW, Primary Sequence
  - Intact mass, peptide map
  - **Sequence variants**
- N-Glycans (G0, G0F, G1F, Man5)
- O-Glycans, Lys hydroxylation, Linker mods
- Truncations, extensions, clips, terminii
- PTM – typically function of molecule
- Signal peptide processed
- Dimers, trimers and High order aggregates
- Mispaired chains – homodimer, 1/2
- Disulfide-trisulfide, free thiol, Cys
- Aggregates and high order species

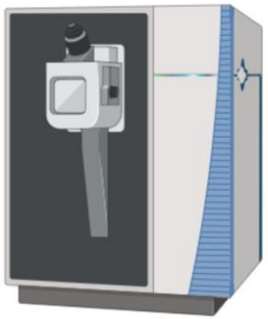
HRAM Mass Spec Platform for Built for Biotherapeutic Characterization



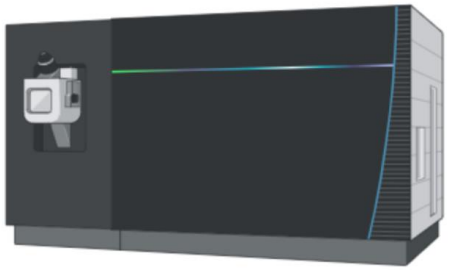
From Aggregates to Peptides



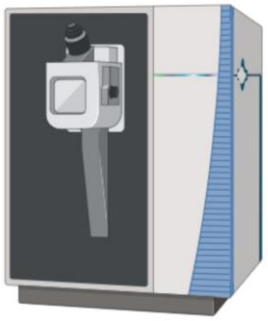
QE UHMR – SEC/MS -Native ESI



OE 480 – Intact Workflows



Neo – nESI FAIMS Eclipse advanced characterization



OE 480 – Peptide Workflows, SVA

- Analysis of Large Molecules requires high resolution and accurate mass
- Characterization of aggregates rely on UHMR optimized to transmit high m/z
- Extended mass range of Exploris offer flexibility for intact to peptide applications.
- Reduced footprint of Exploris ideal for close spacing next to robotics.
- Common format for sample lists and raw data format facilitates scripts to automate steps
- Users training and experience, familiarity with Xcalibur, Chromeleon, Vanquish LC

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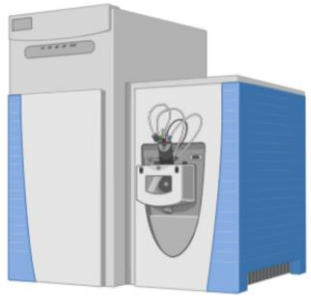
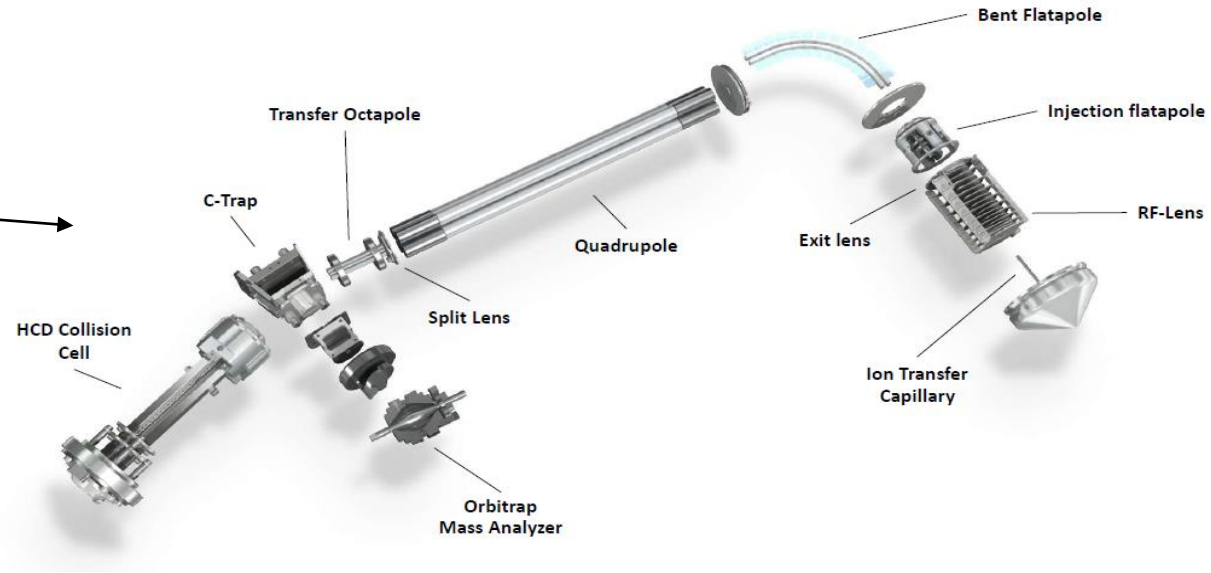
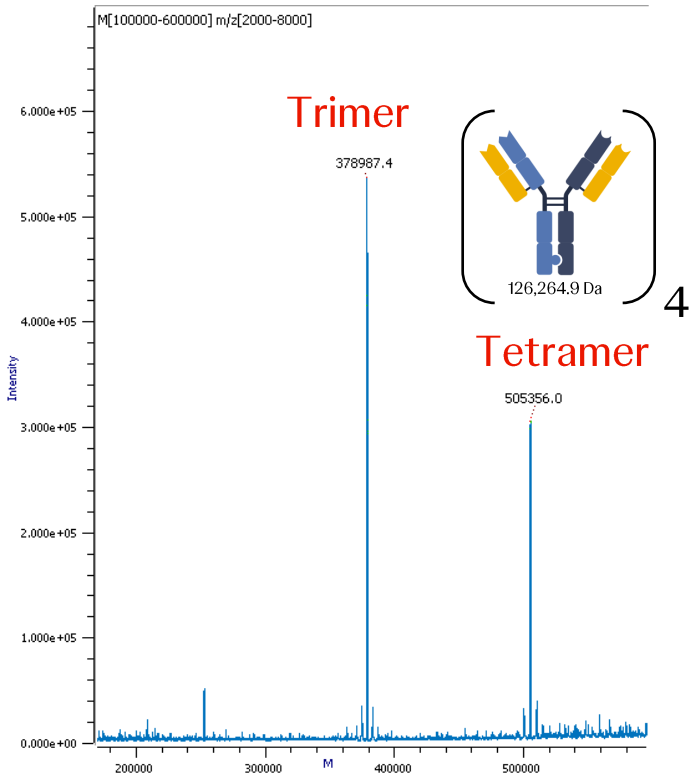
QE UHMR Optimized for High Mass







Lower RF frequencies on all ion routing elements to improve Ion Transmission

- Injection Flatapole
- Bent Flatapole
- Quadrupole
- Transfer Multipole
- C-Trap
- HCD cell

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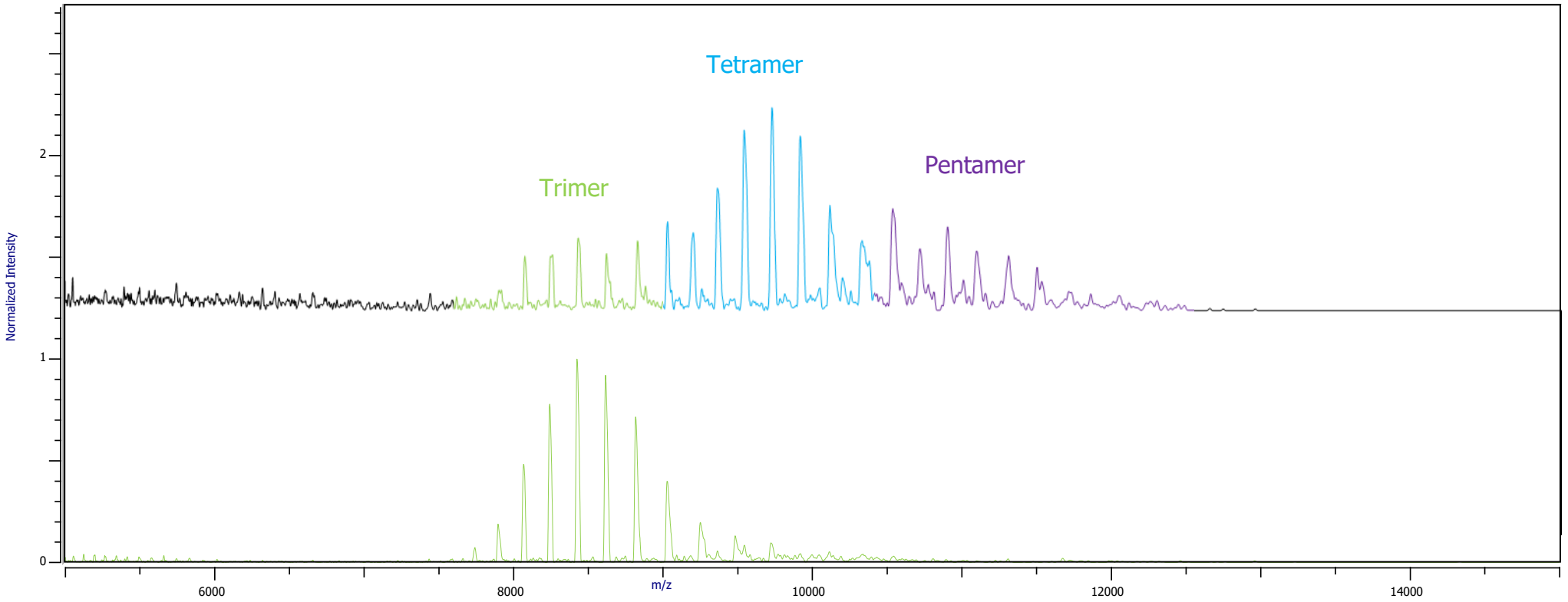
Native SEC-MS – Isolated High MW Fraction Tetramer and Pentamer



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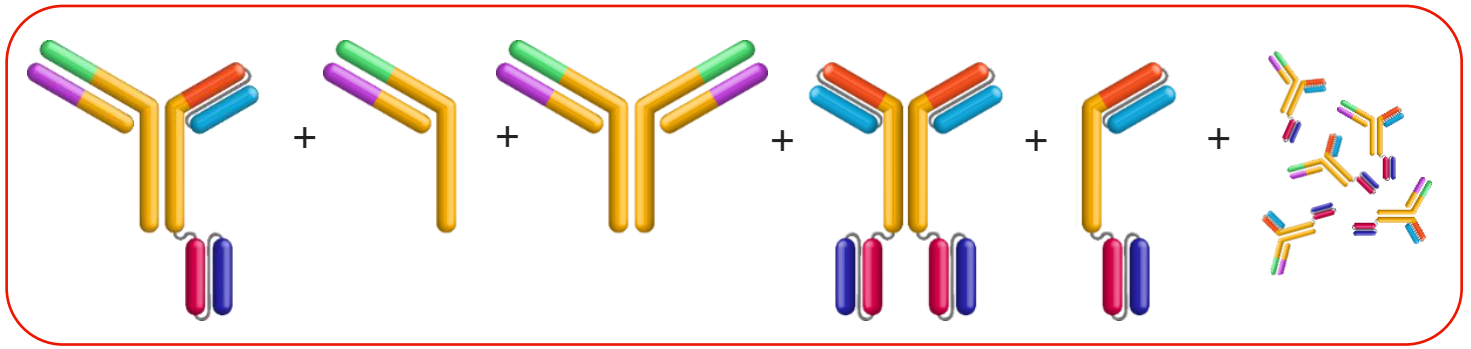
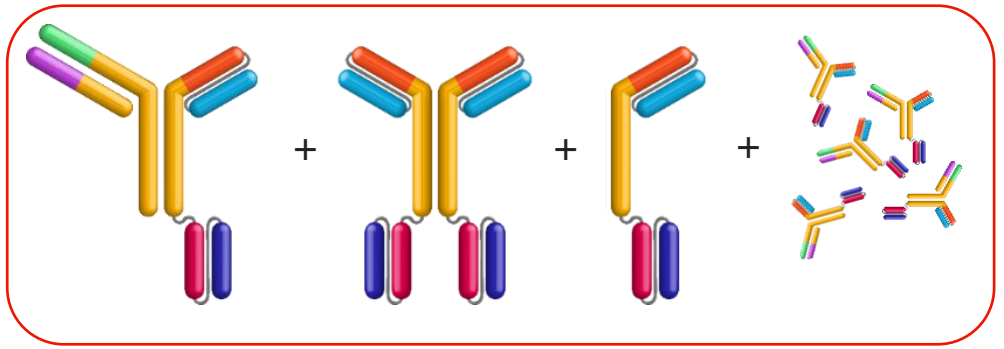
Manufacturing Challenges of Multispecif Ab

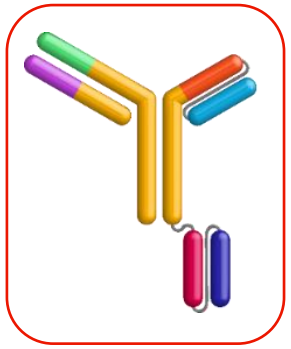
Concurrent Expression

ProA Purification

Further Polishing



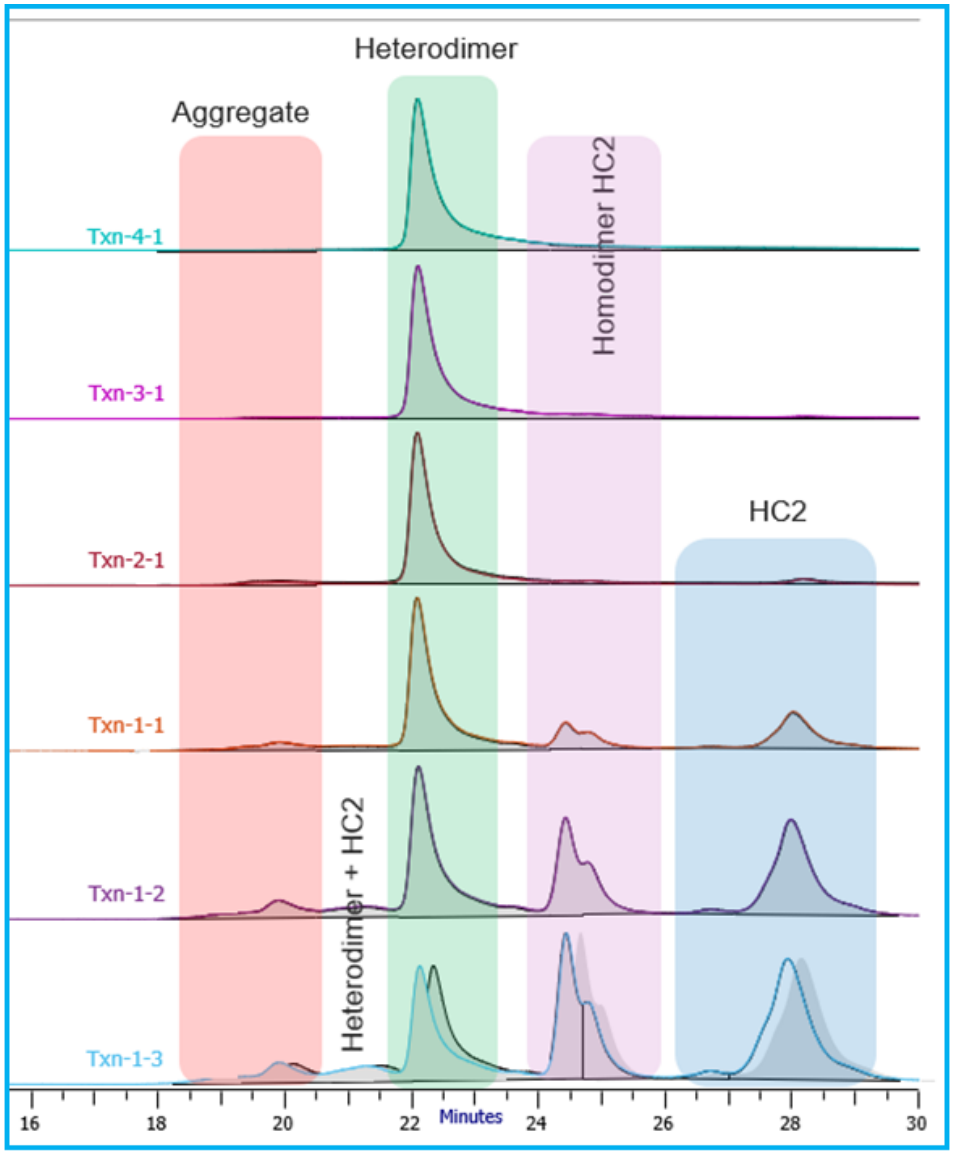


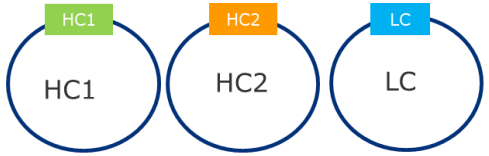


Control excess HC2 through transfection ratios.

Aggregates are difficult to control through expression conditions.

Identification of Trispecific Impurities SEC-MS & Quantitation SEC-FLR

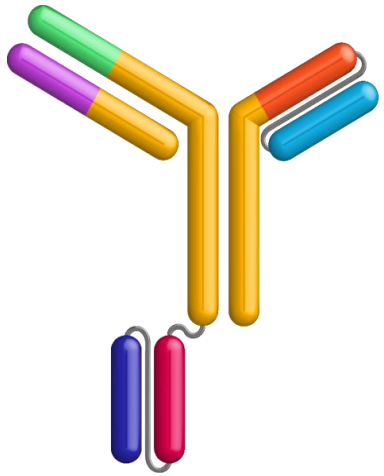




1 : 1 : 2

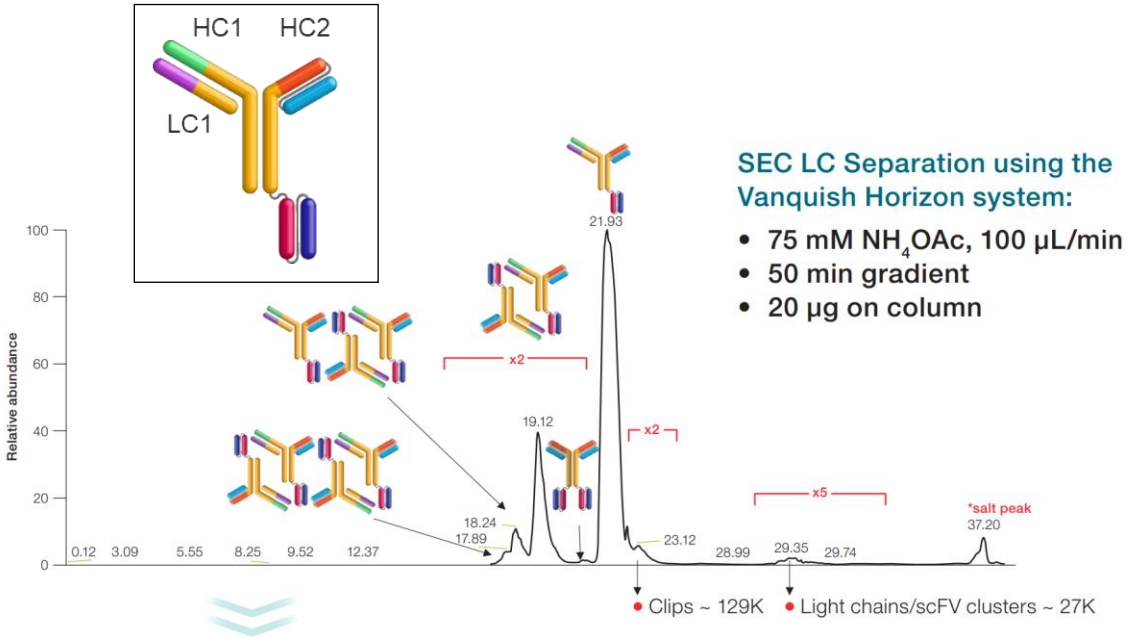
Overexpress HC1

Overexpress HC2



Ret. Time.	19.9	21.8	22.1	24.4	24.8	26.7	28.0
ID	Dimer and *Heterodimer +HC2 Dimer	Dimer and *HC2 Tetramer	Heterodimer	HC2 Dimer		HC2	
Txn-4-1	0.4	1.4	98.3	0.0	0.0	0.0	0.0
Txn-3-1	2.0	2.4	95.6	0.0	0.0	0.0	0.0
Txn-2-1	3.7	2.9	77.0	5.6	0.0	0.0	10.8
Txn-1-1	*5.6	*4.0	57.7	6.7	5.1	0.0	20.9
Txn-1-2	*6.6	*4.9	35.0	13.7	8.3	1.0	30.5
Txn-1-3	*6.7	*5.4	23.8	17.6	9.8	1.2	35.4

Elucidating the Mechanism of Multispecific Antibody Aggregation through Subunit Analysis



SEC LC Separation using the Vanquish Horizon system:

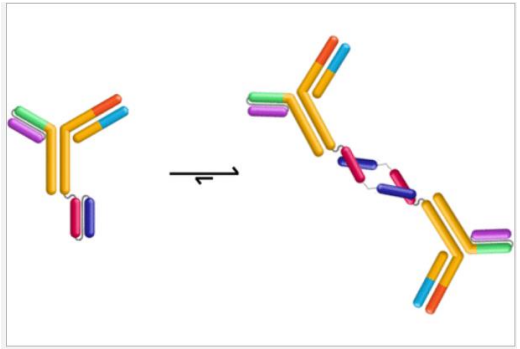
- 75 mM NH_4OAc, 100 µL/min
- 50 min gradient
- 20 µg on column

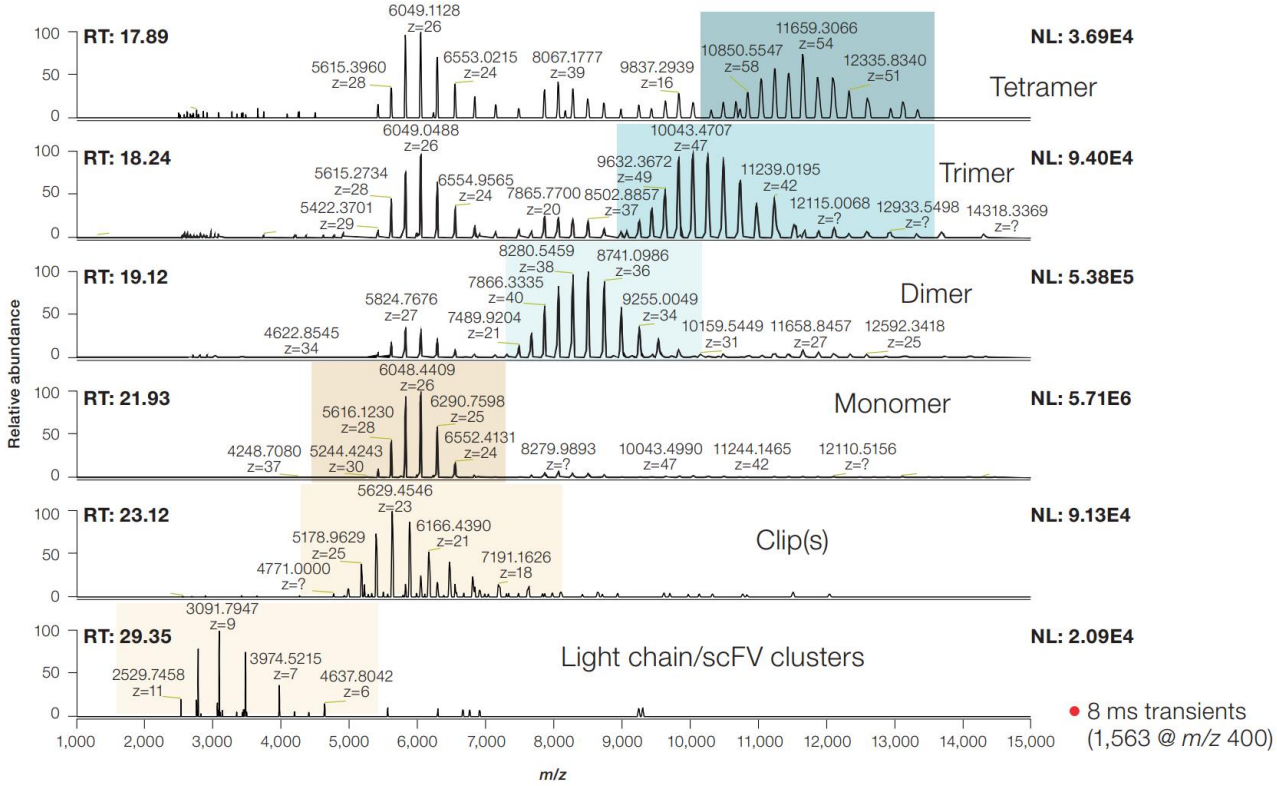
SEC LC Separation using the Vanquish Horizon system:

-75 mM NH_4OAc, 100 µL/min

-50 min gradient

-20 µg on column





J. Am. Soc. Mass Spectrom. 2023, 34, 12, 2654–2661

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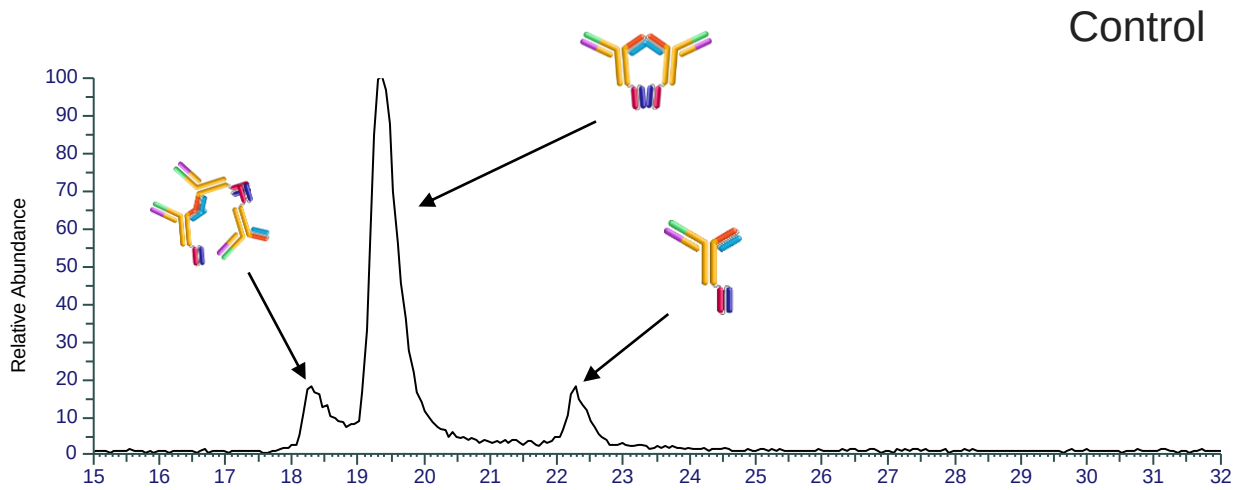
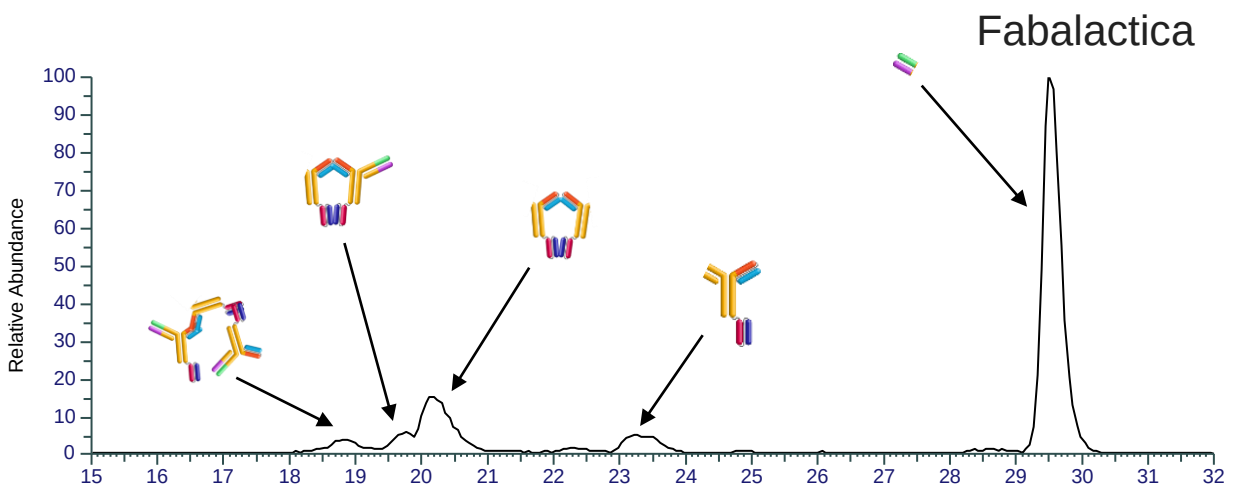
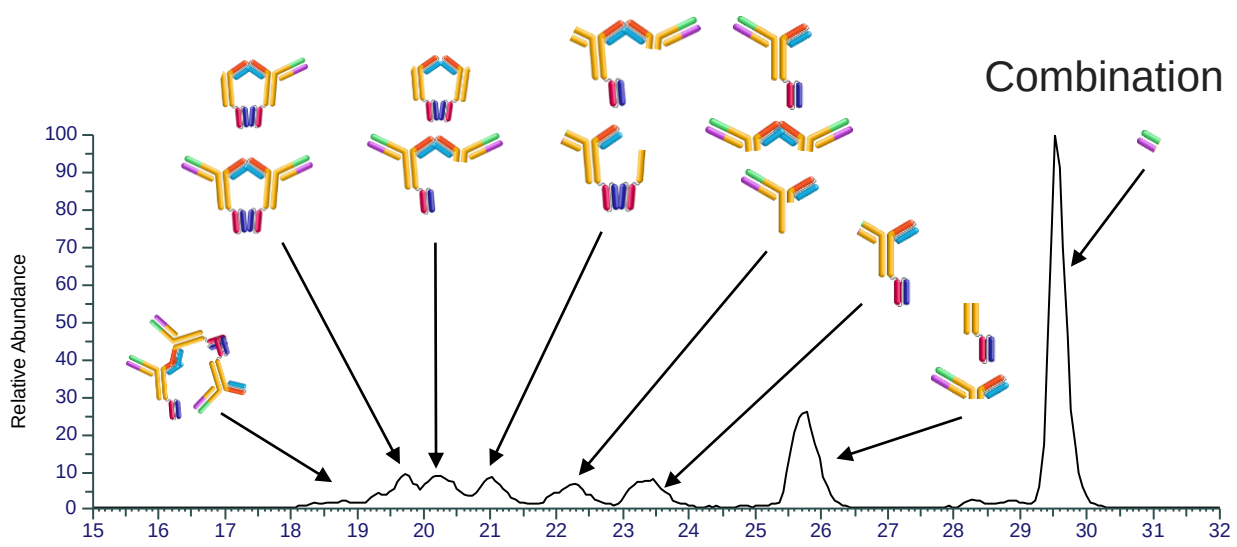
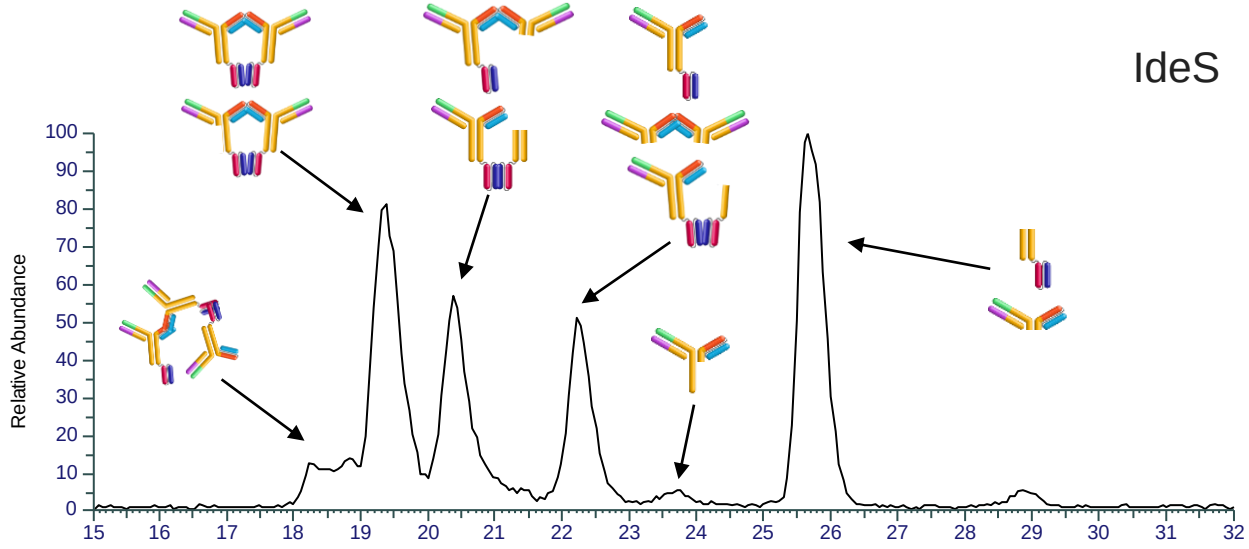
SEC-MS Profiles
Multispecific Subunits









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Intact Workflows

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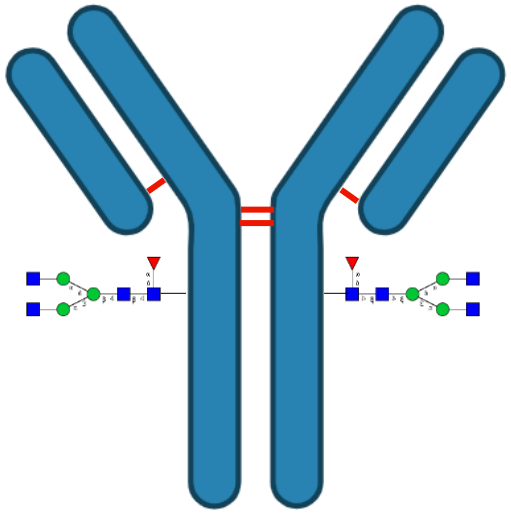
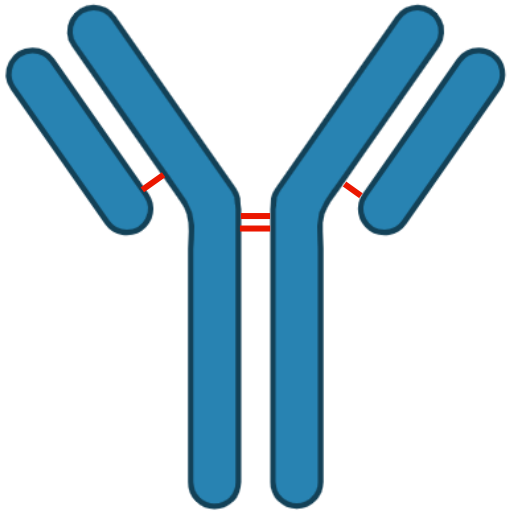
Comprehensive MS Characterization – Top to Bottom

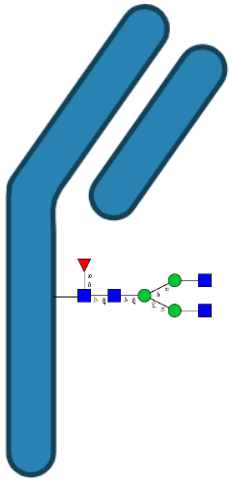
Intact Assays Confirm MW and Assess Global Modifications,



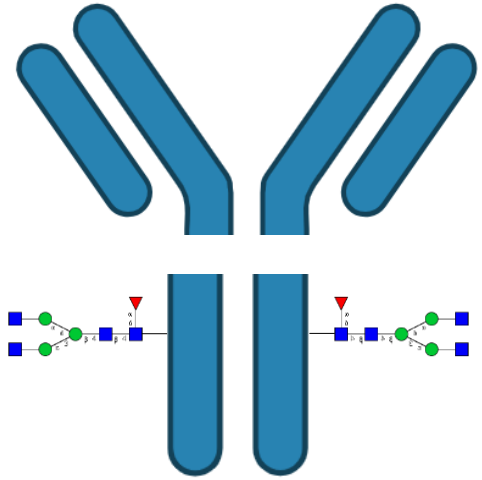
Intact Mass



Degly Intact Mass

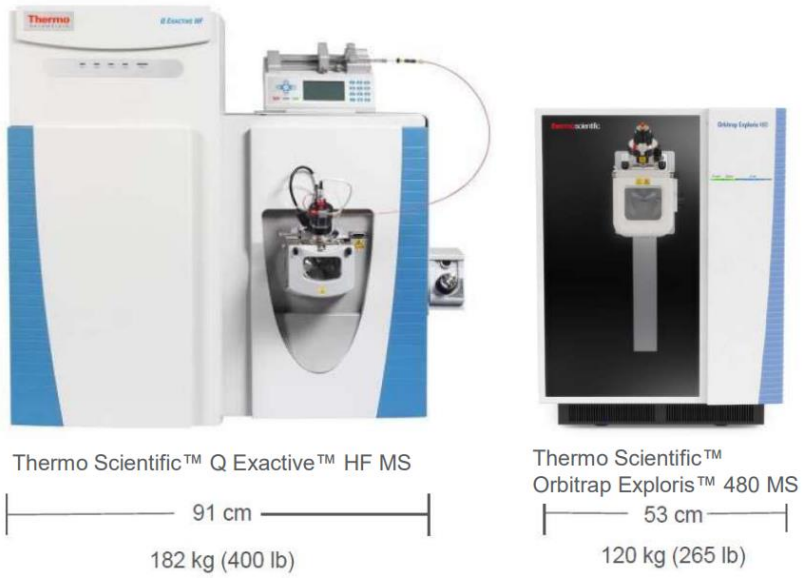


Reduced Mass

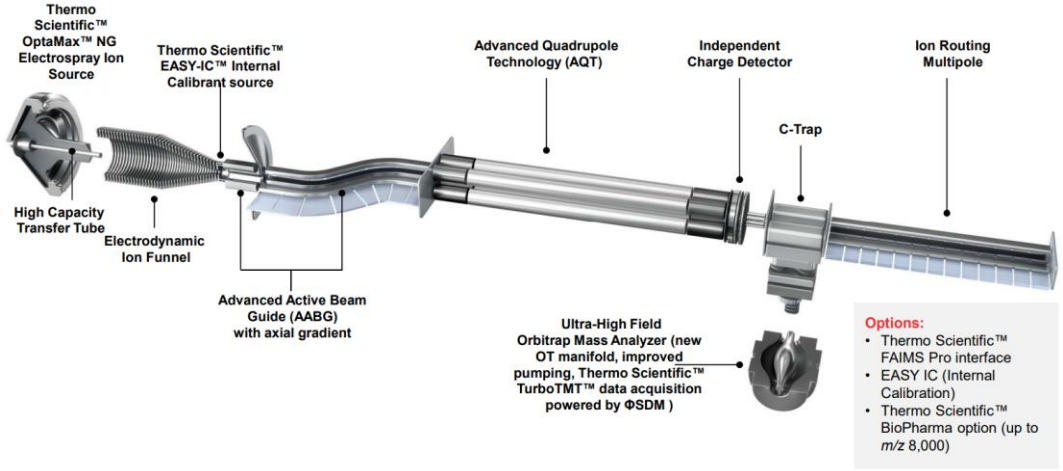


Subunit Mass

Orbitrap Exploris 480



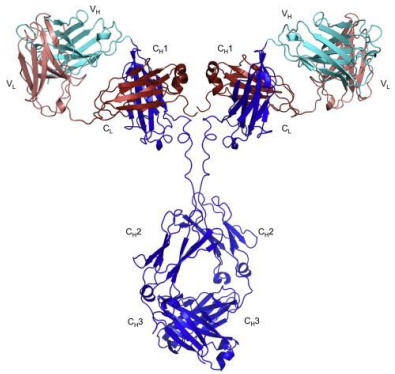
Ion Optics and Mass Analyzers

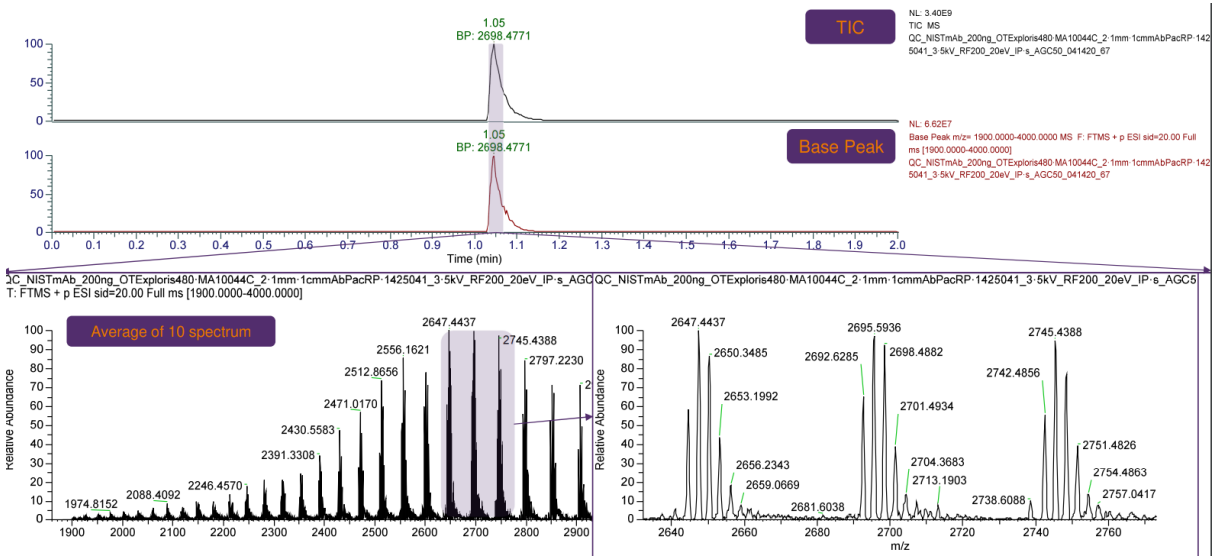


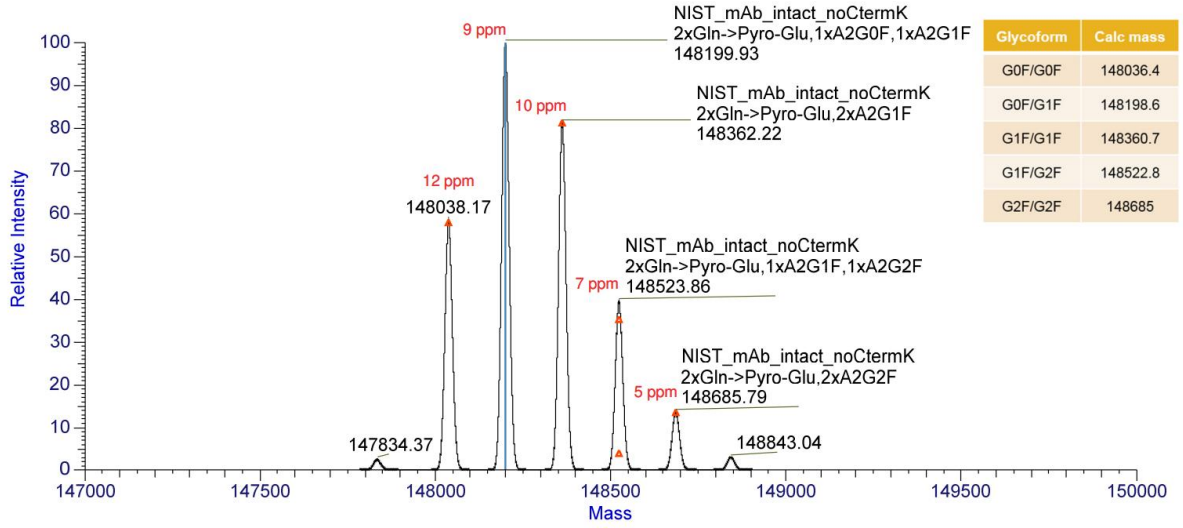
Performance Characteristics	
Resolution	480,000 at *m/z* 200
Mass range	*m/z* 40–6,000 or up to *m/z* 8,000 with BioPharma option
Scan rate *	Up to 40 Hz at resolution setting 7,500 at *m/z* 200
Mass accuracy *	< 3 ppm with external calibration < 1 ppm using internal standard, lock masses
Sensitivity	MS/MS: 100 fg reserpine on column S/N 150:1 SIM: 50 fg reserpine on column S/N 150:1
Dynamic range	> 5,000 within a single spectrum
Polarity switching	One full experimental cycle acquired in at >1.4 Hz where the cycle consists of acquiring one full scan MS in positive and negative polarity at a resolution setting of 60,000
Multiplexing	Up to 20 precursors per scan

- Analysis of Large Molecules requires high resolution and accurate mass
- Characterization of aggregates rely on UHMR optimized to transmit high m/z
- Extended mass range of Exploris offer flexibility for intact to peptide applications.
- Reduced footprint of Exploris ideal for close spacing next to robotics.

Intact Mass on OE480 with Biophama Option







Glycoform	Calc mass
G0F/G0F	148036.4
G0F/G1F	148198.6
G1F/G1F	148360.7
G1F/G2F	148522.8
G2F/G2F	148685

200 ng NIST Ref. Std on OE 480, 12 ppm

Intact Assays

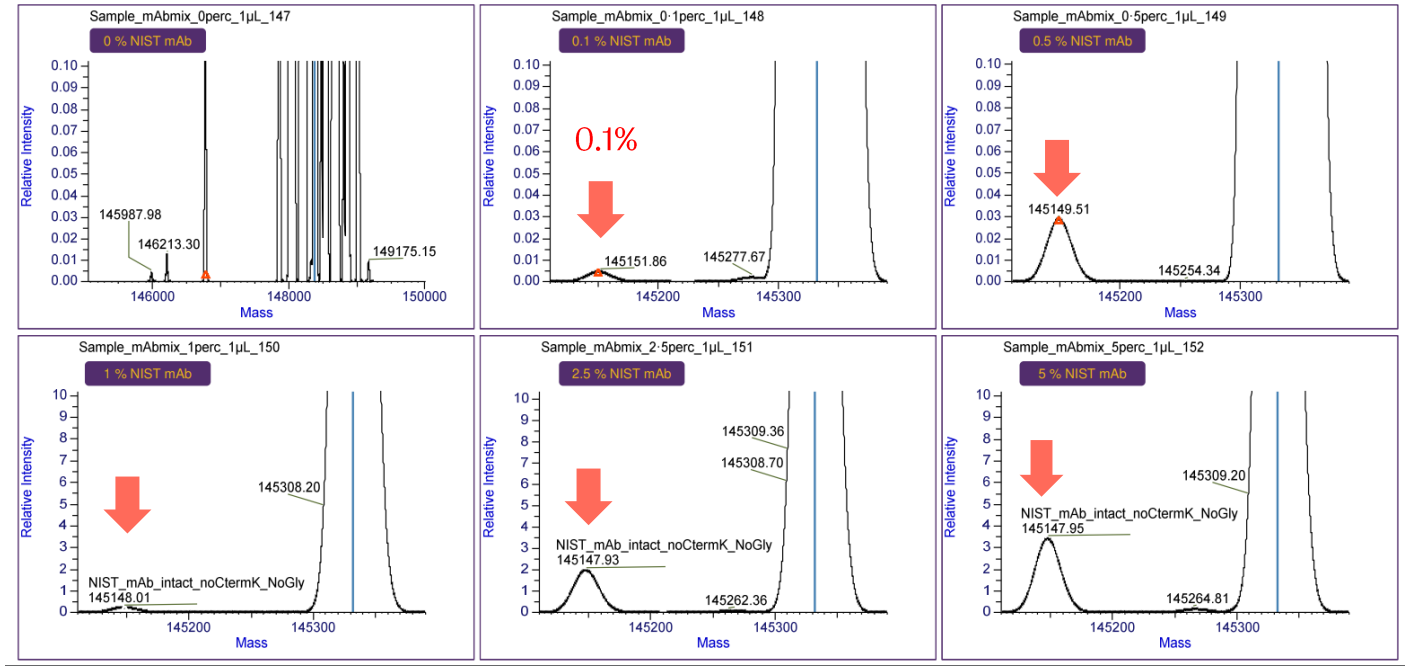
- Confirm MW within 15 ppm
- Glycan profile
- Assess PTMs, Clips
- Excellent tool for 1st pass at purity, undesired species
- Require only ~ 100 ng of material

Intact Spiking Experiments to Evaluate Instrument Dynamic Range and Recovery

Testing ability to detect low level species

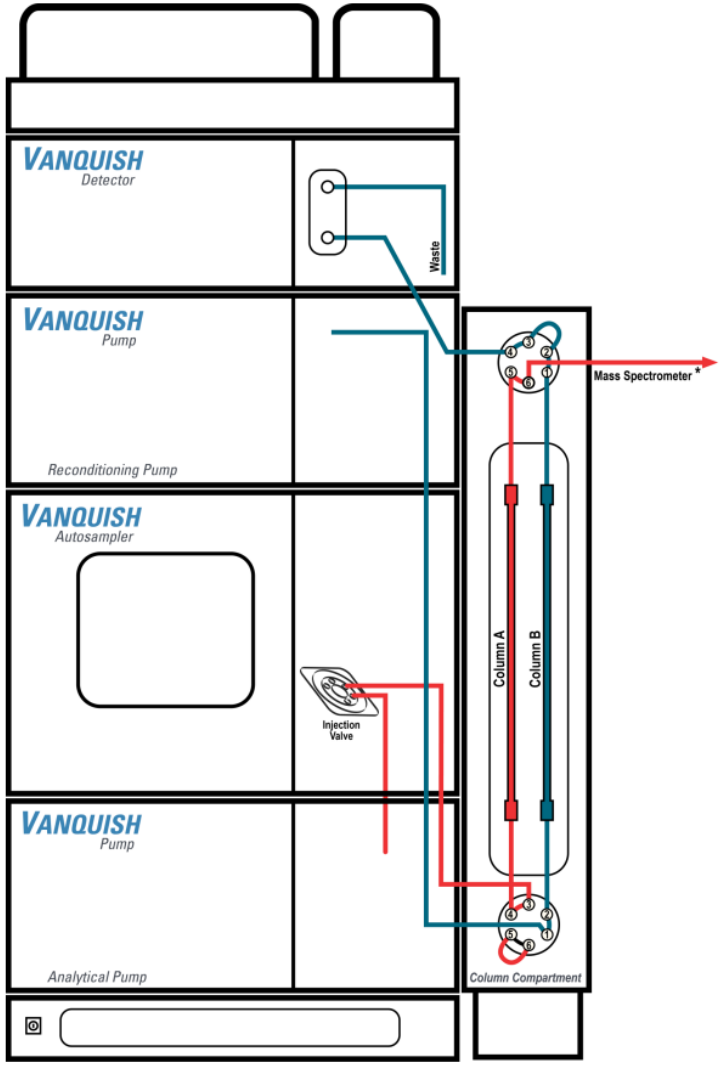
- Experiment Details
  - Spiked NIST mAb (145,149 Da) into Waters mAb
  - Levels: 0, 0.1, 0.5, 1.0, 2.5 and 5.0%
  - Deglycosylated with PNGase F
  - Evaluate ability to detect and % recovery

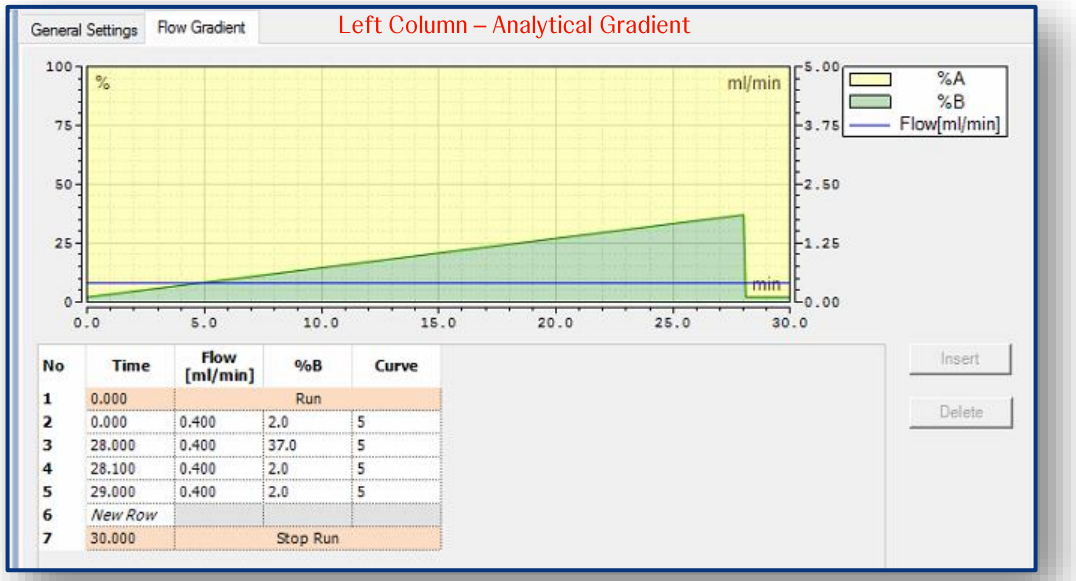
- Results
  - Detected lowest level spike, 0.1%
  - Recovery linear with spike level
  - Within 80 – 120%



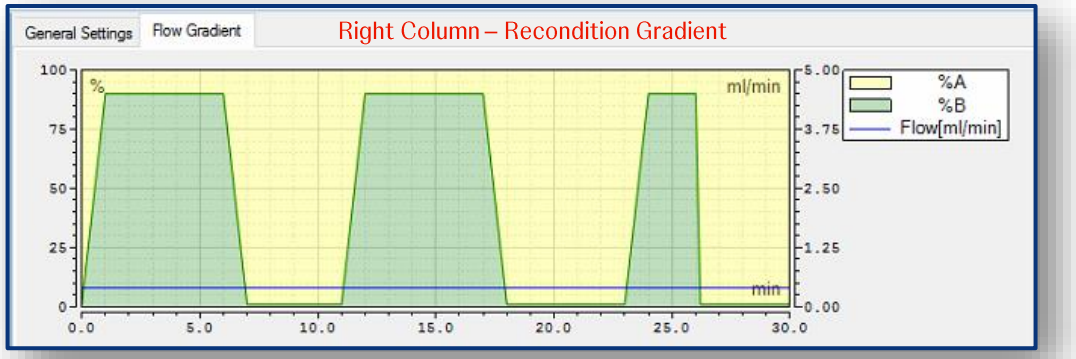
Vanquish Duo UHPLC

Increased sample throughput 2x !





No	Time	Flow [ml/min]	%B	Curve
1	0.000	Run		
2	0.000	0.400	2.0	5
3	28.000	0.400	37.0	5
4	28.100	0.400	2.0	5
5	29.000	0.400	2.0	5
6	*New Row*			
7	30.000	Stop Run		



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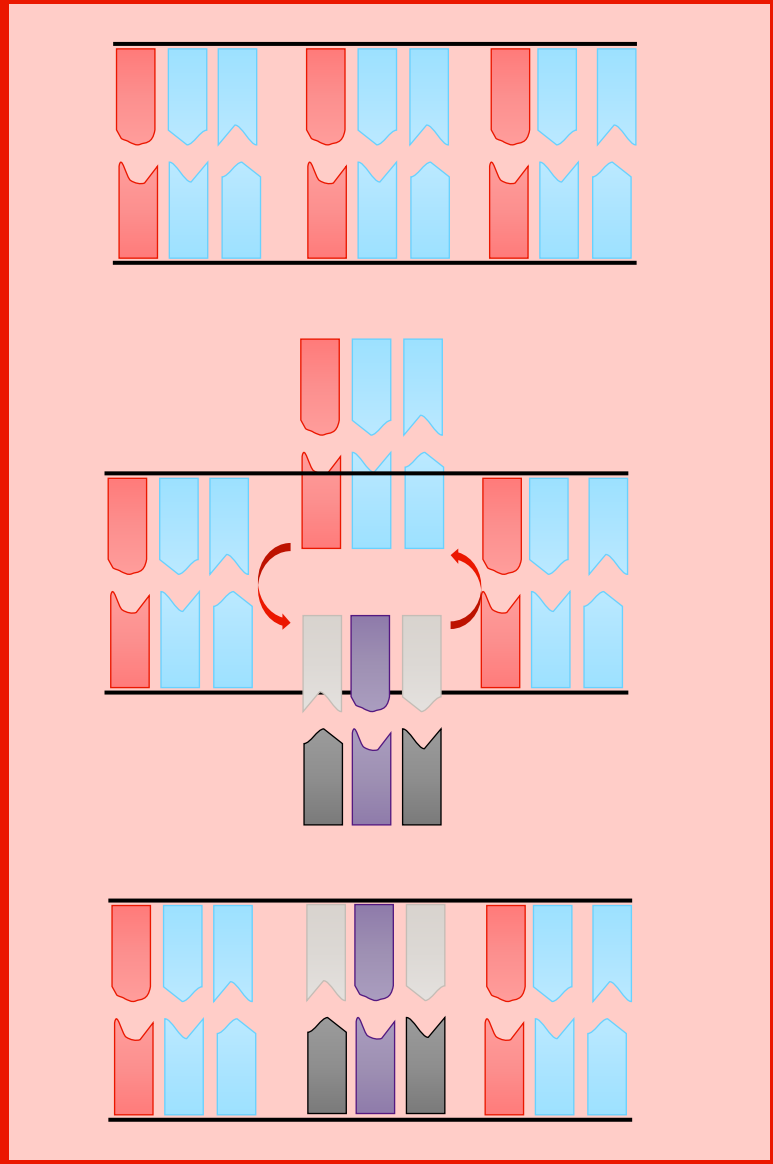
Sequence Variants



DNA Substitution – Genetic Mutation

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Importance of a Robust Sequence Variant Analysis Workflow

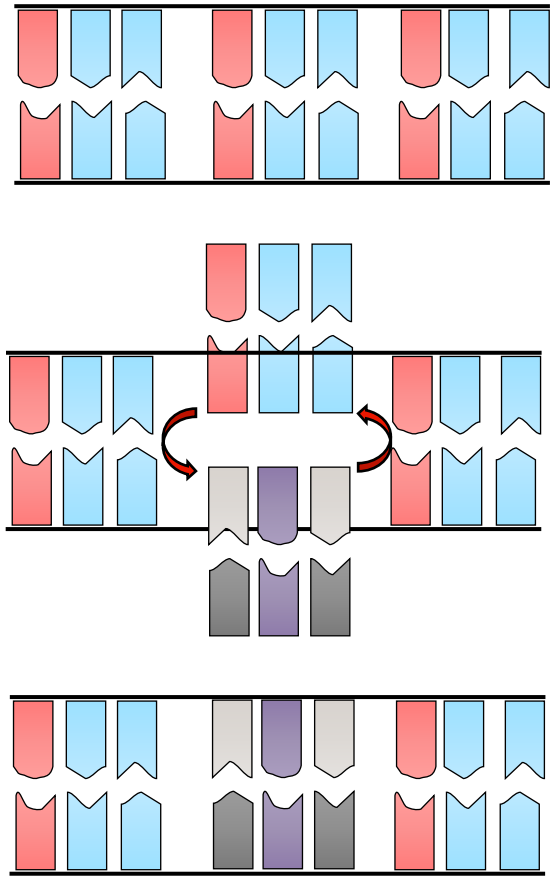
- Goals
  - Screen clones leading into final selection, avoid clones with SVs
  - Detect early and eliminate from group in genetic (or unknown)
- OE 480 LC/MS Peptide Map
  - Multiple protease digests and LC/MS acquisitions to provide orthogonal methods to validate
  - Test SV method with spiking study
  - Filtering method and scoring techniques to evaluate true positive vs. false ID

Sequence Variants

Important to Monitor during CLD

Undesired or unintentional amino acid substitutions, deletions, or insertions during protein biosynthesis

Genetic Mutations – Errors during DNA replication



Mistranslations and Misincorporations

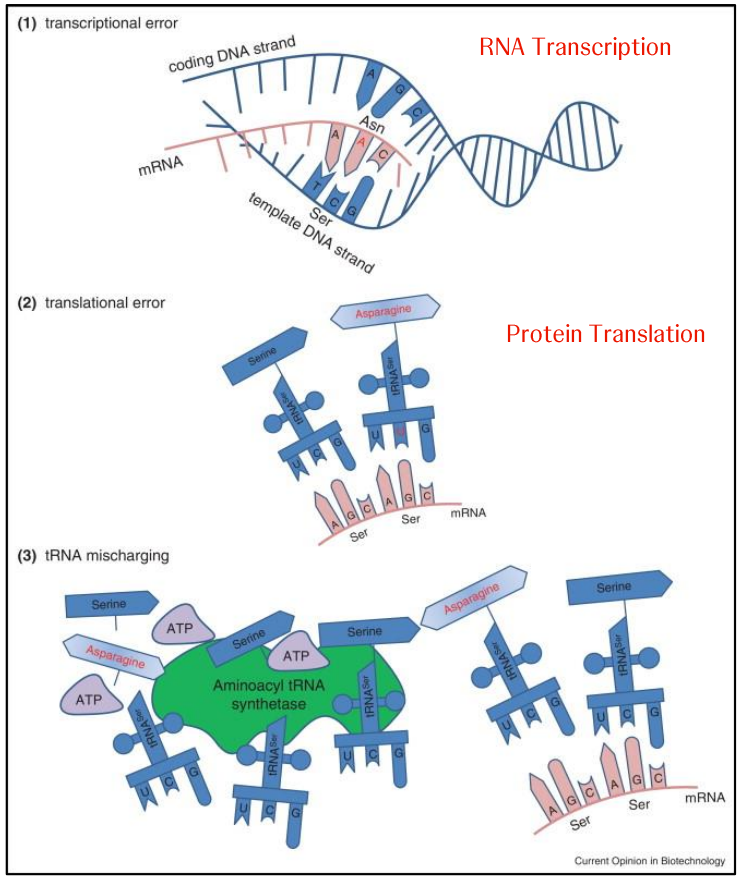


Figure from: Harris, R.P., Kilby, P. M, Amino acid misincorporation in recombinant biopharmaceutical products, Current Opinion in Biotechnology, Vol 30, Dec. 2014

Detection Strategies

- LC/MS peptide map with SV search algorithms
- Next Gen Sequencing (NGS)
- Amino Acid Analysis

Peptide Spiking Experiments to Evaluate SVA Method and Instrument

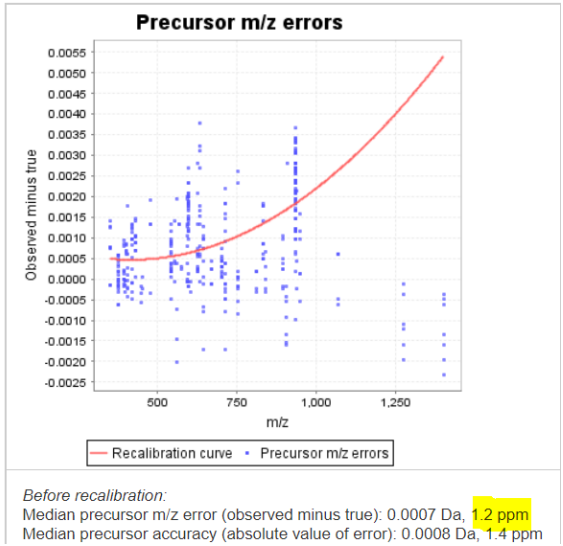
Testing ability to detect and quantitate SV above and below threshold of 0.1%

- Experiment Details
  - Spiked 10 NIST synthetic SV peptides into NIST digests
  - Levels: 0, 0.5, 0.1, 0.2, 0.5 and 1.0%
  - LC/MS/MS peptide map
  - Search: Byos- PMI
  - Evaluate ability to detect and % recovery
  - Compared instruments, gradients, search engines, fragmentation
- Results
  - Detected lowest level spike, 0.05%
  - Provided confidence to set threshold of 0.1% rel.
  - Recoveries were within 80 – 120%

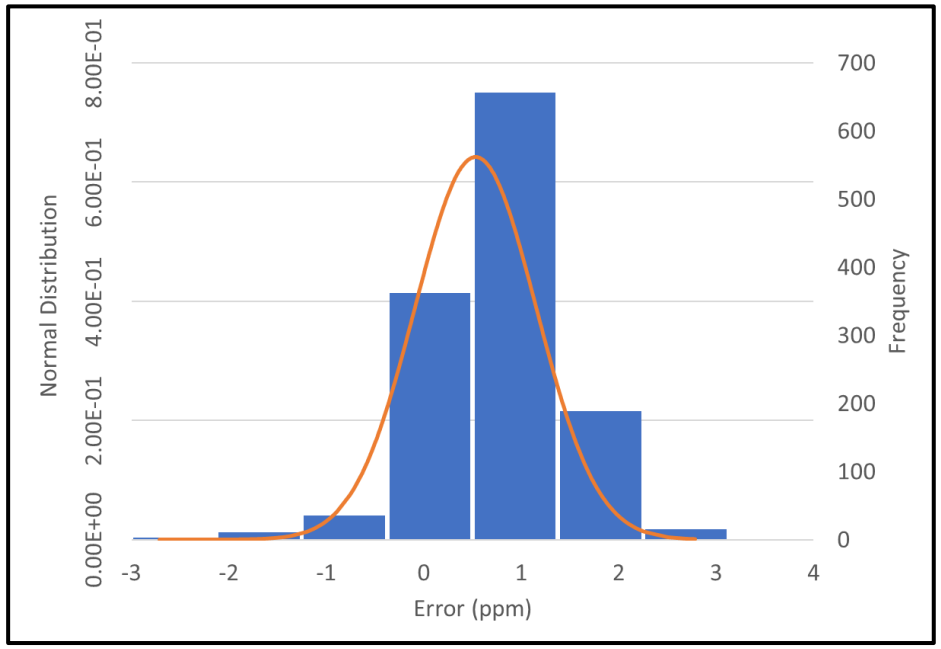
Sequence Variant	Instrument	Gradient	0.00%	0.05%	0.10%	0.20%	0.50%	1.00%
ALP[-26]APIEK	Exploris	30min	0.00	0.09	0.18	0.34	0.81	2.07
DSTYS[+27]LSSTLTLSK	Exploris	30min	0.03	0.07	0.10	0.19	0.42	1.32
FNWYVDG[+58]VEVHNAK	Exploris	30min	0.01	0.04	0.08	0.23	0.48	1.17
GFYPSDIA[+30]VEWESNGQPENNYK	Exploris	30min	0.00	0.01	0.03	0.07	0.15	0.39
LASGV[+14]PSR	Exploris	30min	0.00	0.06	0.13	0.29	0.59	1.48
STSGGTAALGC[+60.1]LVK	Exploris	30min	0.00	0.05	0.09	0.21	0.41	1.08
TTPPVLDSDGSFFLYS[+27]K	Exploris	30min	0.04	0.08	0.11	0.27	0.48	1.36
VDNALQSG[+58]NSQESVTEQDSK	Exploris	30min	0.00	0.07	0.14	0.32	0.72	2.98
VTNMDPADTATYYC[+60.1]AR	Exploris	30min	0.00	0.01	0.03	0.08	0.16	0.49
VVSV[+14]LTVLHQDWLNGK	Exploris	30min	0.02	0.02	0.04	0.12	0.39	1.19

Bo Zhai

Importance of Mass Accuracy in Peptide Map

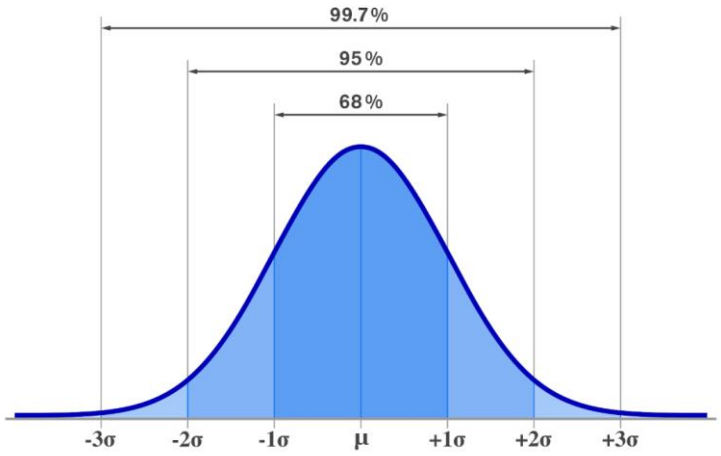


Mass measurements from all peptide-spectrum matches to a theoretical sequence. Median precursor m/z error is 1.2 ppm on Exploris



99.7% of peptides are within 3σ or 1.86 ppm

Max	2.78
Min	-2.71
Average	0.53
Stdev	0.62



- Predictable, stable and consistent mass accuracy is valuable tool in data analysis
- Mass calibrations stable for > 1 week
- Internal calibrants built into Exploris methods keep ppm error low (1-2 ppm)
- Knowledge of expected mass accuracy and a traceable sign of instrument performance and health
- Valuable attribute to rule out potential false positives from search engines

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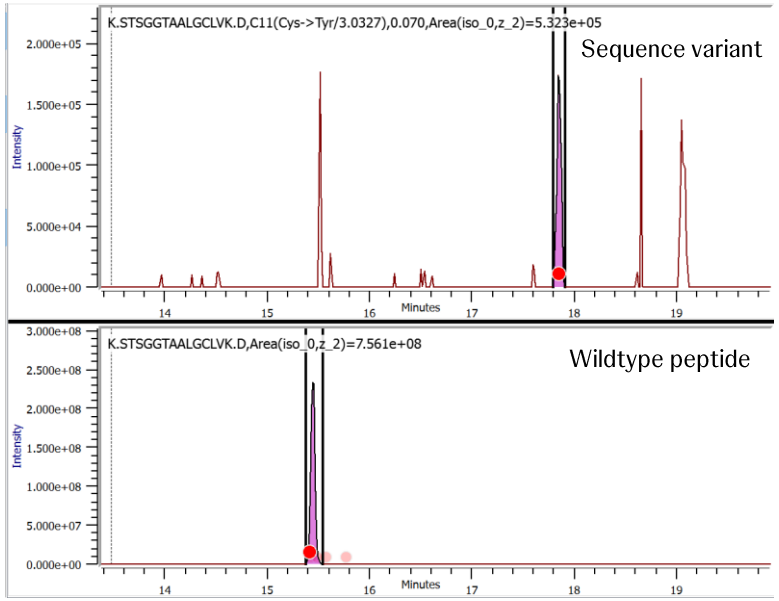
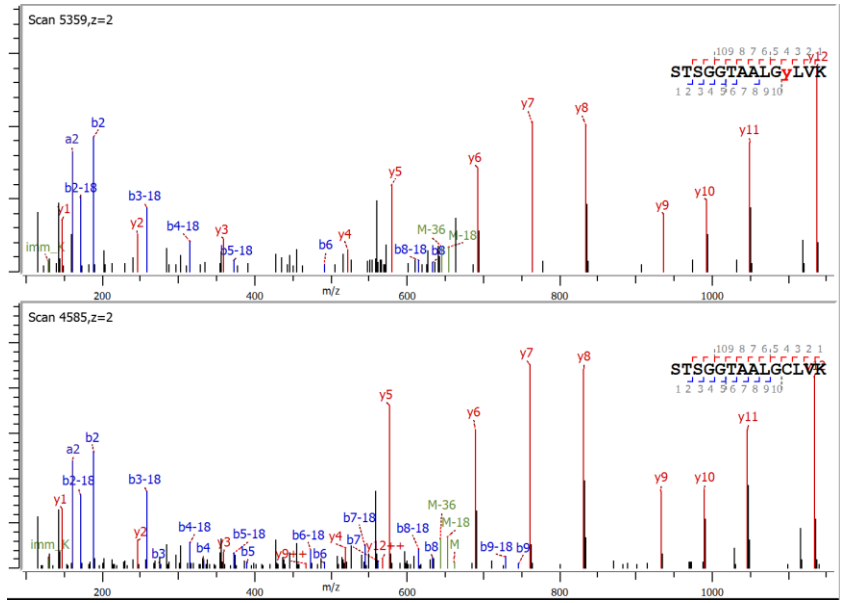
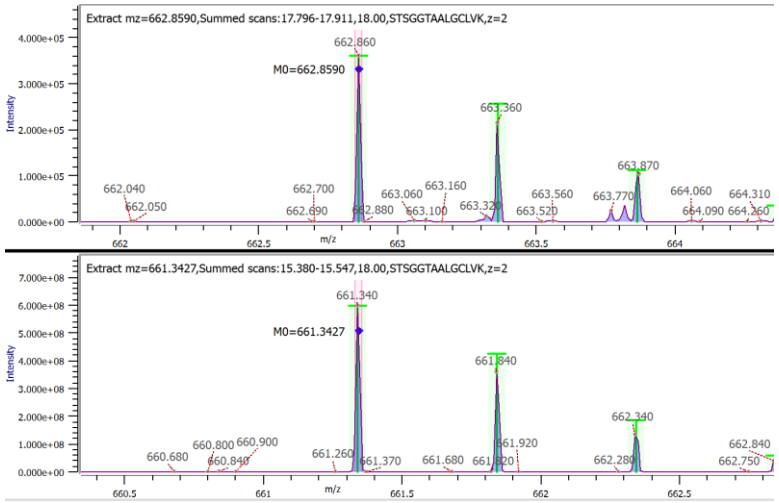
Cys-> Tyr Sequence Variant

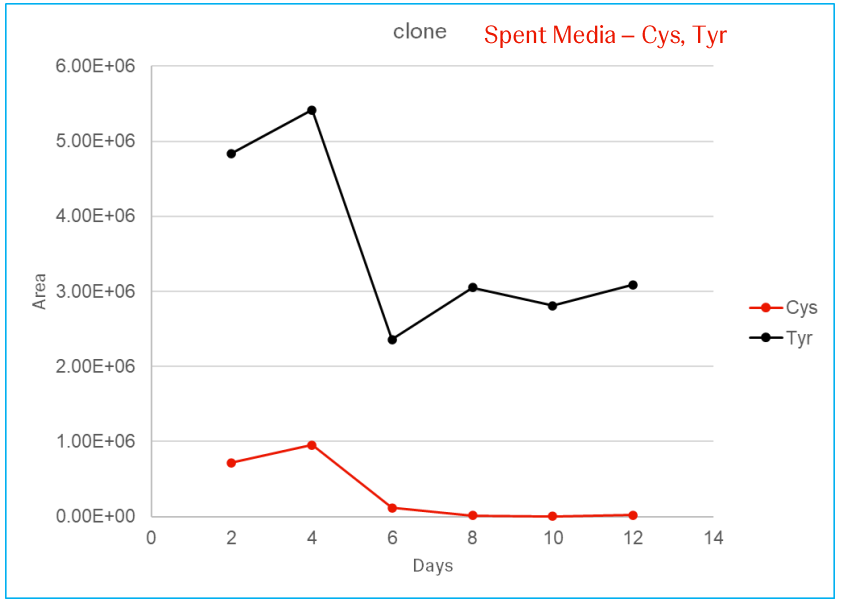


MS2 – Product Ion Spectra



MS1 – Precursor Ion Spectra





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ETD Based Disulfide Analysis

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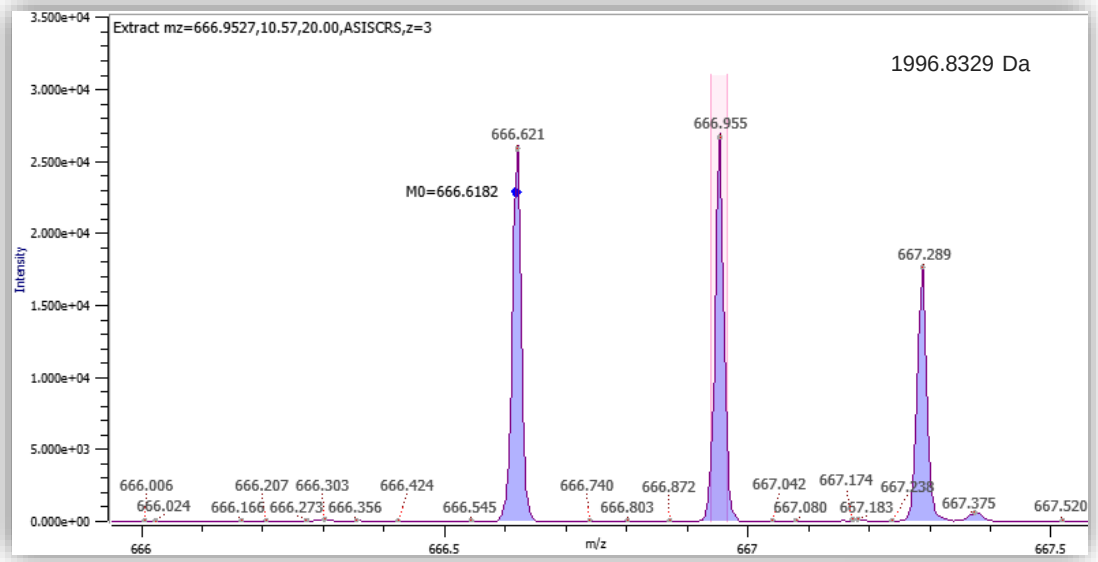
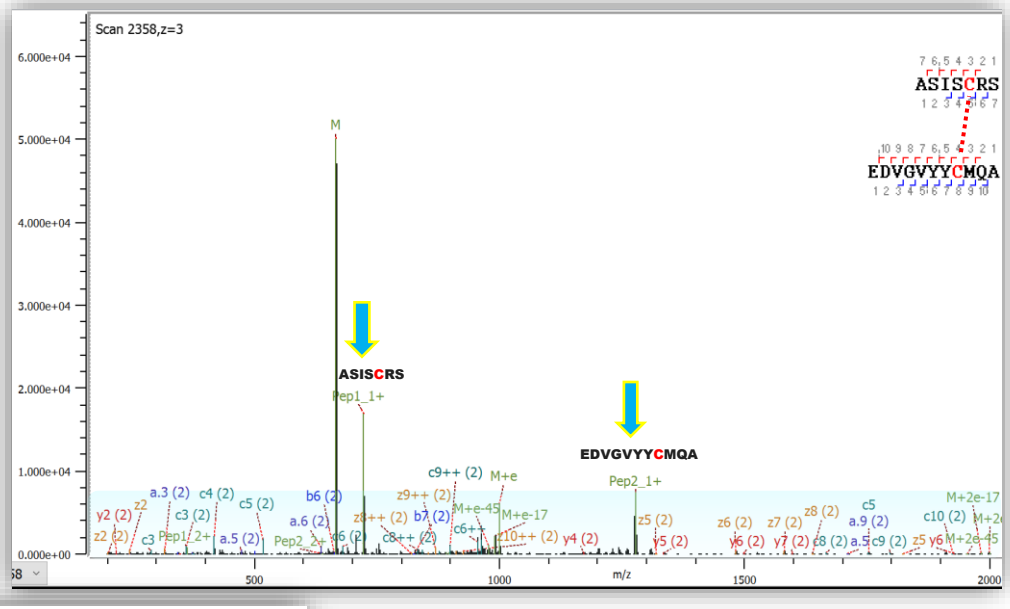
Identifying Disulfides by Mass Spec – NR Disulfide Map

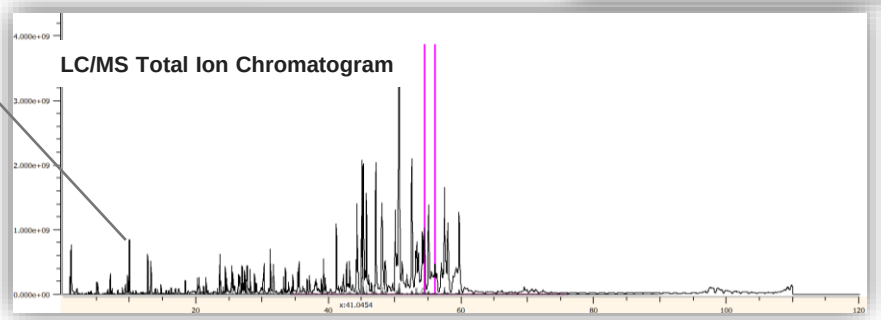
1. Mass of disulfide complex
2. Mass of each peptide
3. Mass of the fragments of each peptide

MS1

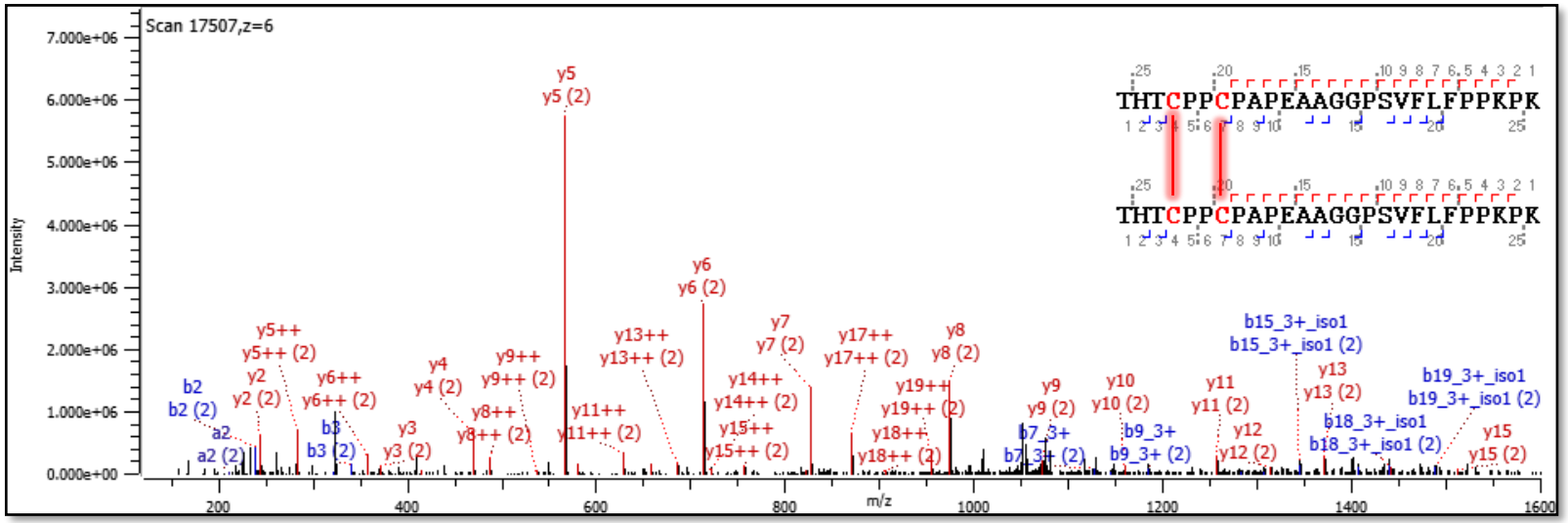


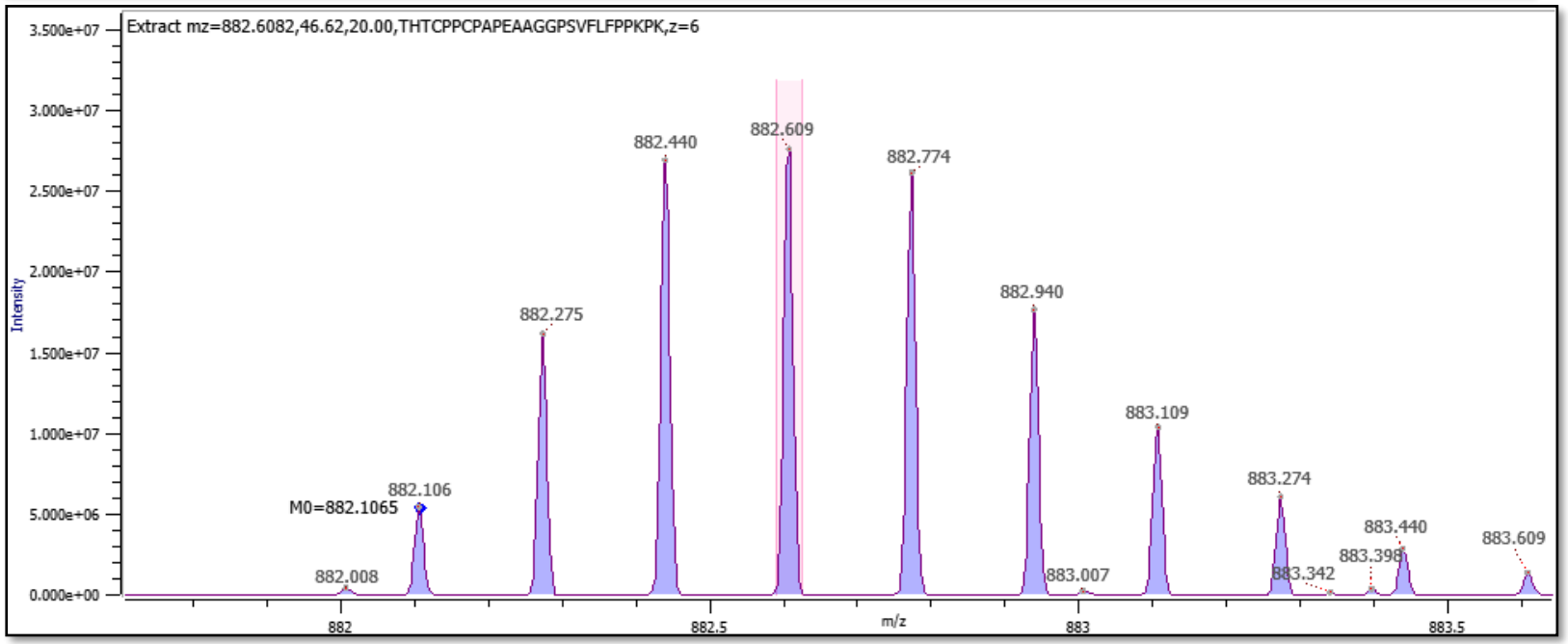
MS2

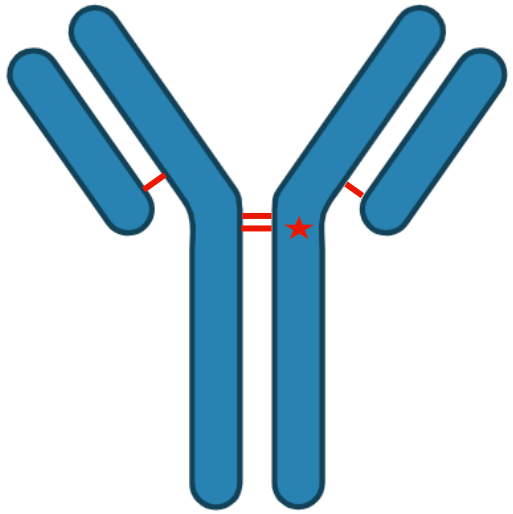




Expected Hinge HC1 – HC2 Disulfide







Intra-chain Disulfides

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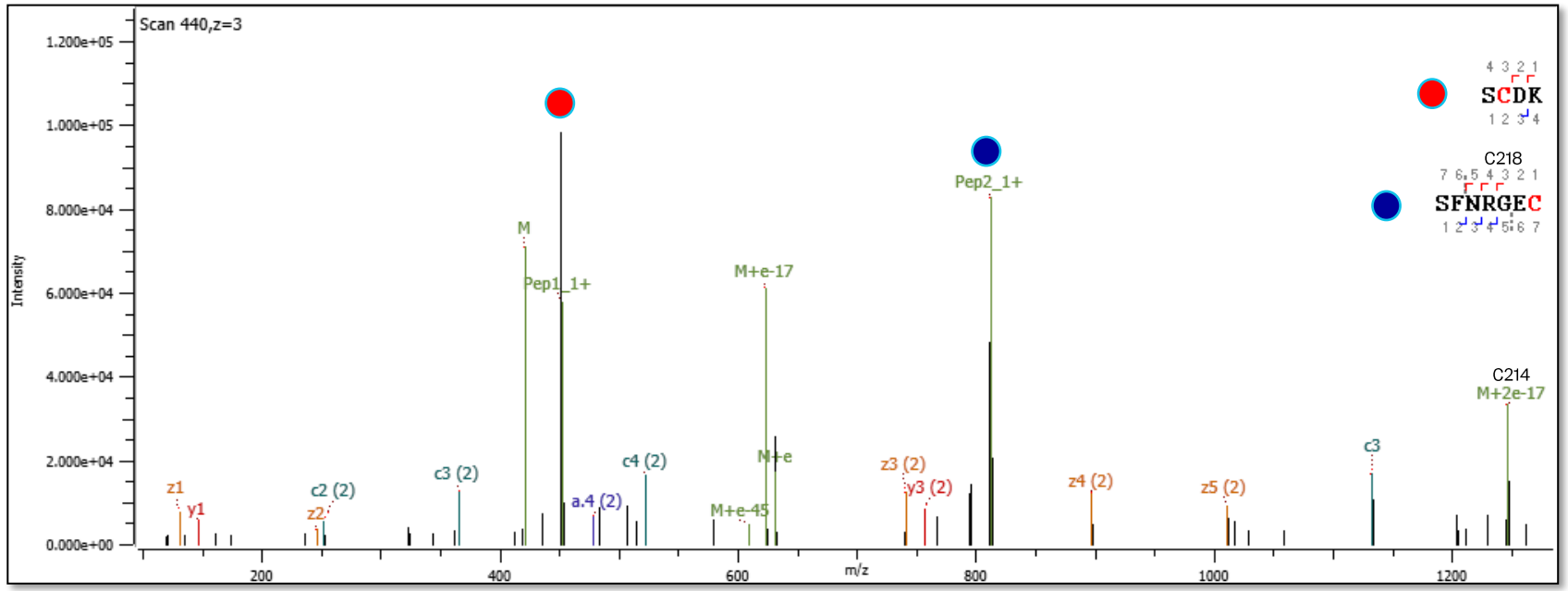
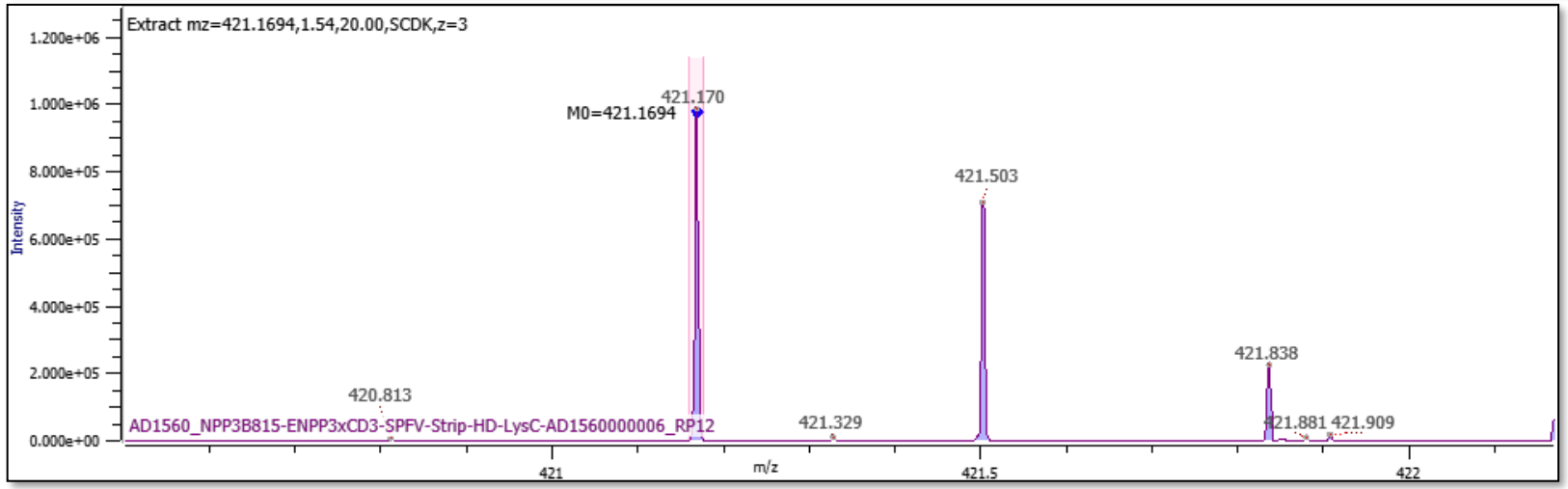
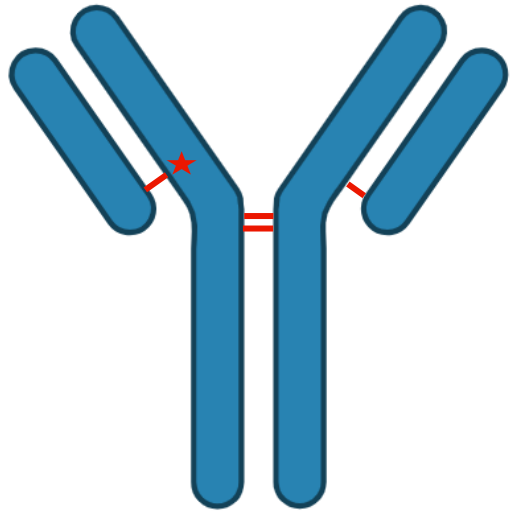
Inter-chain HC-LC Disulfide







Intra-chain Disulfides

Role of Automation in Analytical Sciences

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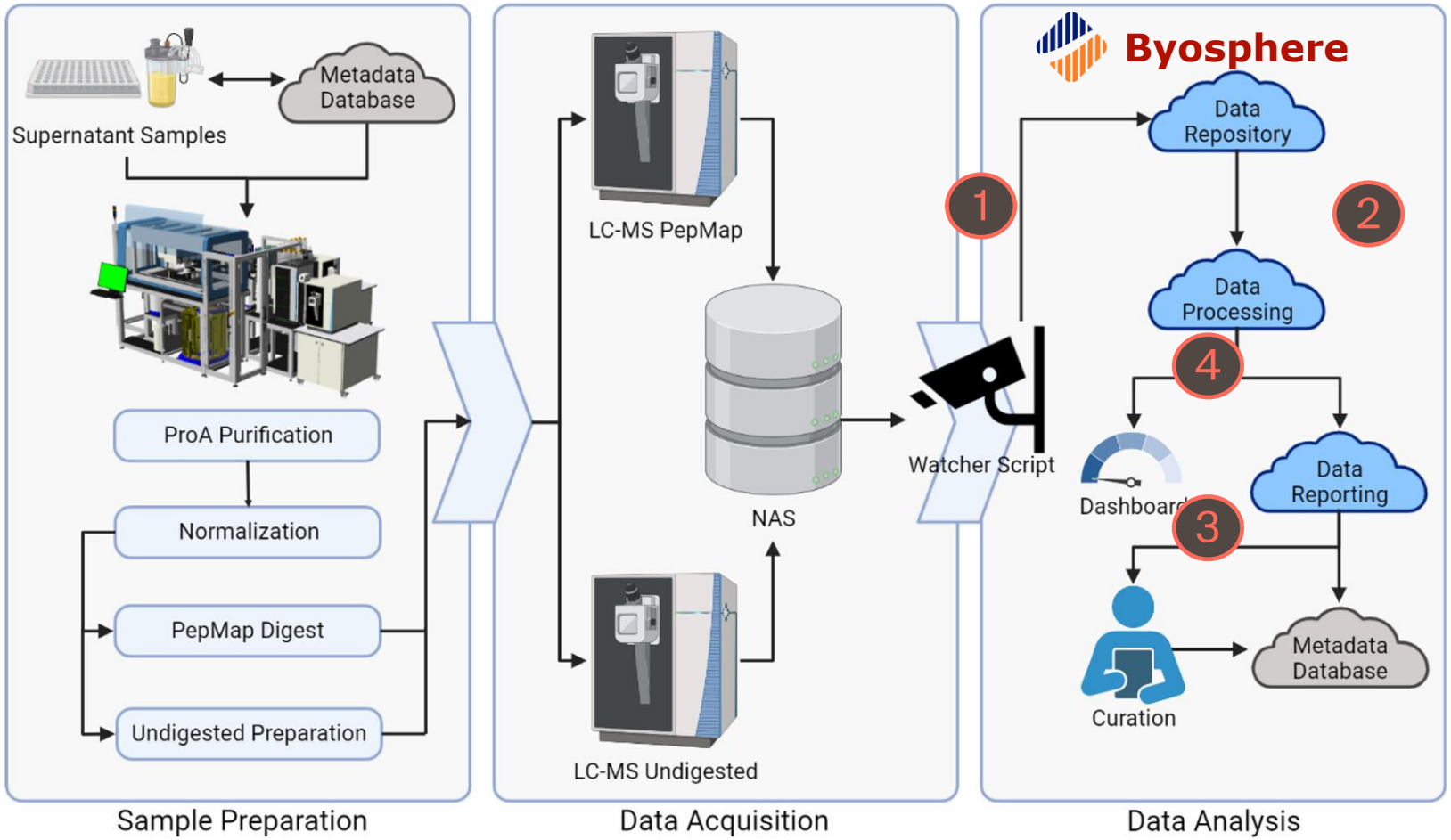
End to End Pipeline: Real-time data processing & report generation assists the scientist in validating results and reduces turn-around-time



1. **Watcher:** script automatically sweeps "raw" files into the data repository
2. **Processing:** "raw" files are processed using the correct workflow (e.g. intact, pep map, etc)
3. **Curation:** Scientists can easily inspect project files and validate reports
4. **Dashboards:** meta-data used to query results across all runs/projects

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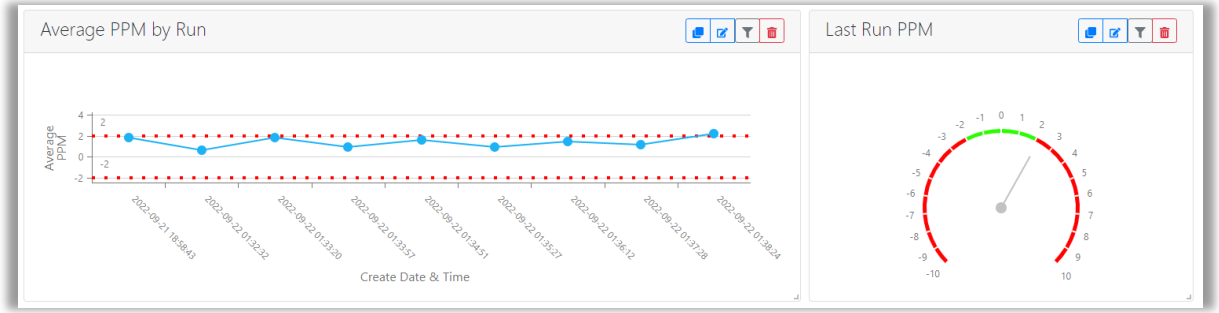
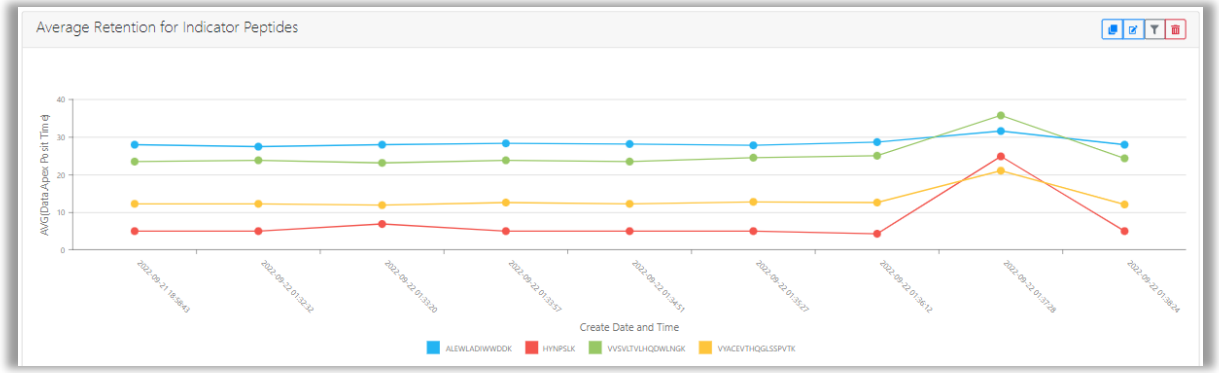
Waldenmaier, H., et al. Journal of the American Society for Mass Spectrometry 2023 34 (6), 1073-1085

QC Dashboard Example: Monitoring System Performance With Each Project

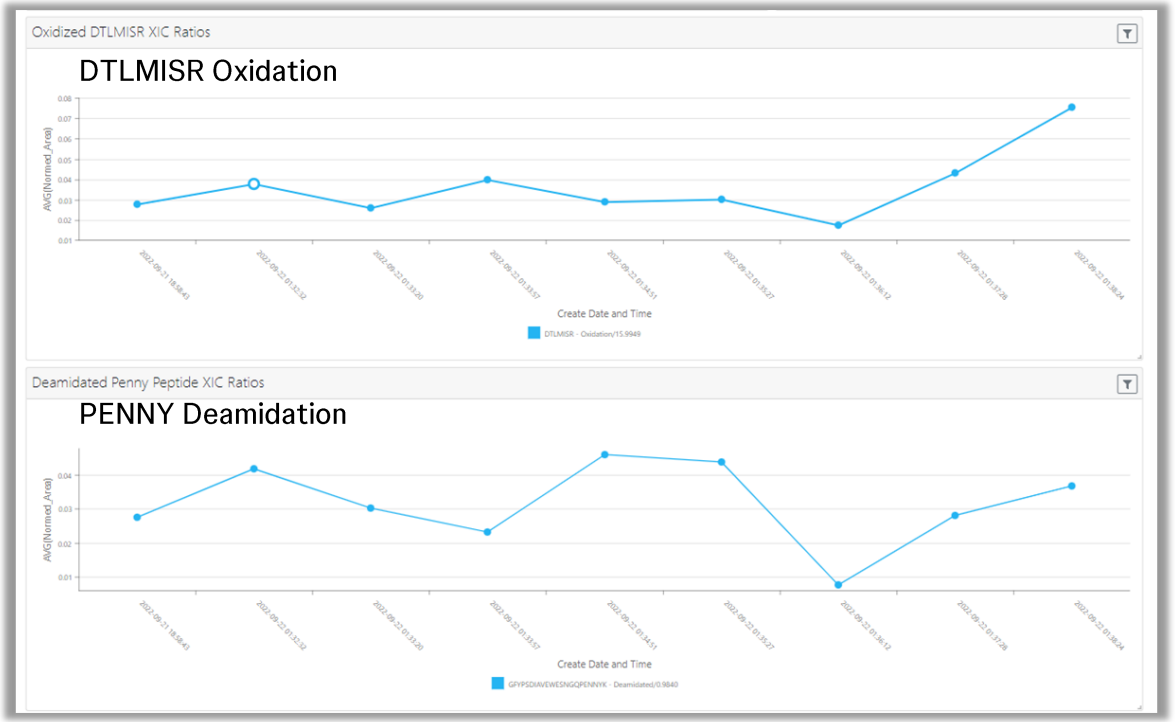
Instrument (LC-MS) Performance





- Track PPM accuracy, configure tolerances and send notifications if last run in is out-of-spec
- Monitor LC performance for RT consistency

Sample Processing Performance



- Look for consistency in peptide mapping digests
- Low-level of modifications in the system suitability sample

NIST mAb digest performed with every analytical request as system suitability

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Example of a full dashboard view

- Mass accuracy (ppm)
- Post Translational Modifications
- Glycoform Profile
- Mis-cleavages / Semi-specific digest

Process Supernatant Plates Directly From Cell Culture

Purification

Job Input

Sample Info:
name, molecule ID, titer

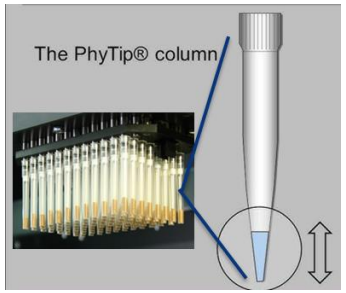
Tube Barcode	Sample Name	C Code (if available/relevant)	SCL ID (if available/relevant)	Octet Titer (g/L)	ProA/G Titer (g/L)	Abso
HTAT-04181	CDS22B002	C5828A	CDS22B002		2.155	
HTAT-04182	CDS22B003	C5829A	CDS22B003		2.095	
HTAT-04183	CDS22B004	C5830A	CDS22B004		2.357	
HTAT-04184	CDS22B005	C5831A	CDS22B005		2.722	
HTAT-04185	CDS22B012	C5838A	CDS22B012		2.669	
HTAT-04186	CDS22B014	C5840A	CDS22B014		2.542	
HTAT-04187	CDS22B022	C5828A	CDS22B022		2.751	
HTAT-04188	CDS22B025	C5831A	CDS22B025		2.564	

or LIMS

Sample Plate

clarified supernatant

Purification



Phynexus:
proA column
pipette tips.

96 samples in parallel (~2hrs total)

Hamilton Vantage system

Normalization

Measure and normalize concentration to prepare for next step

★ Skip purification if samples already purified

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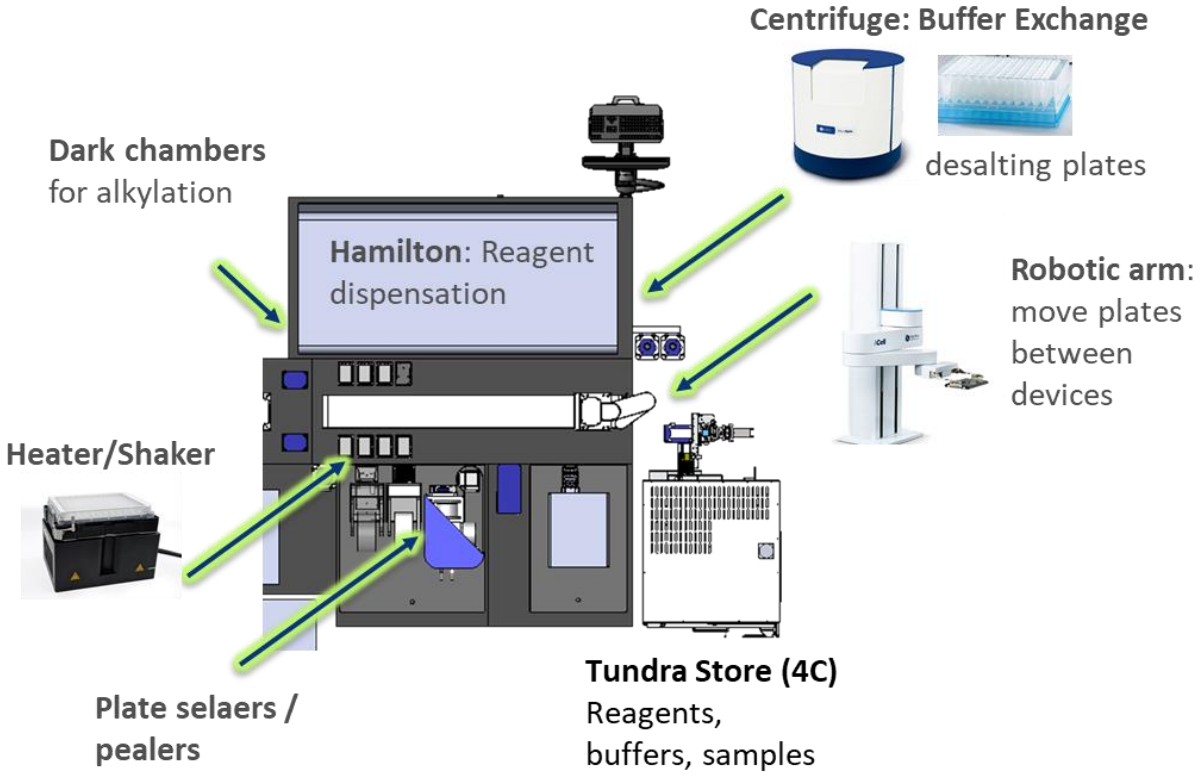
Lab Operations to Add Reagents, Exchange Buffers, Incubate… available on deck

Sample Prep

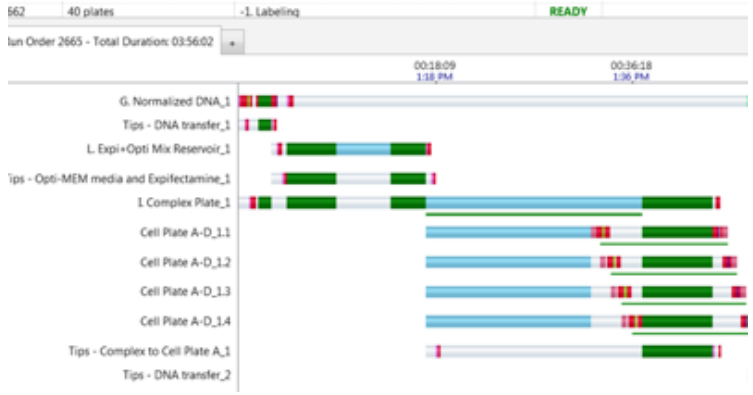


Flexibility to modify or add treatment protocols

Current MS Assay List

Assay	Process Requirements
Intact mass	Untreated
Deglycosylated	PNGaseF, incubation
Reduced	DTT, short incubation
Peptide Map Digest (Trypsin & Chymotrypsin)	Denature/reduce, alkylation, incubation, buffer exchange, enzyme, quenching

Scheduling Software: Manages Multiple Processes



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Comparing Semi-automated (SA) vs Fully-automated (FA) process

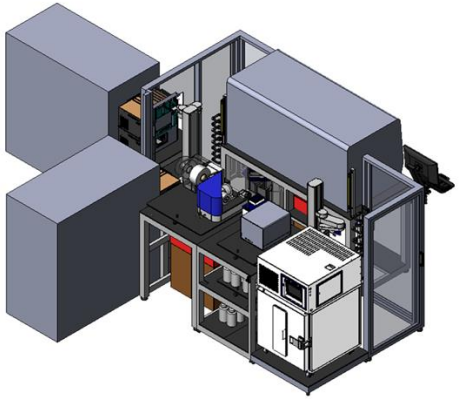
Semi-Automated (SA) Process

proA purification

Reagent & Buffer dispensation for pep map, degly, reduction

Non-automated Incubations, Buffer Exchange

Fully Automated (FA) Process

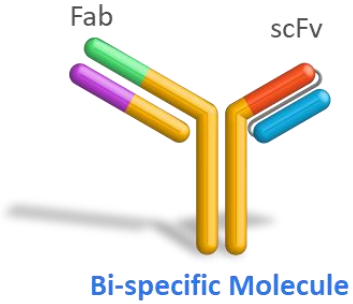


APIS

mAb

NIST mAb & mAb-1

- 10 replicates
- Peptide Mapping / PTM & Digest Parameters



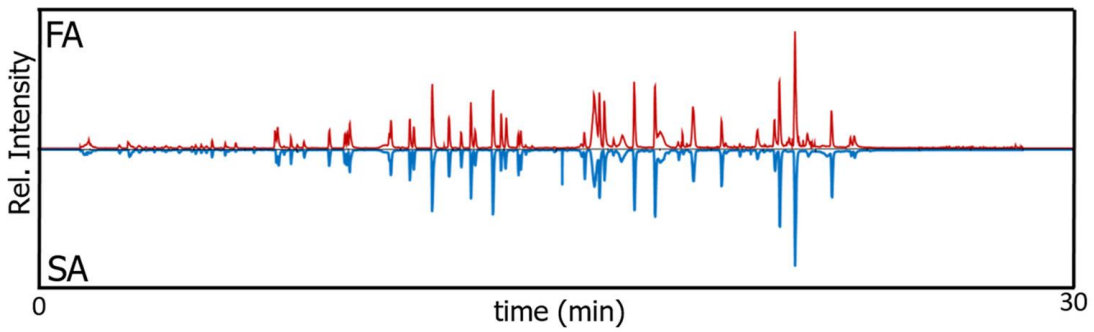
Bi-specific Molecule

48 cell lines from ambr bioreactors

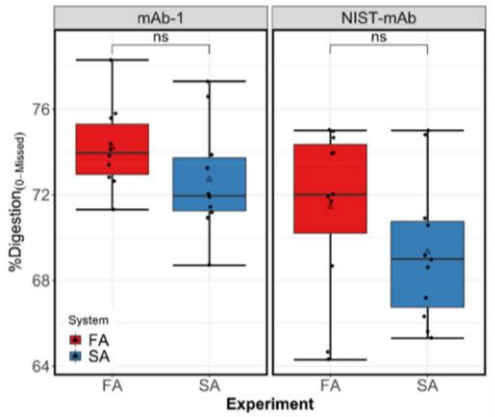
- Intact, degly, reduced mass assays:
- - spectra quality
- - glycan profile

Comparing FA & SA peptide mapping results.
10 technical replicates for NIST and mAb-1

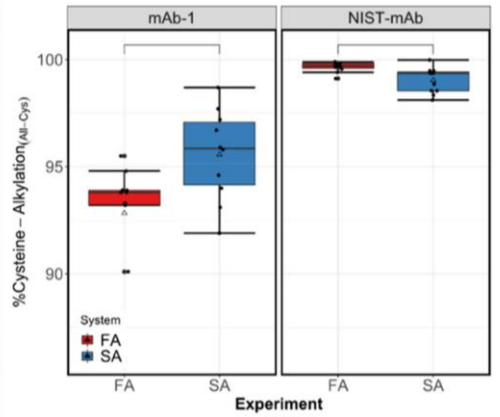
TIC Profile



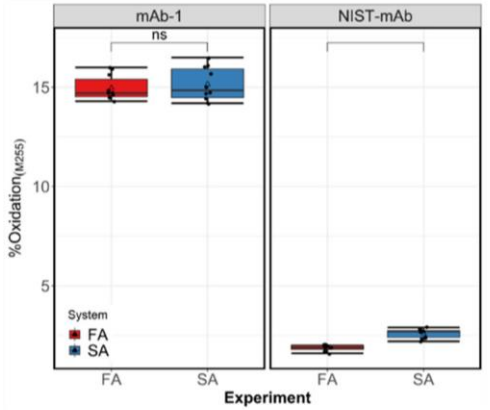
0-Miscleavages



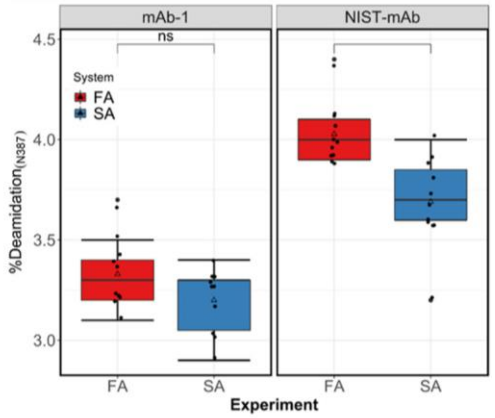
Cys Alkylation



Oxidation (DTLMISR)



Deam ('PENNY')



- TIC Profile similar between both preparation
- Slight differences in Oxidation and Deamidation
  - FA shows less variability
- Fewer miscleavages in FA
- Alkylation 95-100% for both processes, lower variability in FA

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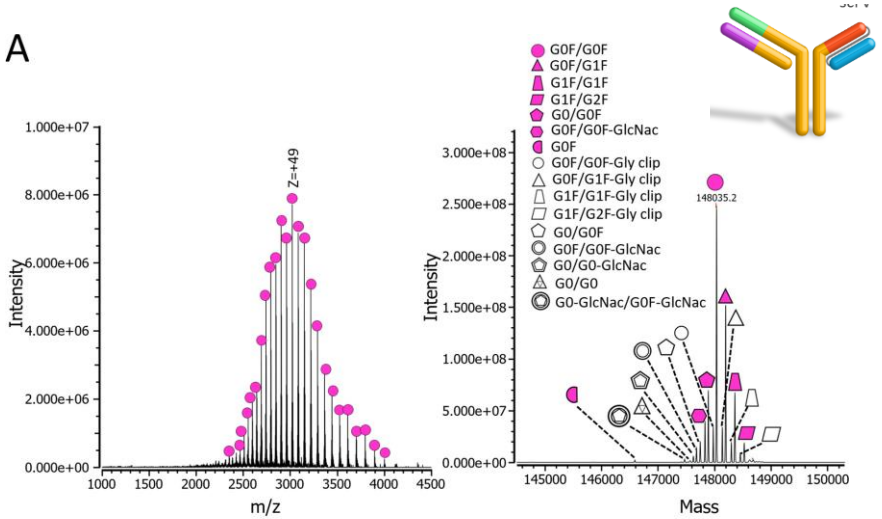
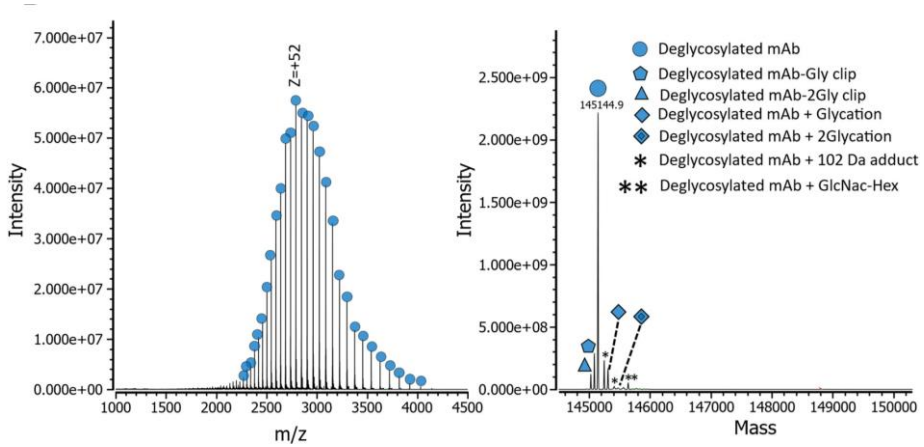
48 clonal cell lines for bi-specific antibody. Representative intact, PNGase from FA process

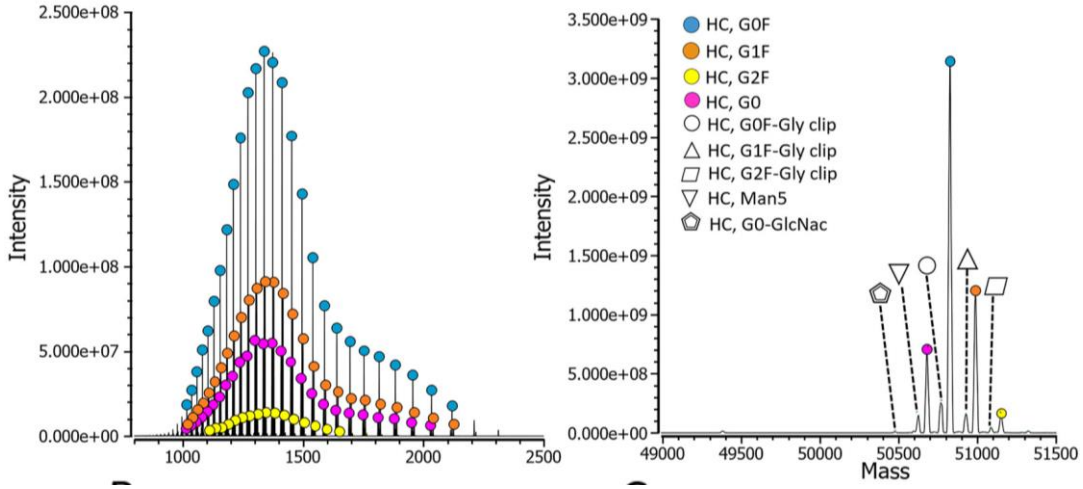




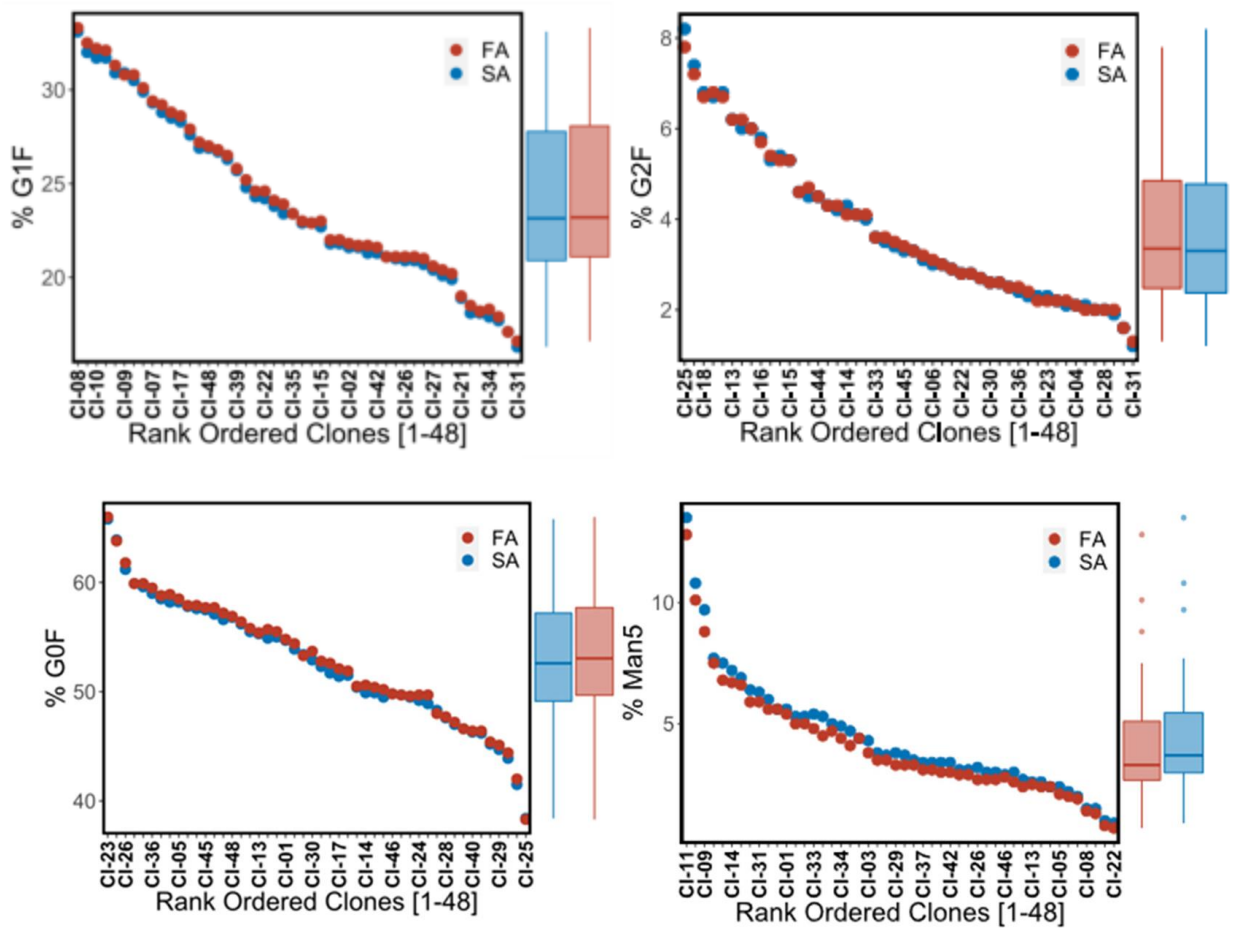
Intact mass shows high sample quality with the ability to resolve low level glycan species and C-terminal clipping

Complete PNGAseF digestion produces a fully deglycosylated protein

Comparing glycosylation profile of FA and SA process using reduced mass material



Representative reduced spectra for bsAb



Highly similar glycan profiles between both processes. Including low level Man5 and G2F species

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Summary

Next Generation Mass Spec Instruments for Today's Complex Biotherapeutics

- The valuable role for High Resolution Accurate Mass Instruments in Cell Line Development strengthened by overall strategy of dedicated methods and workstreams.
- With a steady workstream of similar sample types, dedication of MS Instrument to specific platform assays (intact, peptide, native) key to efficiency
- SEC-MS Assay fills a valuable role in balancing ratio expression and determining multispecific impurities and characterizing product quality of native complexes
- A pair of workhorse Exploris instruments designed to work in parallel on same sample performing different assays
- True end to end automation was shown to advance from crude cell harvest through purification, sample prep., sample list creation, sample acquisition on MS, data analysis and final reporting

Acknowledgements

Thank you - Teamwork Makes the Dream Work

CEAS – MS Group

- Chris Sauer
- Elsa Gorre
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- Hans Waldenmaier
- Eric Beil
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- Abby Chiang

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- Min Du
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- Reiko Kiyonami

Protein Metrics

- Eric Carlson
- Jing Li
- Alex Zhai

PROTEIN METRICS

CEAS Management

- Partha Chowdhury
- Hirsh Nanda
- Tom Kelly