sanofi

Intact Mass Characterization of Multispecific Therapeutic Antibodies

Kalie Mix

Principal Scientist, Large Molecule Research

Outline

- Large Molecule Research Group at Sanofi
- Multi-specific antibodies: formats and analytical methods
- Mass spec for high-throughput samples (early-stage screening)
- Mass spec for medium-throughput samples (protein production QC)
- Technology development and ongoing challenges

Large Molecule Research at Sanofi

Supporting early-stage antibody discovery research for therapeutic areas across the company

Biologics Characterization, Expression, & Purification (BiCEP)

- Antibody and antigen production
- Protein QC & binding assays

Molecular Engineering & Screening Technologies

- DNA synthesis
- High-throughput screening assays

Protein Engineering

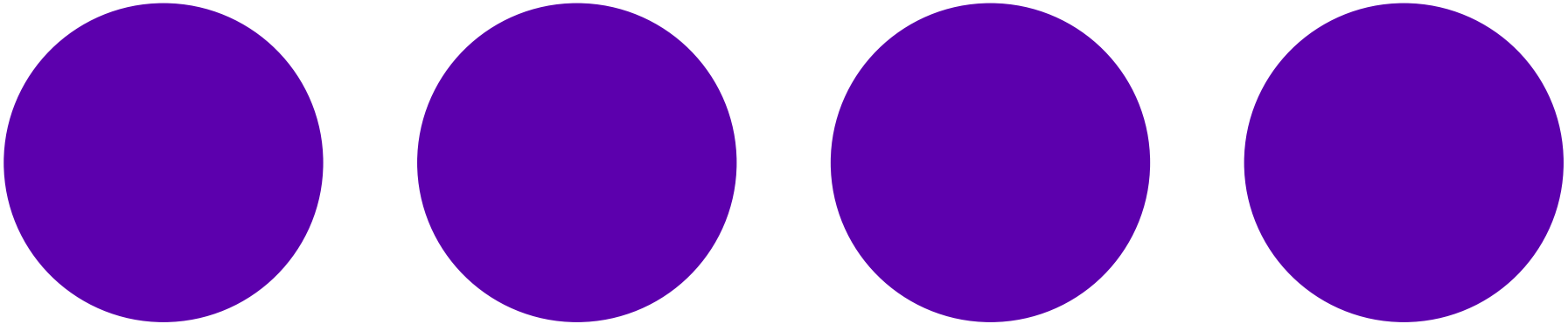
- Developability analysis
- Engineering optimal function & eliminating liabilities

Antibody Discovery

- Phage display
- Single B-cell cloning

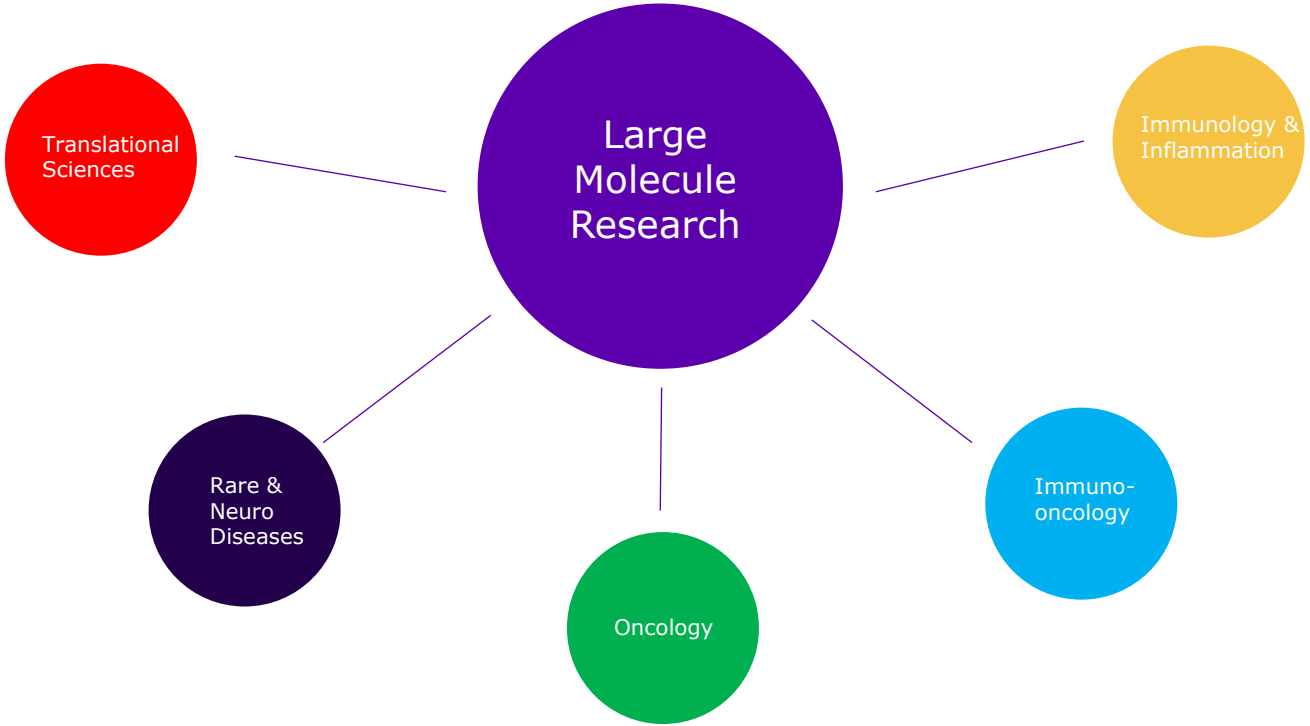
Large Molecule Research at Sanofi

Supporting early-stage antibody discovery research for therapeutic areas across the company



Large Molecule Research at Sanofi

Supporting early-stage antibody discovery research for therapeutic areas across the company



Intact Mass Analysis of Therapeutic Proteins within Protein Production Group

Role in our workflow:

- Mass spec data is used to evaluate protein quality and decide which proteins might make good therapeutic candidates

Instrumentation: Agilent 6545XT Q-TOF

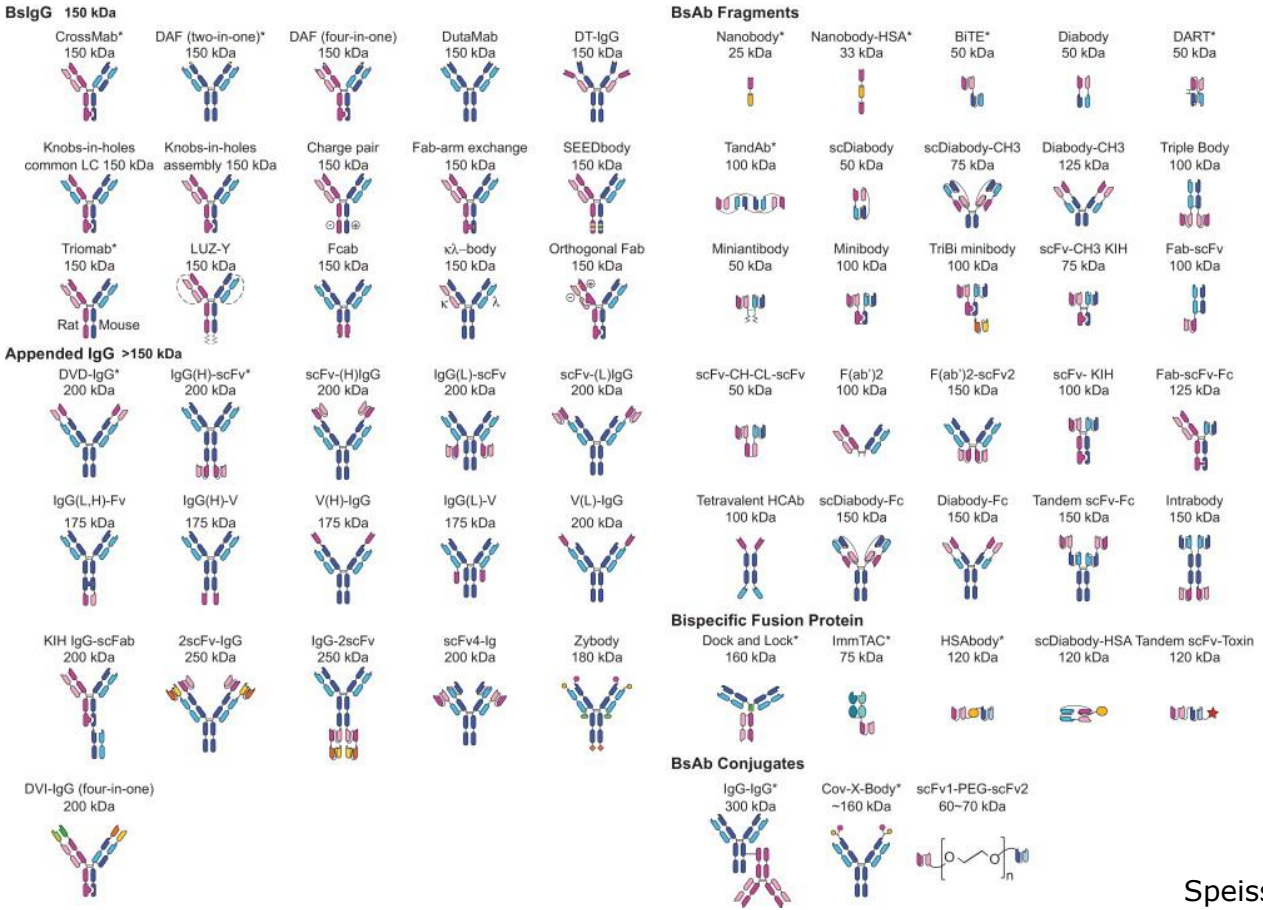
- Designed especially for characterization of large proteins
- Can also be used for released glycans, subunits, peptides, small molecules, etc.

Multispecific Antibodies

- Monoclonal antibodies are a well-established drug class that is growing rapidly
- Many complex disease pathways can be modulated more effectively by engaging multiple targets
- Multispecific antibodies can enable novel functions compared to mixtures of parental antibodies
- Many mechanisms of action are possible:
  - cell bridging
  - receptor cross-linking for agonism or inhibition
  - mimicking of cofactors to position enzyme & substrate
  - employing binding event for transport
- >>100 msAbs in clinical development for diverse indications; cancer, immunology & inflammation, hemophilia, diabetes, HIV, osteoporosis…

Labrijn, A.F. *et. al, Nat Rev Drug Discov,* **2019**, *18.*
Xu, L. *et. al, Science* **2017**, *358.*

Advances in Multispecific Antibody Formats: A Plethora of Options



Considerations for format selection:

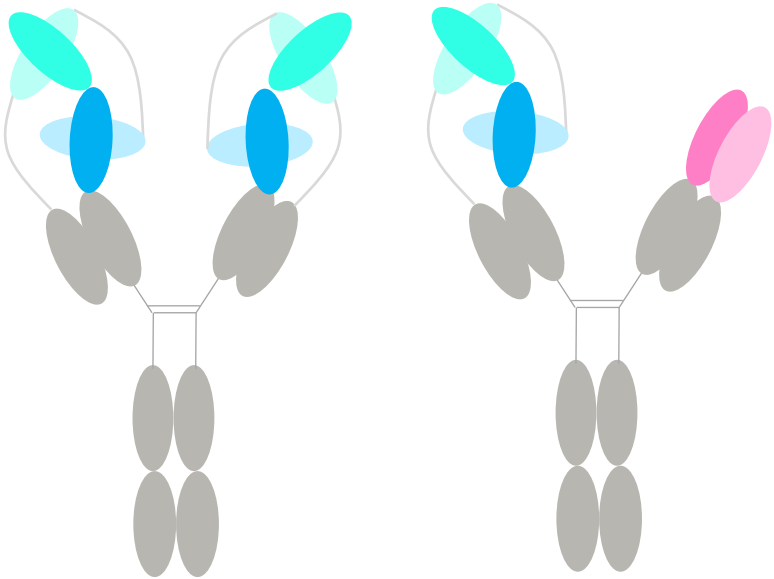
- PK/PD properties
- Fc effector function
- Chain pairing
- Binding valency & geometry
- Developability

Speiss, C. *et. al., Mol. Immunol.* **2015,** *67.*

Sanofi Multispecific Antibody Format: CODV

Cross-**O**ver **D**ual **V**ariable Ig-like Proteins

- Enables binding to 2 or 3 different antigens
- Close similarity to natural IgG
- No N- or C-terminal VH or VL extensions
- Linkers promote defined paratope orientations
- Pharmacokinetic properties and effector functions can be tuned using Fc mutations
- Symmetrical nature of bispecific CODV avoids chain mispairing challenge

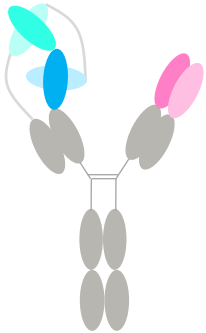


Bispecific CODV

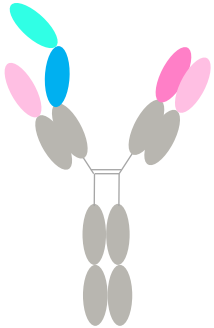
Trispecific CODV

Steinmetz, A. *et. al, mAbs,* **2016**, *8*

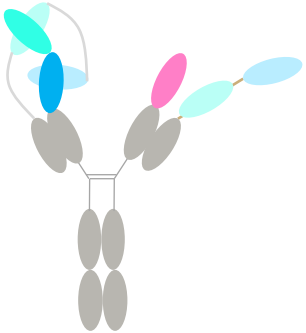
Chain Mispairing in Triab CODVs



Intact triab
~172 kDa

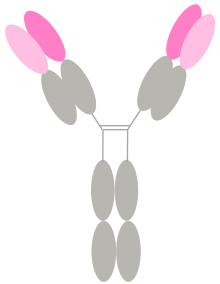


2x mAb LC
~158 kDa

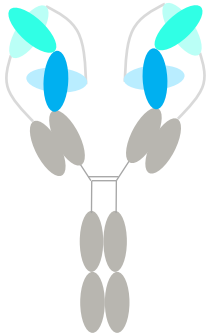


2x CODV LC
~183 kDa

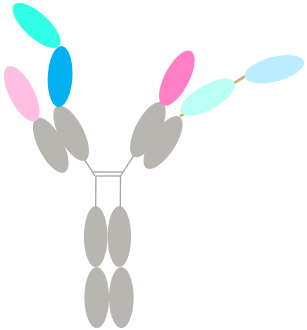
Half CODV
~99 kDa



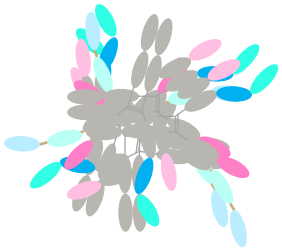
mAb homodimer
~144 kDa



CODV homodimer
~199 kDa



Isobaric mispaired triab

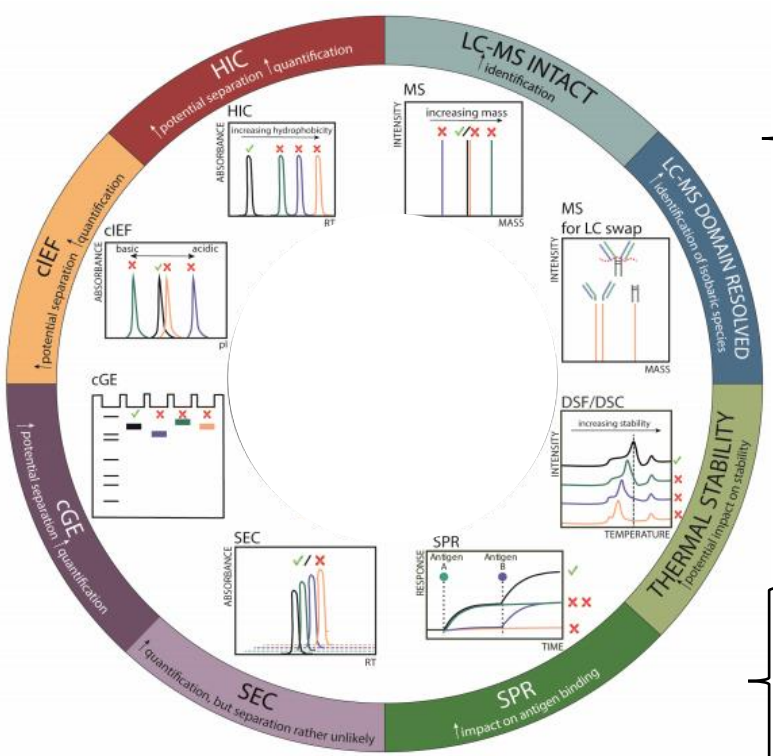


Aggregates

- Mispaired molecules present expression/purification challenges and can hinder downstream assays
- Many internal efforts under way to optimize engineering, expression, and purification
- Identification and quantification of each molecule is critical for identifying best candidates and optimizing production

Characterization of Triab CODVs

*Separation techniques with A280 protein detection enable **quantification***

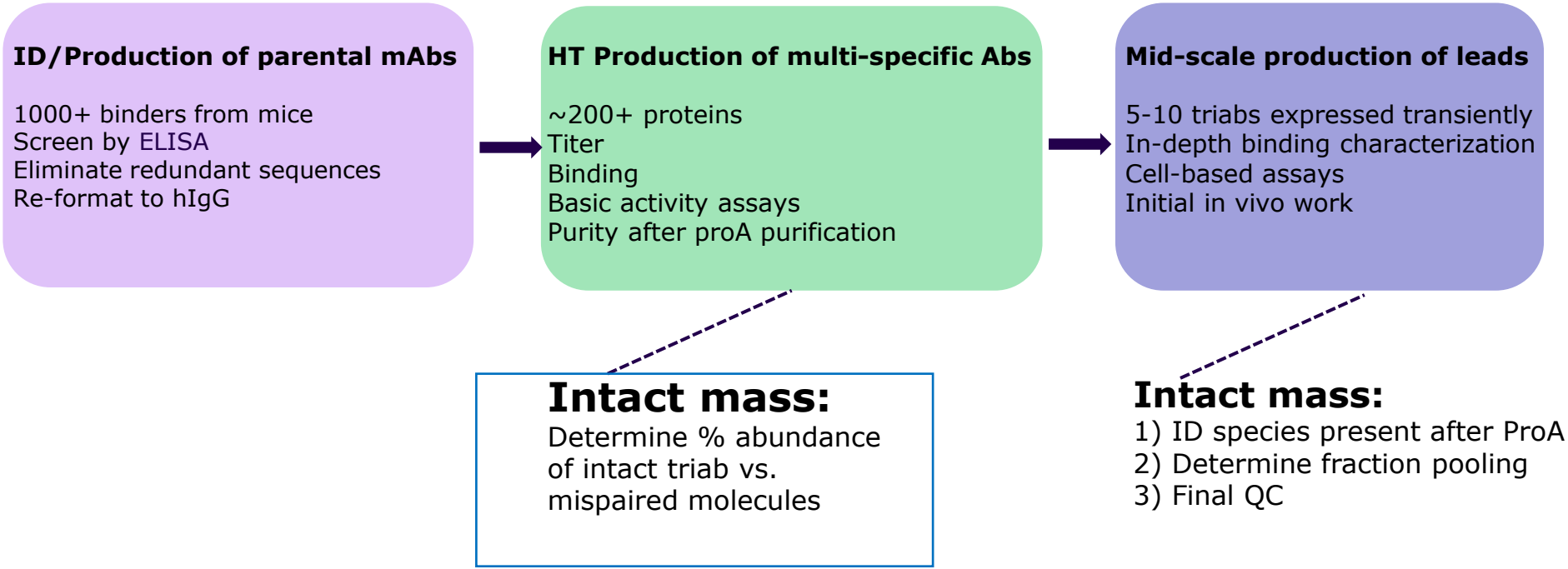


*Mass spectrometry enables **identification***

Binding and stability assays provide information about function

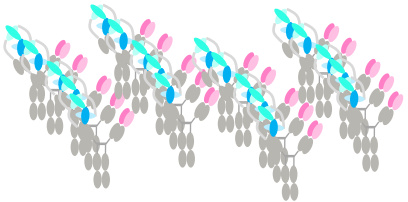
Ercole Rao, *et. al. J. Appl. Bioanalysis* **2020**, *6*.

Multispecific Antibody Discovery Workflow



High-Throughput Production of Trispecific CODV Antibodies

Protein **E**xpression & **P**urification **P**latform



100+ TriAbs
Produced on PEPP

Lead 5-10 TriAbs
Scale up production

- *Expression yield*
- *Binding/functional data*
- ***Purity: aHIC + MS***

Challenge: Importing information for intact + each mispaired molecule
*…**AND** matching it to each raw data file*

HT Triab Analysis using AWS and Byos

Lab Automation Team

- **Task:**

1. Query Genedata Biologics to retrieve sequences for all triabs on a sample list
2. Combine the chains for the intact and mispaired molecules
3. Create an output file that matches the raw data will all the sequences for each triab

Inputs:

MS Path CSV from Byos

Excel file with list of GDB Protein ID#

AWS Gateway

Output:

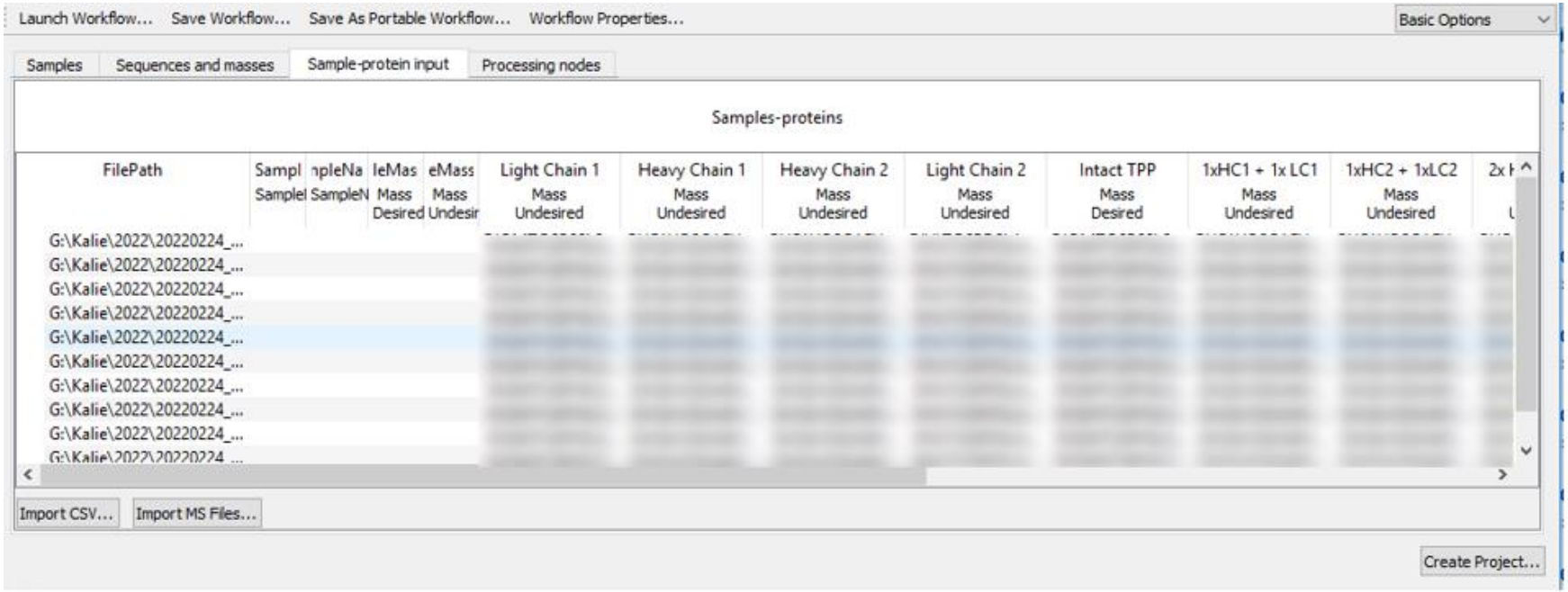
Raw data location

Sequence of each chain

Sequences of intact + mispaired triabs

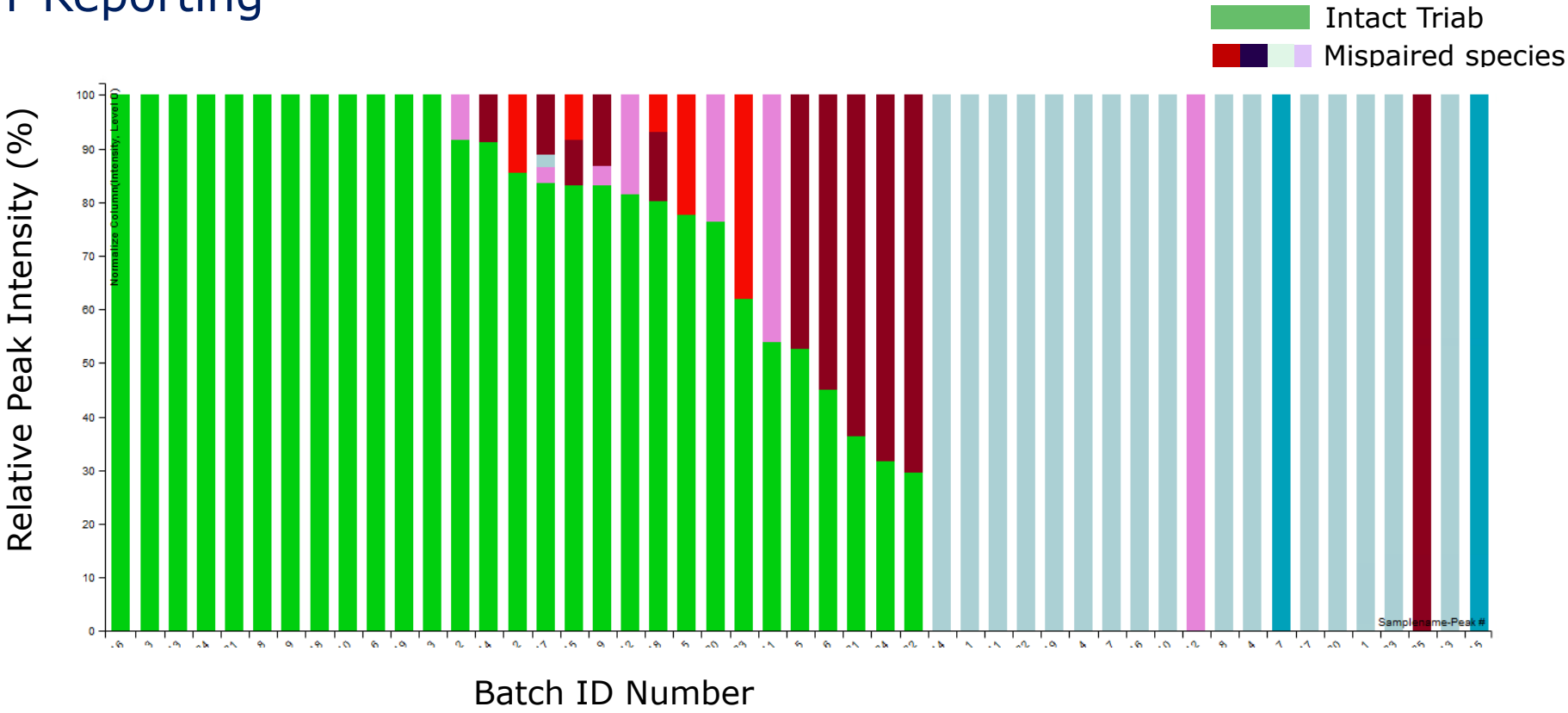
	1	2	3	4	5	6	7	8	9	10	11	12	13
1	ColumnNa	FilePath	Light Chai	Heavy Cha	Heavy Cha	Light Chai	Intact Tria	1xHC1 + 1:	1xHC2 + 1:	2x HC1, 2x	2x HC2, 2x	1x HC1, 1x	1x HC1, 1x
2	Expectation		Undesirec	Undesirec	Undesirec	Undesirec	Desired	Undesirec	Undesirec	Undesirec	Undesirec	Undesirec	Undesired
3	ColumnType		Mass	Mass	Mass	Mass	Mass	Mass	Mass	Mass	Mass	Mass	Mass
4		C:\Users\I0368846\Desktop\Raw data for Triab aut											
5		C:\Users\I0368846\Desktop\Raw data for Triab aut											
6		C:\Users\I0368846\Desktop\Raw data for Triab aut											
7		C:\Users\I0368846\Desktop\Raw data for Triab aut											
8		C:\Users\I0368846\Desktop\Raw data for Triab aut											
9		C:\Users\I0368846\Desktop\Raw data for Triab aut											
10		C:\Users\I0368846\Desktop\Raw data for Triab aut											
11		C:\Users\I0368846\Desktop\Raw data for Triab aut											

Project Creation Options for HT Analysis



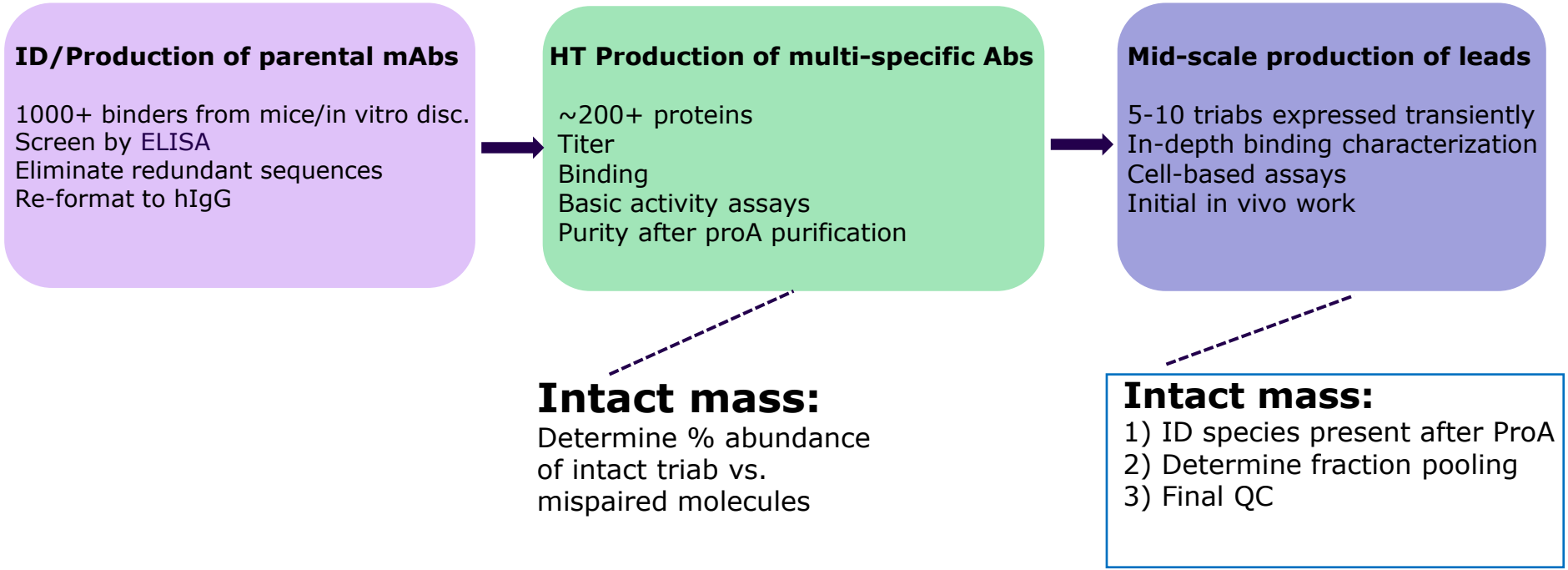
- *Sample-protein input tab in Intact is used to import CSV created by Lab Automation workflow*

HT Reporting



- *Excel report with peak intensities, mass error, plots also generated*
- *Data enables project teams to select best lead candidates*
- *Stacked bar chart view provides visualization for finding trends*

Multispecific Antibody Discovery Workflow



QC of Larger-Scale Triab Productions

Challenge: Importing information for intact + each mispaired molecule

Inputs:

FASTA file with 2 LC's + 2 HC's

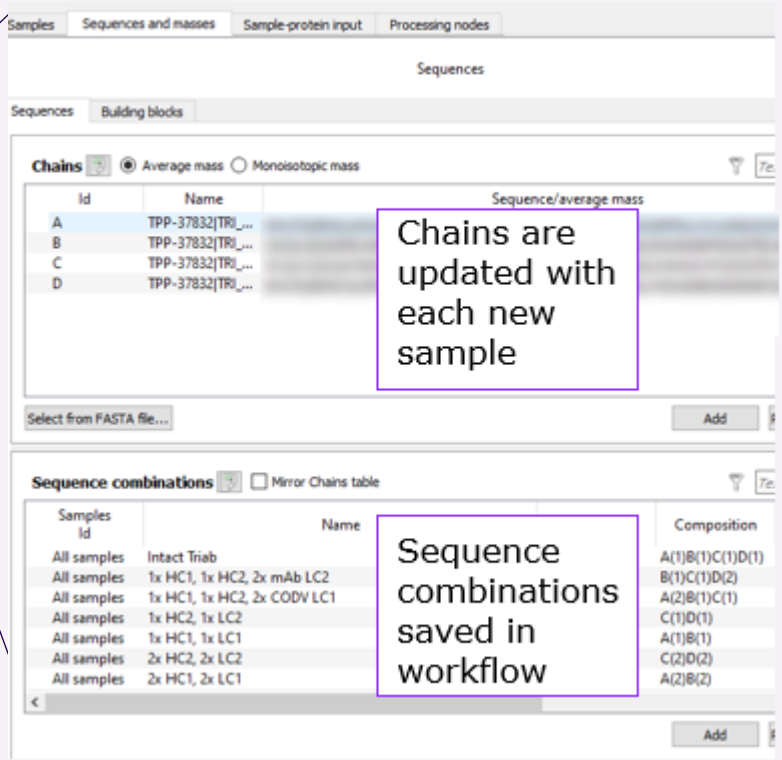
Raw data (*.d) file

Byos Workflow:

Triabs

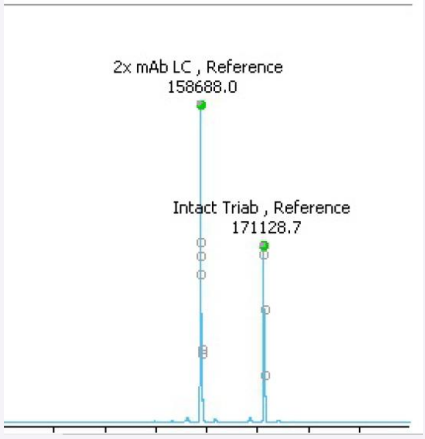
Identification, characterization, and relative quantification of large molecules

Project Creation Options:



Result Reports for End Users: Excel + PDF

- End users are not mass spec analysts: annotations need to be clear
  - Peak annotation is flexible
  - All tabs in PDF reports have free text option to add explanations



	A	B	C	D	E	F	G
1	Sample name	ID confirmed	Mass	Expected mass	Delta mass from calc.	Delta Mass ppm	Outcome
2	PPB-1234	Yes	144500	144495	5	34.6	Passed
3	PPB-1235	Yes	49000	48999	1	20.4	Passed
4	PPB-1236	Yes	145097	145090	7	48.2	Not Passed
5	PPB-1237	Yes	145000	144993	7	48.3	Ambiguous
6	PPB-1238	Ambiguous	146318	146317	1	6.8	Passed
7	PPB-1239	Yes	23001	22998	3	130.4	Passed
8	PPB-1240	Yes	201000	200996	4	19.9	Passed

Results need to be uploaded to Genedata Biologics

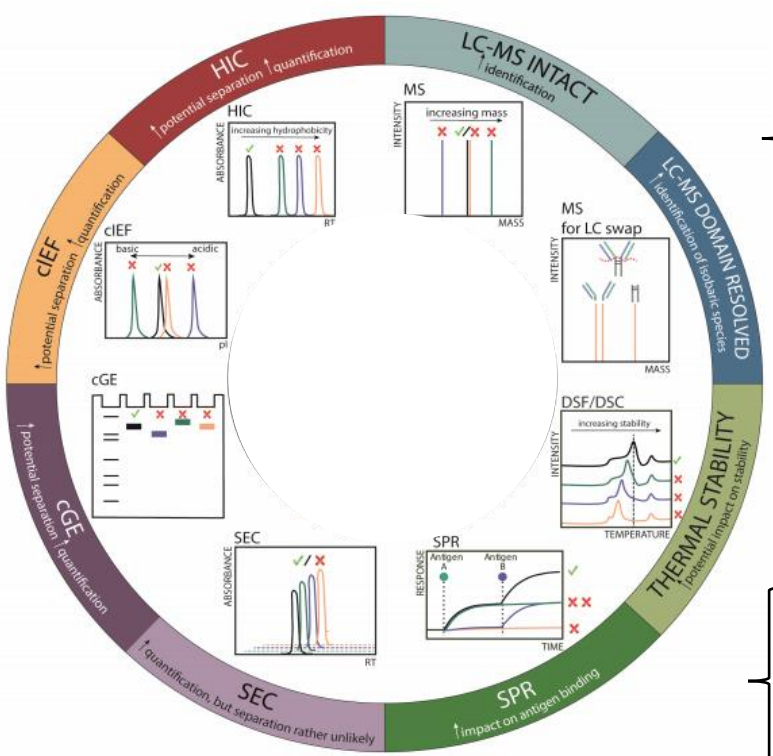
- Report export to Excel w/custom headers
- Multi-doc report accommodates samples analyzed with different workflows

Outline

- Large Molecule Research Group at Sanofi
- Multi-specific antibodies: formats and analytical methods
- Mass spec for high-throughput samples (early-stage screening)
- Mass spec for medium-throughput samples (protein production QC)
- **Technology development and ongoing challenges**

Characterization of Triab CODVs

*Separation techniques with A280 protein detection enable **quantification***



*Mass spectrometry enables **identification***

Binding and stability assays provide information about function

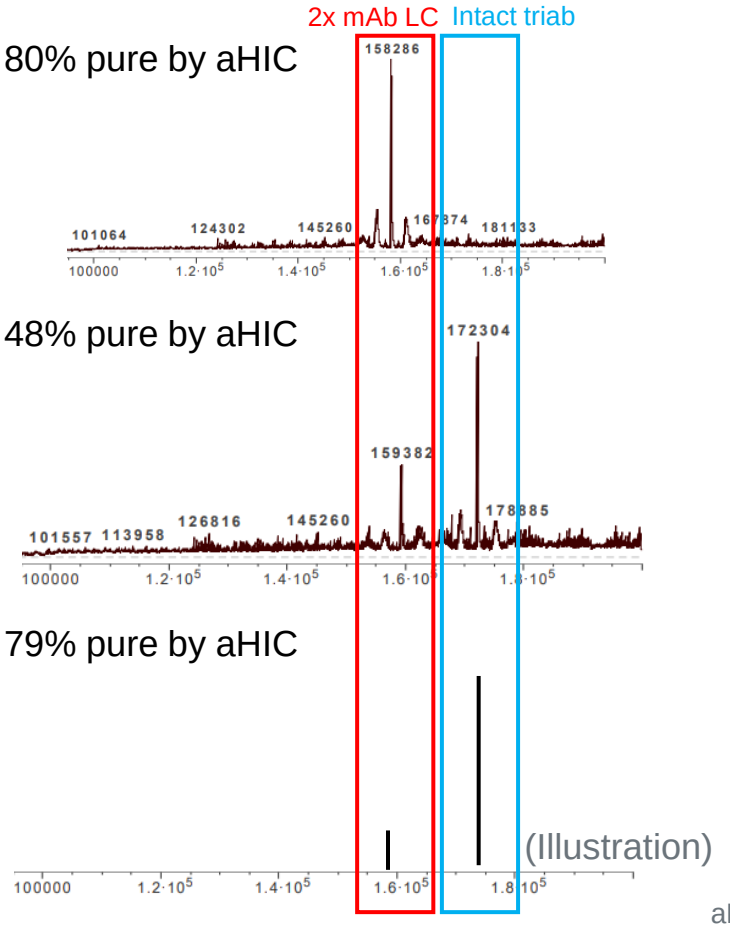
Ercole Rao, *et. al. J. Appl. Bioanalysis* **2020**, *6*.

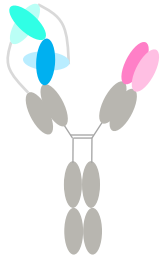
Candidate Selection Using aHIC with MS

HIC MS

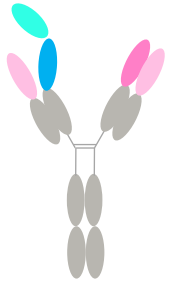
HIC MS

HIC MS





Intact triab



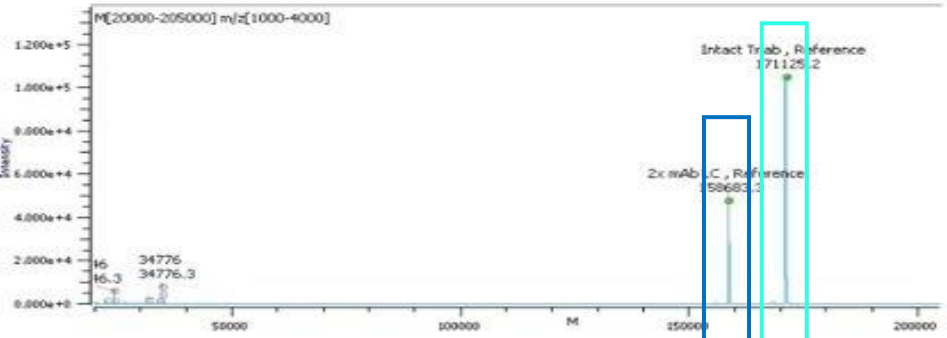
2x mAb LC

aHIC data from Megan Salemi, Sagar Kathuria[22]

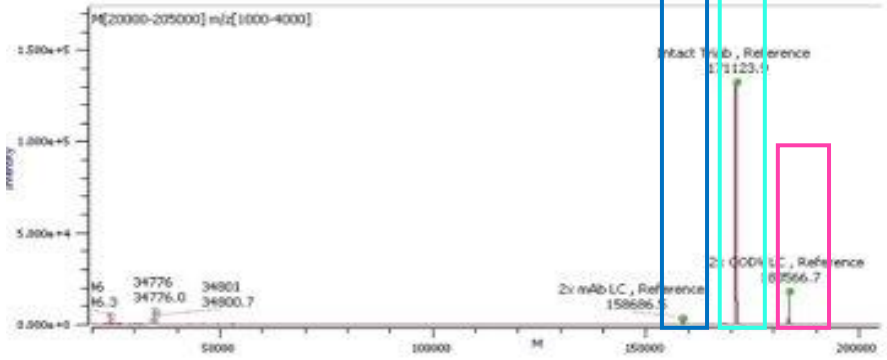
Technology Development with aHIC and MS

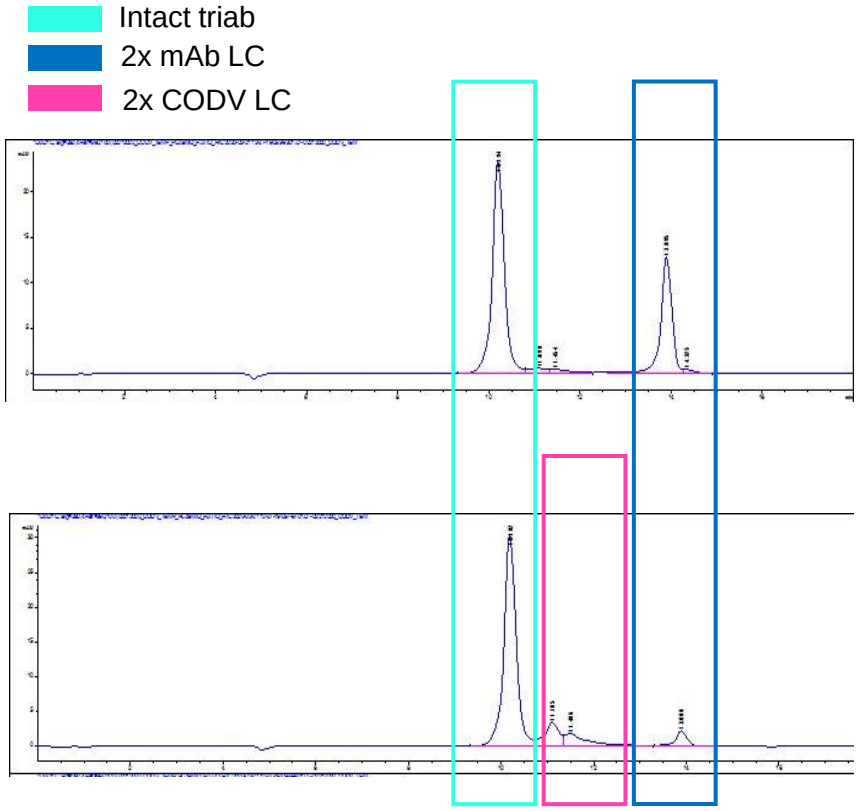
- *Current setup: Assignment of aHIC peaks is tedious and unreliable*
- *Test ability of 2D LC-MS (aHIC x RP) to enable identification and quantification in single experiment*

Triab A
Expression condition 1:



Triab A
Expression condition 2:



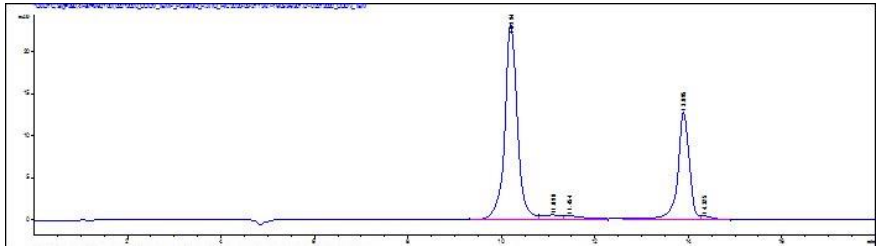


Challenge: Many Separation Techniques Do Not Use MS-compatible Buffers

Solution must be volatile, low salt, and conductive to be ESI-MS compatible

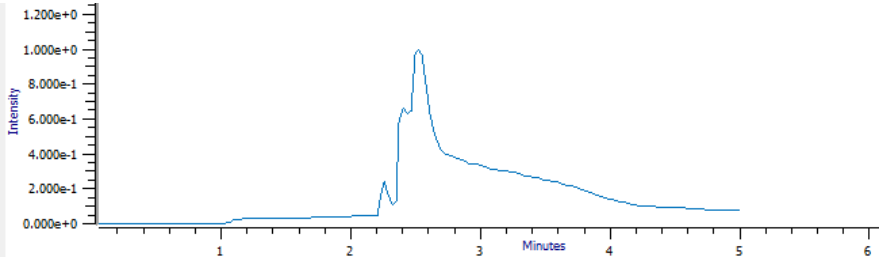
Triab aHIC chromatogram:

Mobile phase: 1.5 M ammonium sulfate/25 mM sodium phosphate



Triab reverse-phase LC TIC chromatogram:

Mobile phase: Water/acetonitrile + 0.1% formic acid



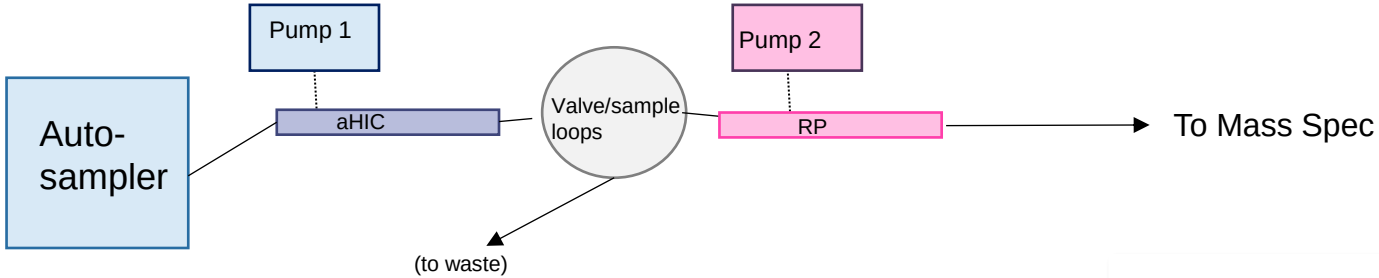
Concentration of common buffer components that reduces the MS signal by 50%:

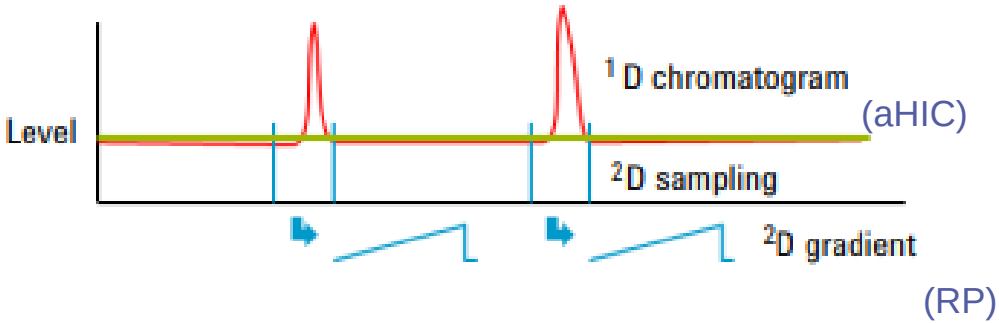
Type	Component	SC_{50}
Salts	Sodium chloride	1.5 mM
	Magnesium chloride	25 µm
	Ammonium sulfate	1.1 mM
Chaotropes	Guanidinium chloride	38 µm
	Urea	600 µm
Detergents*	SDS	1.7 µm
	Triton X-100	4.2 µm
	Tween 20	1.3 µm
Buffers	Tris base	91 µm
	HEPES	600 µm
	PBS	31 µm
	Antibody formulation	3.4 µm

Donnelly, D.P. *et al. Nature Methods*, **2019**, 16

One Potential Solution: 2D LC-MS

- 1st LC column separates proteins (ie using an aHIC column); 2nd LC column buffer-exchanges each peak (ie using a standard reversed-phase column)





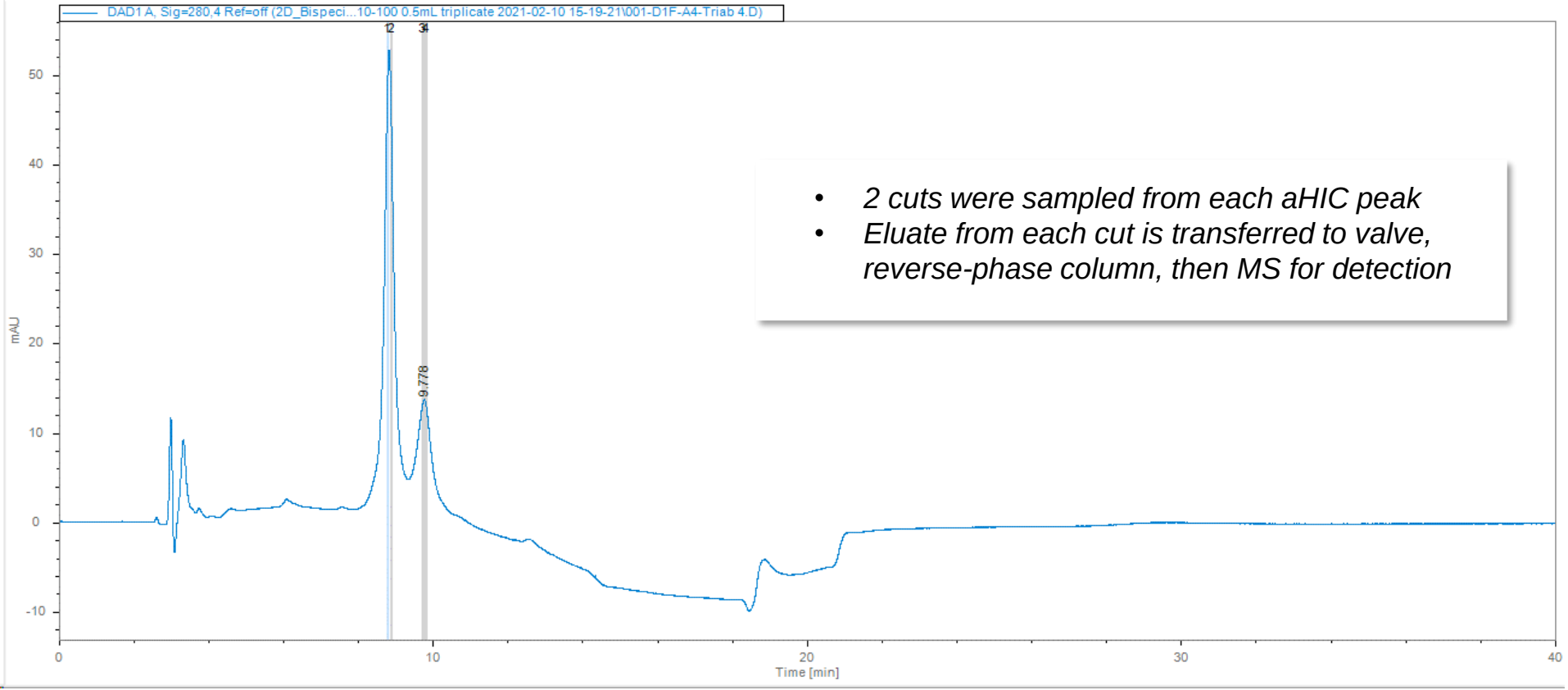
- *A280 Detection from 1st dimension (aHIC) enables protein quantification*
- *MS detection after RP desalting enables protein identification*
- *Collaboration with Agilent demo lab to evaluate this method*

Image credit: Agilent 2D LC User Guide

2D LC-MS Demo with Agilent

- Sent 4 triabs from different projects for analysis
- 1st column: Agilent AdvanceBio HIC column (4.6x100 mm) with 1 M ammonium tartrate, 50 mM sodium phosphate running buffer
- 2nd column: Agilent desalting cartridge & PLRP-S column (2.1x50 mm)
- Mass spec: 6545 XT qTOF
- ***Evaluate whether this method would enable us to better interpret the aHIC data by assigning peaks using MS***
- ***Determine whether mass peak intensity is a valid estimate for intact triab abundance by comparing to A280 quantification***

aHIC Chromatogram from Demo Triab #1

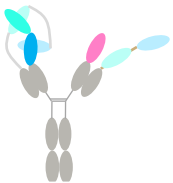


- *2 cuts were sampled from each aHIC peak*
- *Eluate from each cut is transferred to valve, reverse-phase column, then MS for detection*

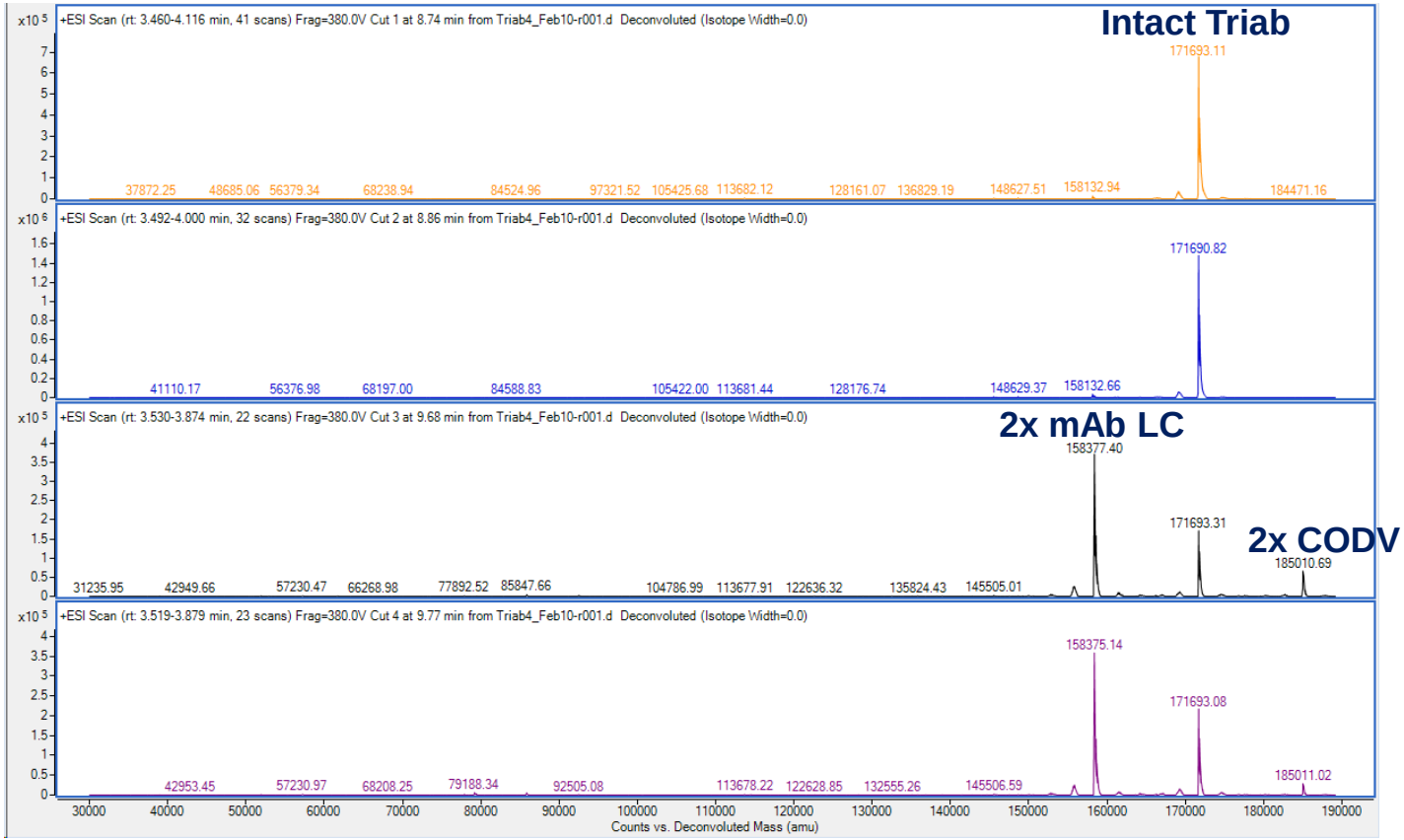
Deconvoluted MS from Each of 4 Cuts:

Intact Triab

2x mAb LC



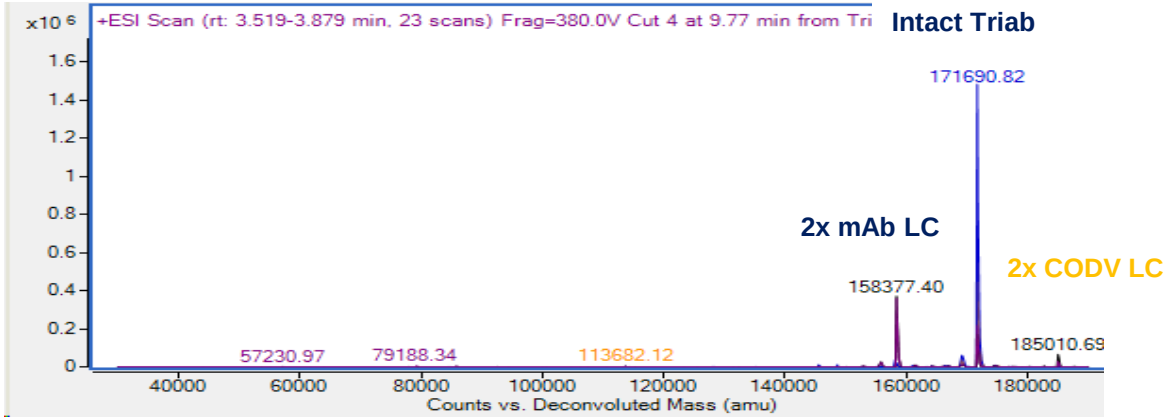
2x CODV LC



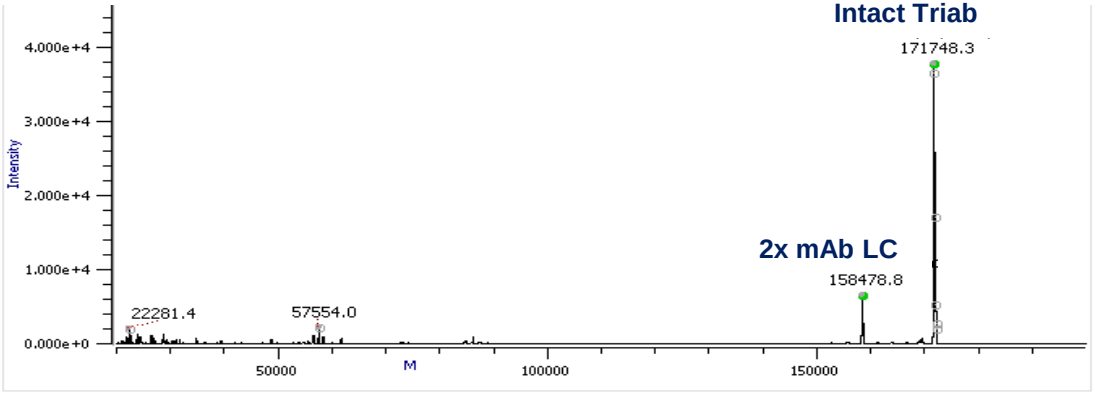
Overlaid 2D Spectra vs Reverse-Phase Spectrum

2D Method enables detection of low-abundance molecules

Overlay of 4 cuts from 2D Method:



Mass Spectrum from Reverse-Phase Method:



SANOFI

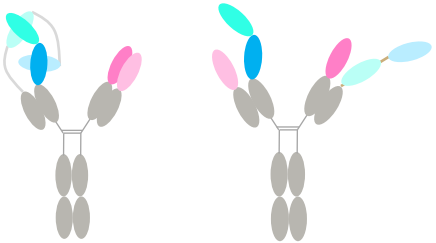
2D HIC Demo Summary

TPP	%Intact Triab-aHIC A280	%Intact Triab- Mass Peak Intensity
Triab 1	76.5	85.1
Triab 2	75.4	71.2
Triab 3	89.2	90

- Relative quantification of intact triab using aHIC chromatogram integration provided similar results as quantification using the mass peak intensities- helpful for HT screening from PEPP
- Co-elution of mispaired molecules on aHIC complicates A280 quantification; might be improved by using a longer aHIC column
- 2D LC-MS enabled detection of low-abundance molecules that were not detected by RP LC-MS
- For in-process analytics, it would still be a helpful method for ID'ing extra HIC peaks

Technology Development for Ongoing Challenges

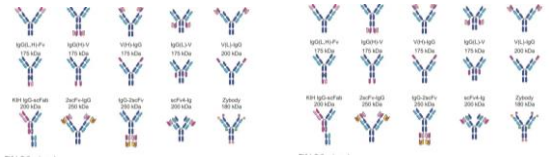
Detection of samples with isobaric mispairing



172 kDa 172 kDa

- *Test ability of HRIM to separate mispaired proteins ion mobility*
- *Additional separation in IM dimension could enable faster data acquisition*
- *Demo with MOBILion in progress*

Analysis of new formats where mispaired molecules are close in expected Mw



- *For certain msAb formats, intact & mispaired proteins differ by <5 Da, making peak assignment ambiguous*
- *Fab digest of large libraries is expensive and time-consuming*
- *Evaluating library design tool to flag molecules with close Mw's before proteins are produced*

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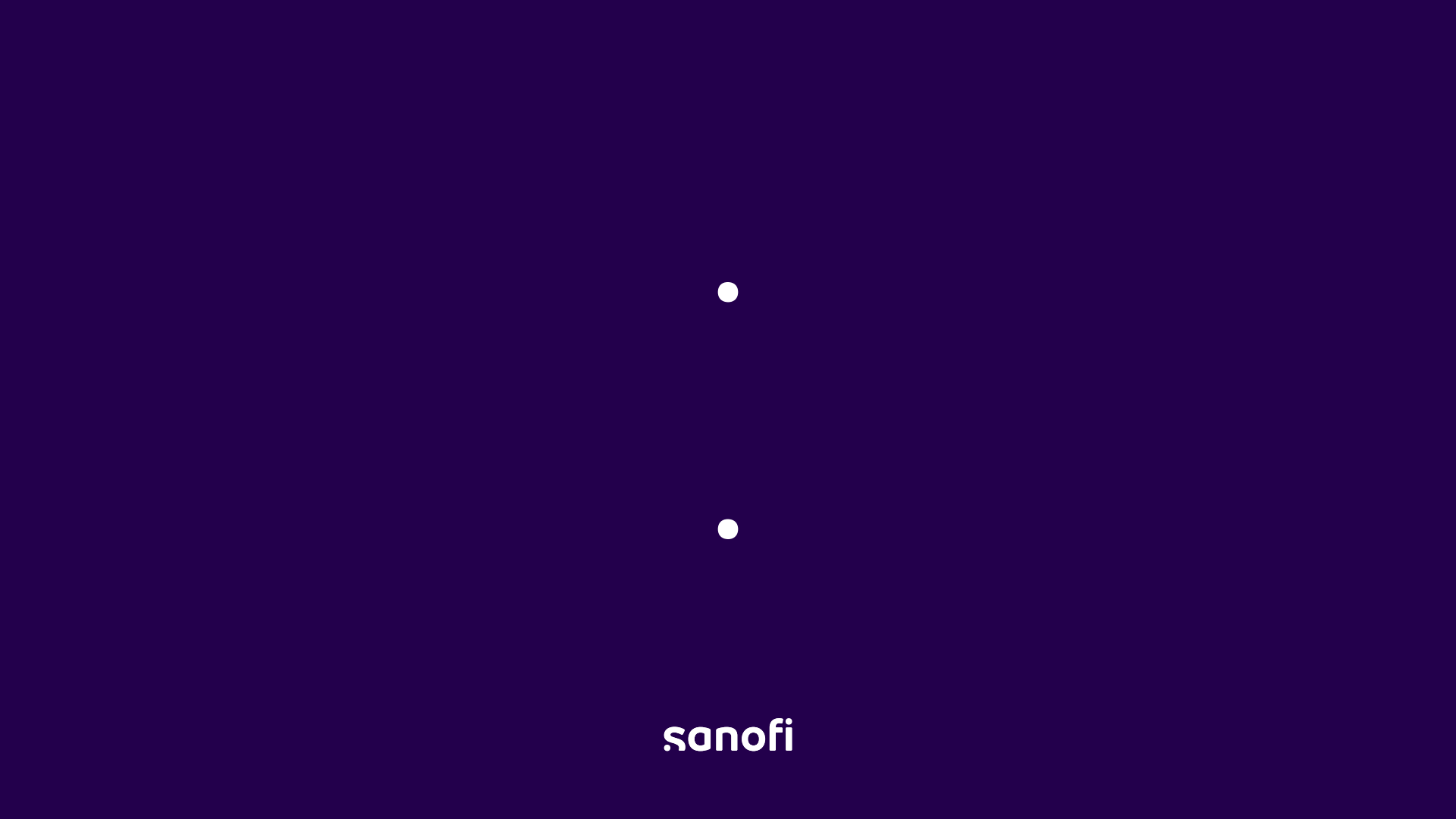
Disclosure statement: Kalie Mix is a Sanofi employee and may hold shares and/or stock options in the company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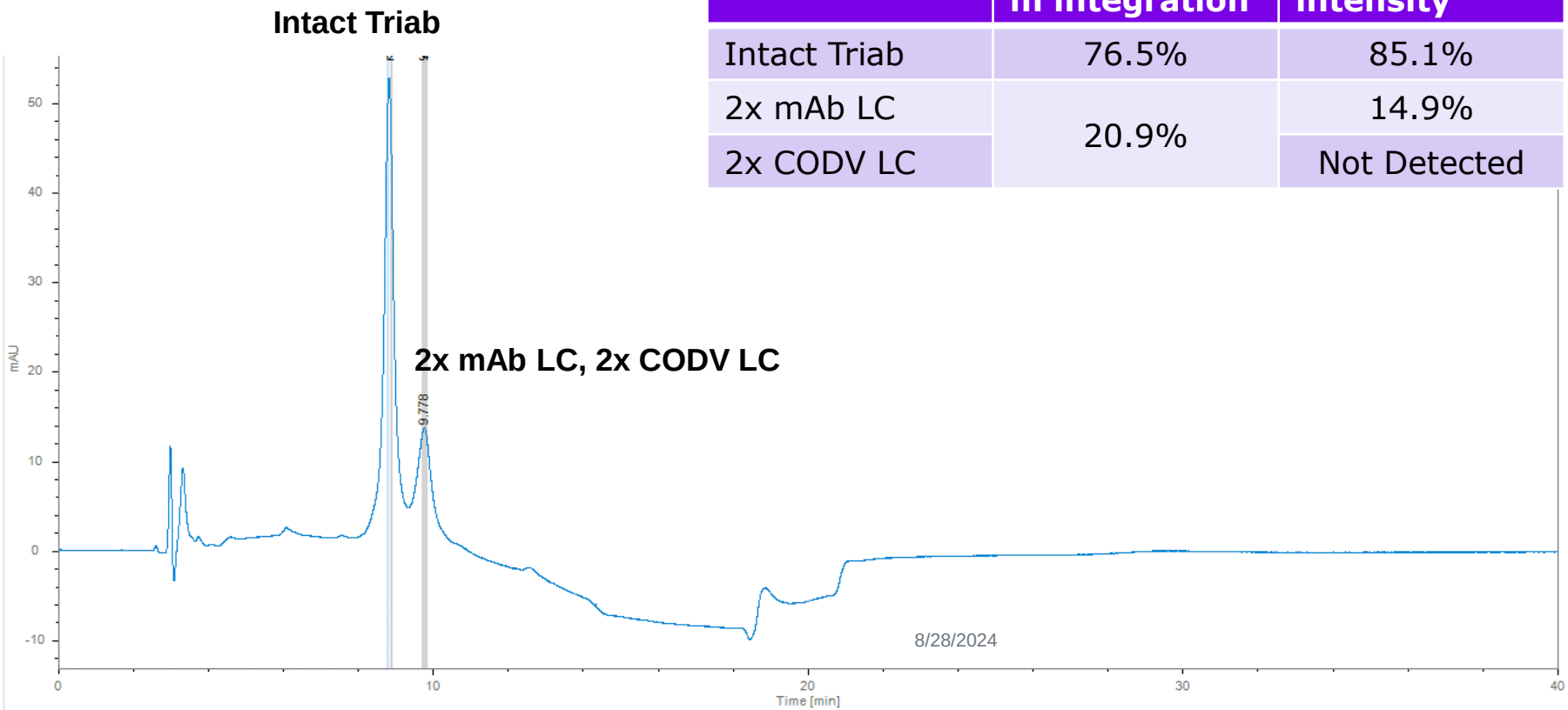
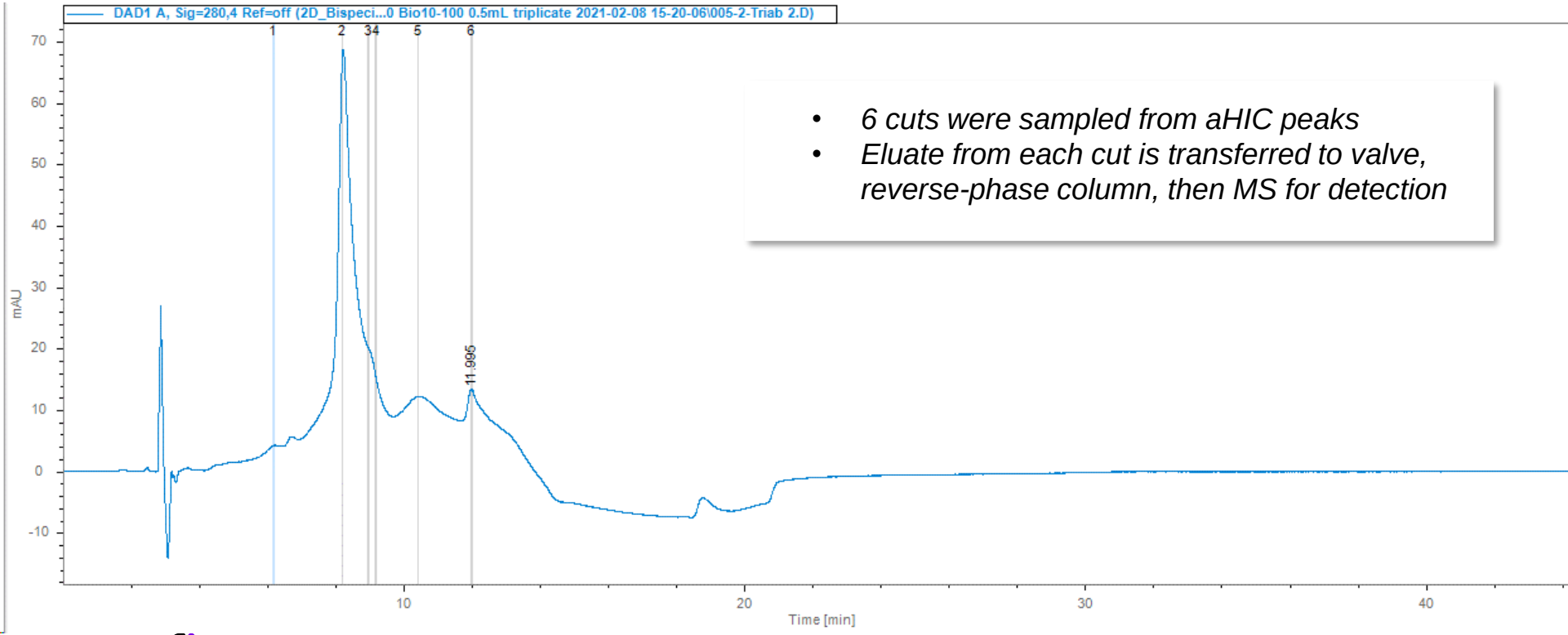
Quantitation Using aHIC Chromatogram integration vs. Mass Peak Intensity

	aHIC Chromatogram integration	RP-LC-MS Mass peak intensity
Intact Triab	76.5%	85.1%
2x mAb LC	20.9%	14.9%
2x CODV LC		Not Detected



aHIC Chromatogram from Demo Triab #2



- *6 cuts were sampled from aHIC peaks*
- *Eluate from each cut is transferred to valve, reverse-phase column, then MS for detection*

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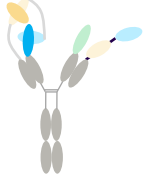
Deconvoluted MS from Each of 6 Cuts

Intact Triab

2x mAb LC



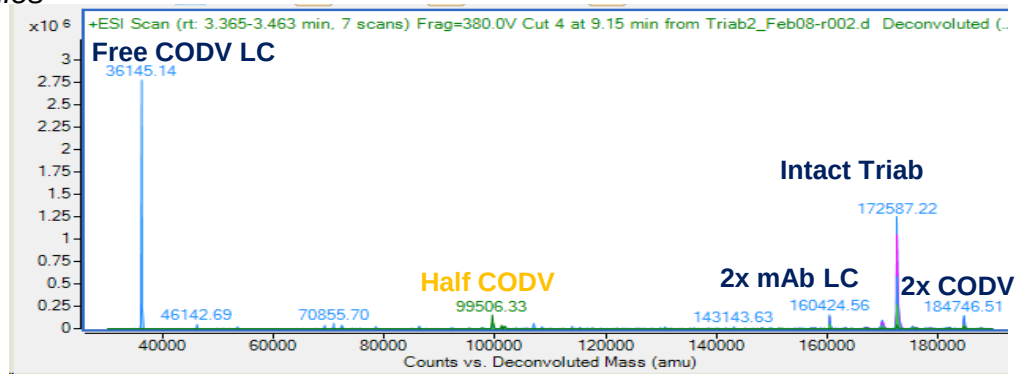
2x CODV LC

Half CODV

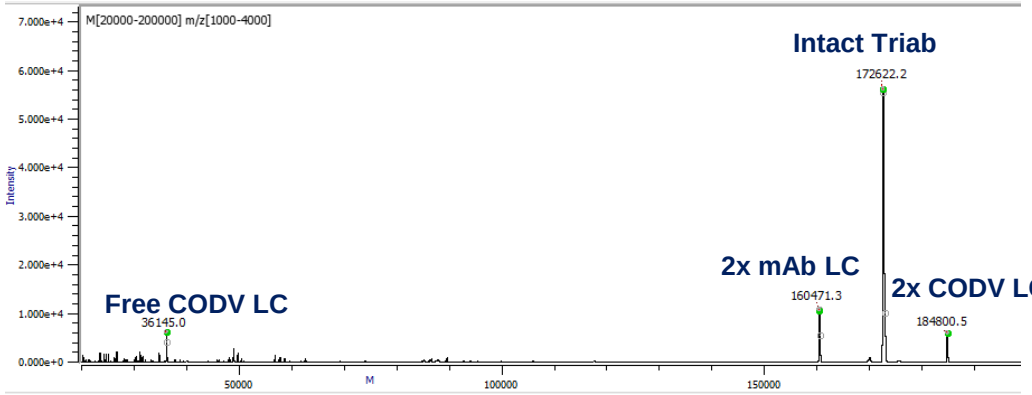
Overlaid 2D Spectra vs Reverse-Phase Spectrum

2D Method enables detection of low-abundance molecules

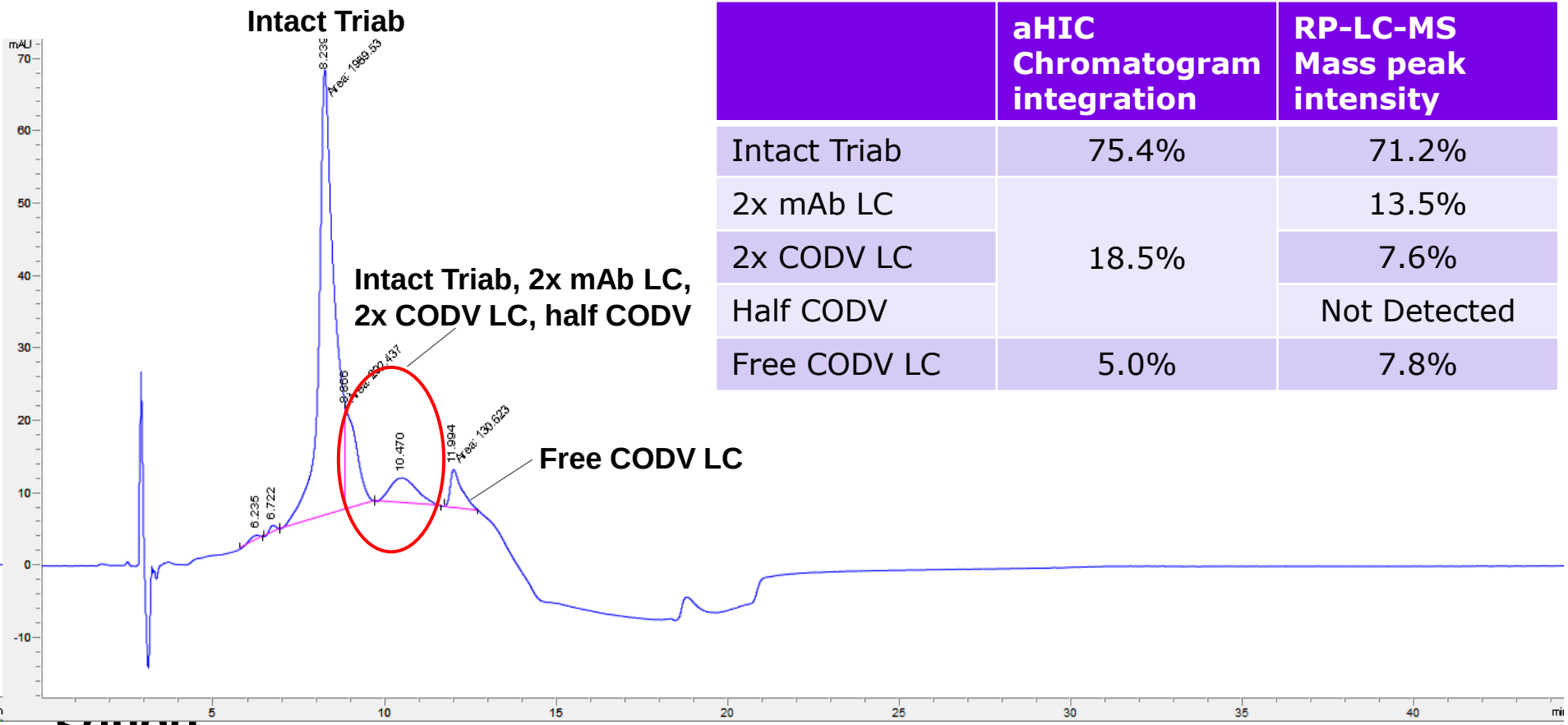
Overlay of 6 cuts from 2D Method:



Mass Spectrum from Reverse-Phase Method:



Quantitation using aHIC Chromatogram Integration vs. Mass Peak Intensity



	aHIC Chromatogram integration	RP-LC-MS Mass peak intensity
Intact Triab	75.4%	71.2%
2x mAb LC	18.5%	13.5%
2x CODV LC		7.6%
Half CODV		Not Detected
Free CODV LC	5.0%	7.8%