

Ultra-Sensitive Intact Monoclonal Antibody Quantification Using AssayMAP Bravo Liquid Handling Platform and 6545XT AdvanceBio LC/Q-TOF Mass Spectrometer

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LC-MS Application Scientist
Agilent Technologies

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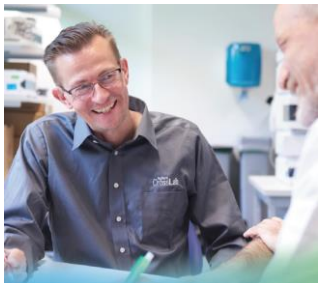
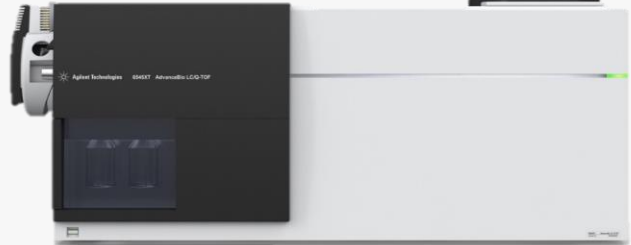
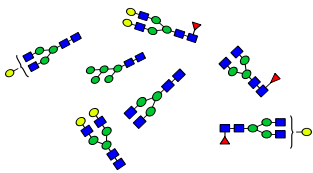
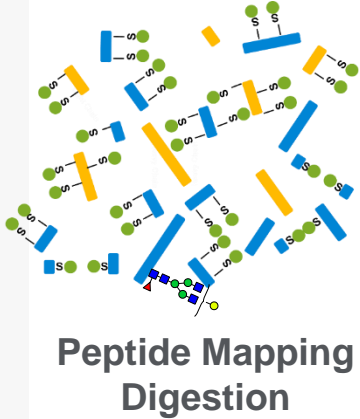
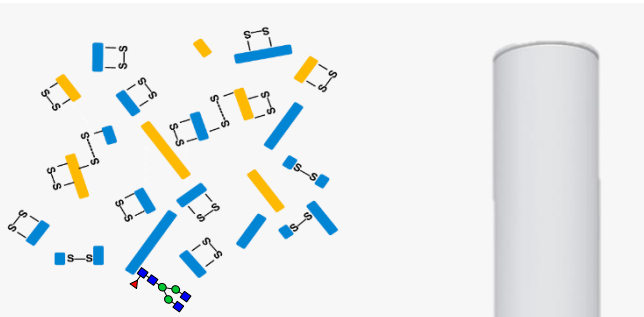
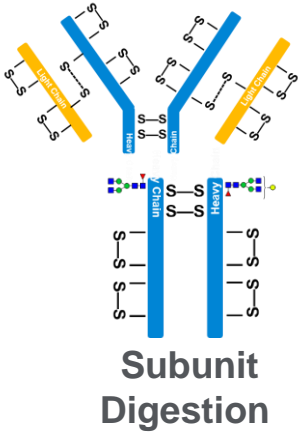
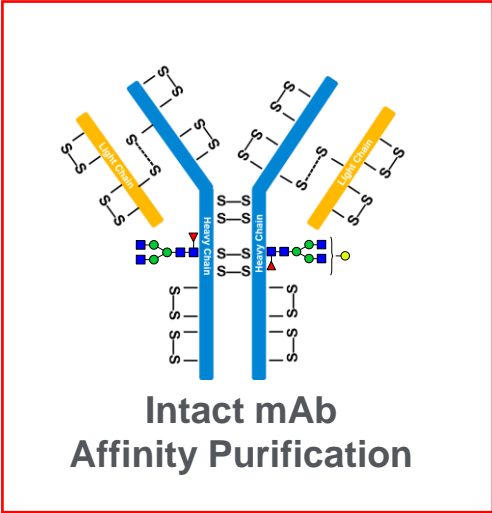
Outline

- 6545XT AdvanceBio Q-TOF: Designed for Biopharma
- 6545XT AdvanceBio Q-TOF: Features
- Intact Protein Analysis Performance on 6545XT AdvanceBio Q-TOF
- Intact protein quantification in biological matrix by 6545XT AdvanceBio Q-TOF
- Live demonstration of 6545XT AdvanceBio Q-TOF



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6545XT AdvanceBio Q-TOF
The centerpiece of multiple
biopharma workflows

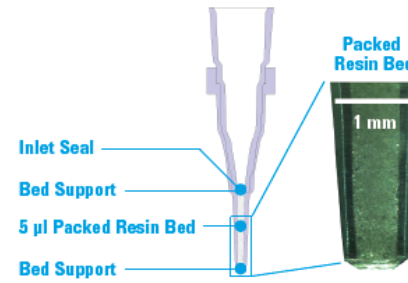
Intact Protein Quantification Workflow

Sample Prep

Separation

Detection

Data Processing & Report



Product	Description	Application	Catalog number
C18 cartridges	1 rack (96 cartridges)	Desalting mixtures of peptides and small proteins prior to analysis by mass spectrometry.	5190-6532
Fe(III)-NTA cartridges	1 rack (96 cartridges)	Phosphopeptide enrichment prior to analysis by mass spectrometry.	G5496-60085
Protein A (PA-W) cartridges	1 rack (96 cartridges)	Purification of monoclonal and polyclonal antibodies and Fc fusion proteins with affinity for Protein A.	G5496-60000
Protein G (PG-W) cartridges	1 rack (96 cartridges)	Purification of monoclonal and polyclonal antibodies and Fc fusion proteins with affinity for Protein G.	G5496-60008
Resin-Free cartridge	1 rack (96 cartridges)	Placeholder when working with sample numbers that are not a multiple of eight.	G5496-60009
Reversed Phase (RP-S) cartridges	1 rack (96 cartridges)	Desalting or fractionating mixtures of peptides and small proteins prior to analysis by mass spectrometry.	G5496-60033
Reverse Phase (RP-W) cartridges	1 rack (96 cartridges)	Desalting of denatured proteins (antibodies) prior to trypsin digestion.	G5496-60086
Streptavidin (SA-W) cartridges	1 rack (96 cartridges)	Generation of affinity cartridges by immobilizing biotinylated molecules. Affinity cartridges can subsequently be used to purify target molecules from complex matrices.	G5496-60010
Strong Cation Exchange (SCX) cartridges	1 rack (96 cartridges)	Fractionating mixtures of peptides and small proteins prior to analysis by mass spectrometry.	5190-6536
TiO2 cartridges	1 rack (96 cartridges)	Phosphopeptide enrichment prior to analysis by mass spectrometry.	G5496-60016

Intact Protein Quantification Workflow



Agilent 1290 Infinity II



Agilent AdvanceBio LC Columns

- PLRP-S 1000 Å
- AdvanceBio RP mAb
- Zorbax SB 300 RRHD
- Poroshell 300

Every protein can behave differently. Agilent has multiple LC columns for protein separation.

Intact Protein Quantification Workflow

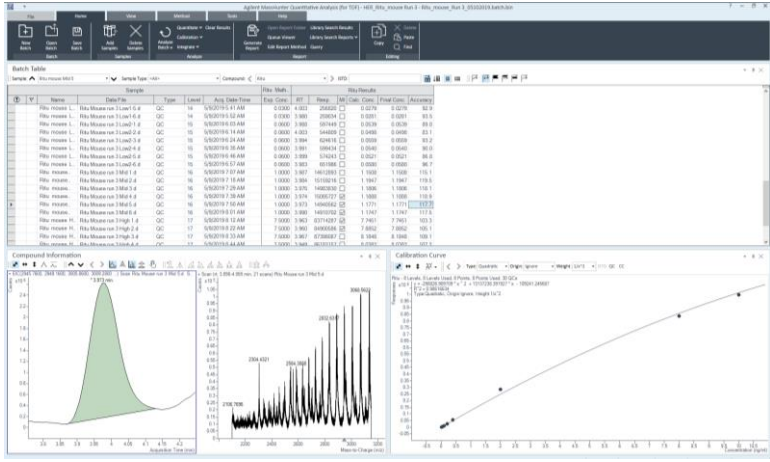


Agilent 6545XT AdvanceBio Q-TOF

Designed to excel at intact protein analysis.

(see features and performance slides later in presentation)

Intact Protein Quantification Workflow

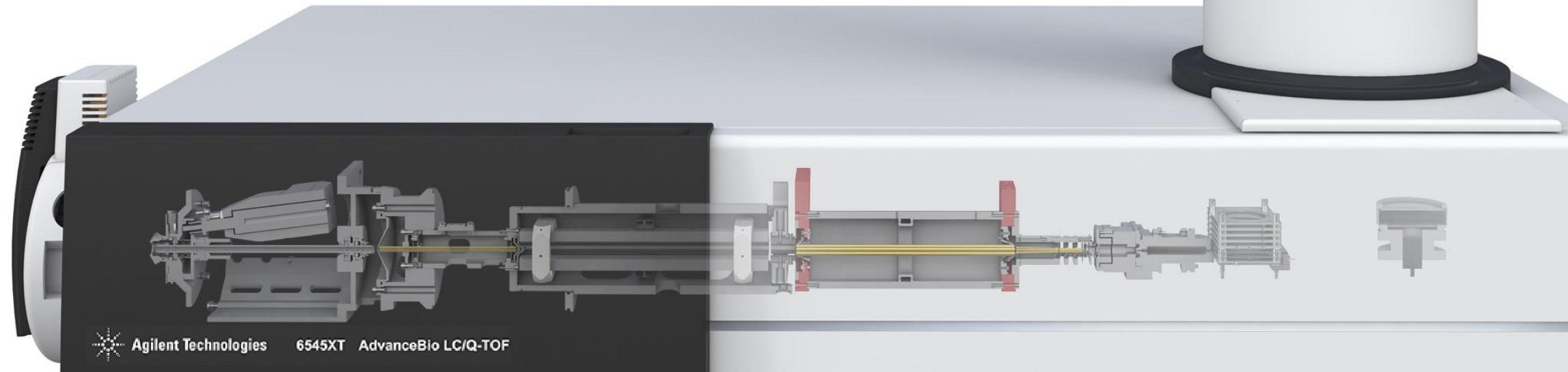


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6545XT Features for Large Biomolecule Analysis

- Excellent protein spectral clarity from ultra-low TOF vacuum
- One-click optimization for large molecules with SWARM autotune
- Capable of analyzing very large molecules, with a variable mass range up to 30k m/z
- 50k resolution from improved beam optics
- Protein performance verification at install, and includes quick-start protein method
- Ease of maintenance with vent-free capillary removal

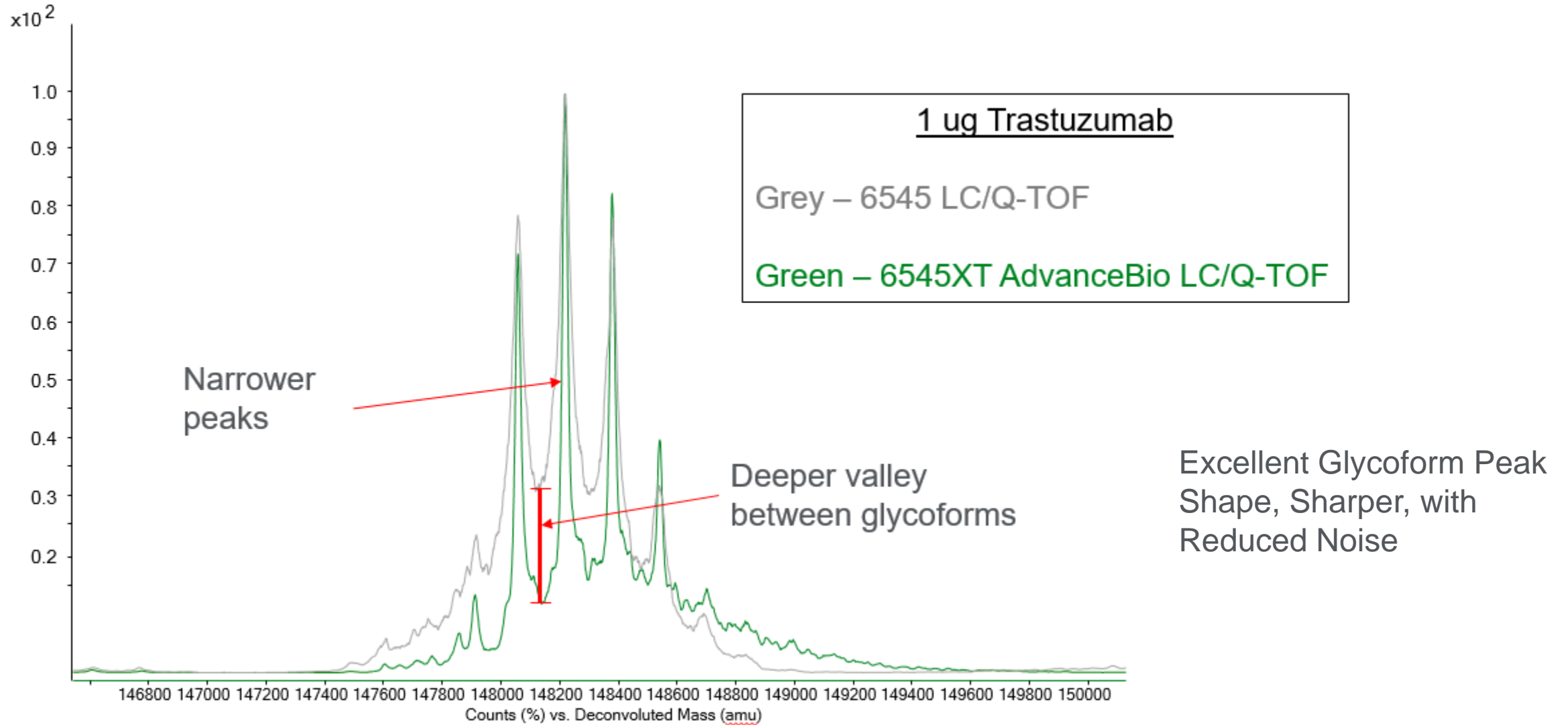
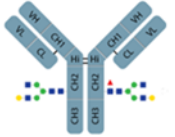


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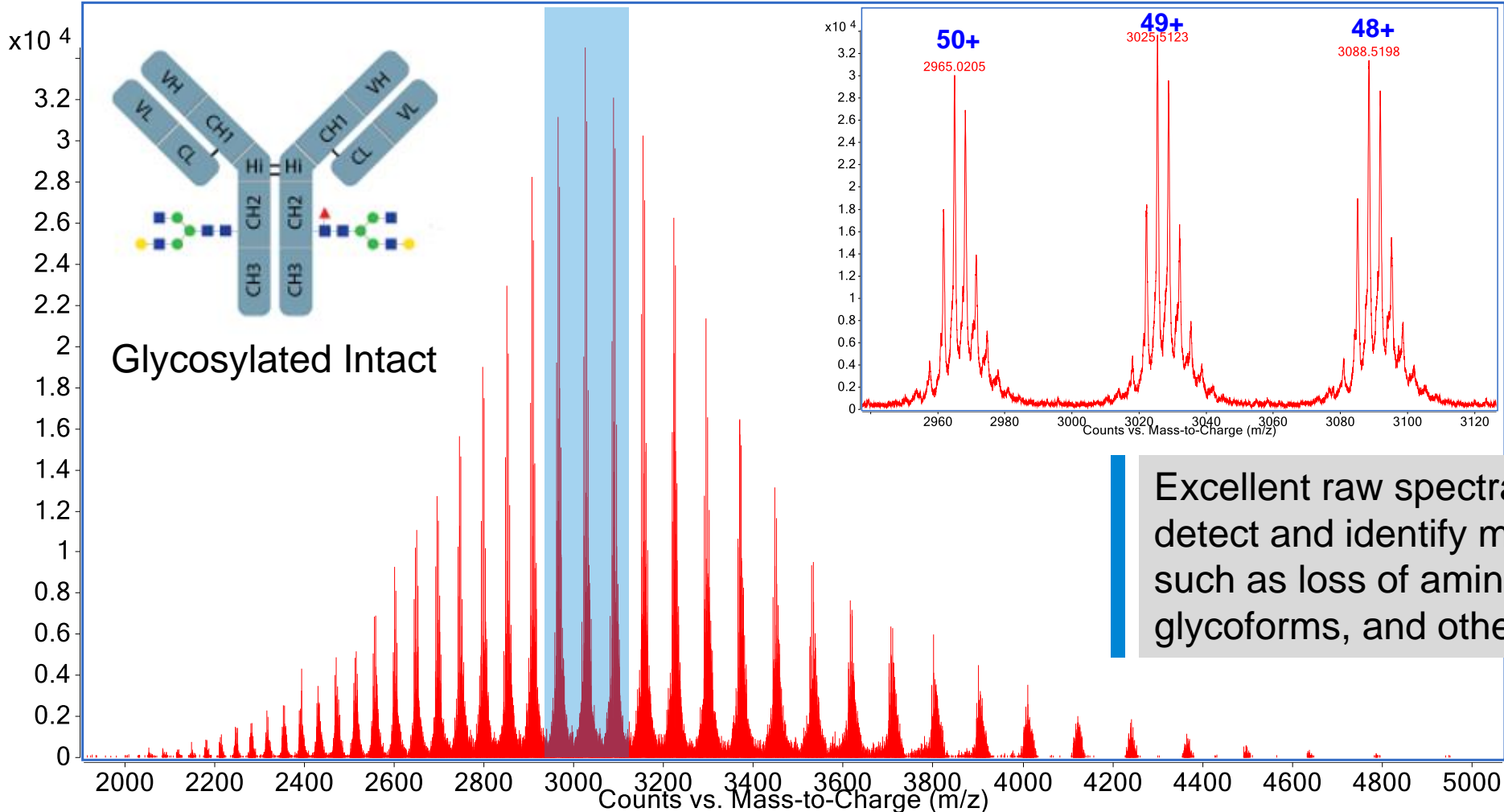
- 6545XT AdvanceBio Q-TOF: Designed for Biopharma
- 6545XT AdvanceBio Q-TOF: Features
- Intact Protein Analysis Performance on 6545XT AdvanceBio Q-TOF
 - Accuracy / Isoform Detection
 - Reproducibility
 - Analytical Sensitivity / Dynamic Range
 - Robustness
- Intact protein quantification in biological matrix by 6545XT AdvanceBio Q-TOF
- Live demonstration of 6545XT AdvanceBio

Agilent 6545XT AdvanceBio Q-TOF - Improved TOF Vacuum

Confident identification of low level of glycan isoform



Intact Protein Raw Spectral Quality

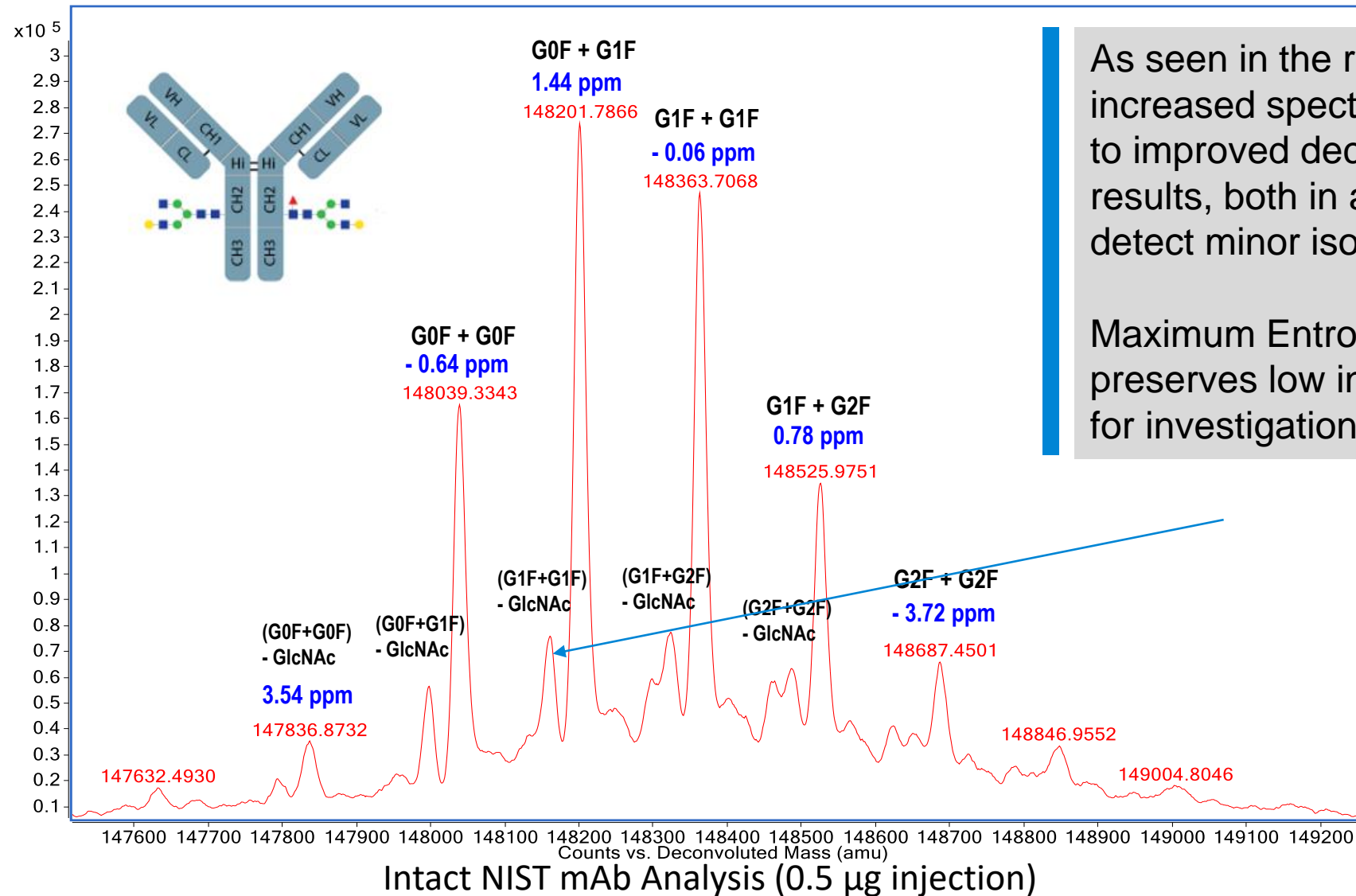


Glycosylated Intact

Excellent raw spectral quality to detect and identify minor isoforms such as loss of amino acids, minor glycoforms, and other PTMs.

Intact NIST mAb Analysis (0.5 µg injection)

Intact Protein Deconvoluted Mass Accuracy and Isoform Detection



As seen in the raw spectra, the increased spectral clarity leads to improved deconvoluted results, both in accuracy and to detect minor isoforms.

Maximum Entropy deconvolution preserves low intensity signals for investigation of heterogeneity.

Know More at the Intact Level

Proteoform	Theoretical Mass (Da)	Observed Mass (Da)		
		Lab 1	Lab 2	Lab 3
1. G0F/Aglycosylated	146591.8	ND	146595.3 (t)	ND
2. G1F/Aglycosylated	146754.0	ND	146756.0 (t)	ND
3. G2F/Aglycosylated	146916.1	ND	146915.8 (t)	ND
4. G0F/G0F – 2GlcNAc	147630.8	147633.1 (t)	ND	ND
5. G0F/G0F – GlcNAc	147834.0	147837.9 (mn)	147837.4 (mn)	ND
6. G0F/G1F – GlcNAc	147996.1	147993.2 (t)	148000.6 (mn)	148000.0 (mn)
7. G0F/G0F	148037.2	148040.5 (M)	148039.3 (M)	148045.0 (M)
8. G0F/G0F + K	148165.3	148154.6 (t)	148164.9 (mn)	148163.0 (mn)
9. G0F/G1F	148199.3	148201.9 (M)	148201.7 (M)	148206.5 (M)
10. G0F/G1F + K	148327.5	148325.5 (t)	148328.1 (mn)	148302.0 (mn)

Proteoform	Theoretical Mass (Da)	Observed Mass (Da)		
		Lab 1	Lab 2	Lab 3
11. G1F/G1F	148361.4	148364.3 (M)	148363.8 (M)	148368.5 (M)
12. G1F/G1F + K	148489.6	148483.7 (t)	148492.4 (mn)	ND
13. G1F/G2F	148523.6	148526.7 (mn)	148526.1 (mn)	148531.0 (mn)
14. G1F/G1F + 2K	148617.8	ND	148624.1 (t)	ND
15. G1F/G2F + K	148651.8	148643.1 (t)	148654.5 (mn)	ND
16. G2F/G2F	148685.7	148688.5 (mn)	148687.5 (mn)	ND
17. G2F/G2F + Hex	148847.7	148847.9 (mn)	148849.4 (mn)	ND
18. G2F/G2F + 2Hex	149010.0	ND	149008.2 (t)	ND

- In a previously published multi-lab study¹ characterizing the NIST mAb, 18 proteoforms are described by three labs using different HRAM systems. **Each lab missed some of the proteoforms, while injecting 5 µg or performing an infusion.**

1. Schiel et al.; *State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization Volume 2. ... ACS Symposium Series; American Chemical Society: Washington, DC, 2015.*

Know More at the Intact Level

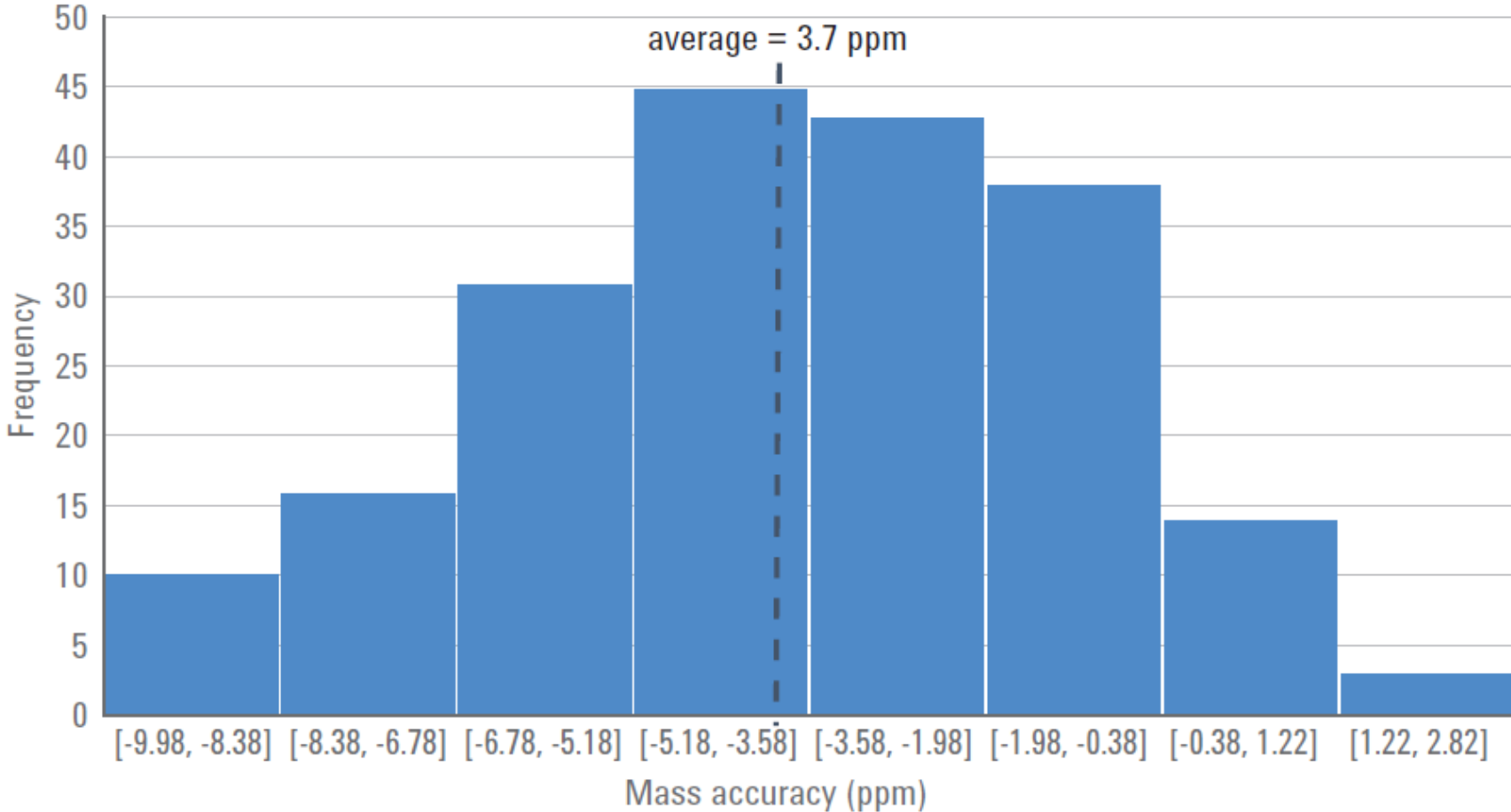
Proteoform	Theoretical Mass (Da)	5.0 µg	0.5 µg
		Missed by:	Agilent 6545XT
1. G0F/Aglycosylated	146591.8	2 Labs	146592.5
2. G1F/Aglycosylated	146754.0	2 Labs	146755.8
3. G2F/Aglycosylated	146916.1	2 Labs	146918.5
4. G0F/G0F – 2GlcNAc	147630.8	2 Labs	147632.5
5. G0F/G0F – GlcNAc	147834.0	1 Lab	147836.0
6. G0F/G1F – GlcNAc	147996.1		147998.5
7. G0F/G0F	148037.2		148038.6
8. G0F/G0F + K	148165.3		148161.9
9. G0F/G1F	148199.3		148200.9
10. G0F/G1F + K	148327.5		148324.7

Proteoform	Theoretical Mass (Da)	5.0 µg	0.5 µg
		Missed by:	Agilent 6545XT
11. G1F/G1F	148361.4		148363.1
12. G1F/G1F + K	148489.6	1 Lab	148488.1
13. G1F/G2F	148523.6		148525.2
14. G1F/G1F + 2K	148617.8	2 Labs	148623.6
15. G1F/G2F + K	148651.8	1 Lab	148652.9
16. G2F/G2F	148685.7	1 Lab	148686.6
17. G2F/G2F + Hex	148847.7	1 Lab	148846.7
18. G2F/G2F + 2Hex	149010.0	2 Labs	149003.5

- The 6545XT AdvanceBio LC/Q-TOF **detects every proteoform** described, while injecting less material, and with the best mass accuracy for 13 of the 18 peaks

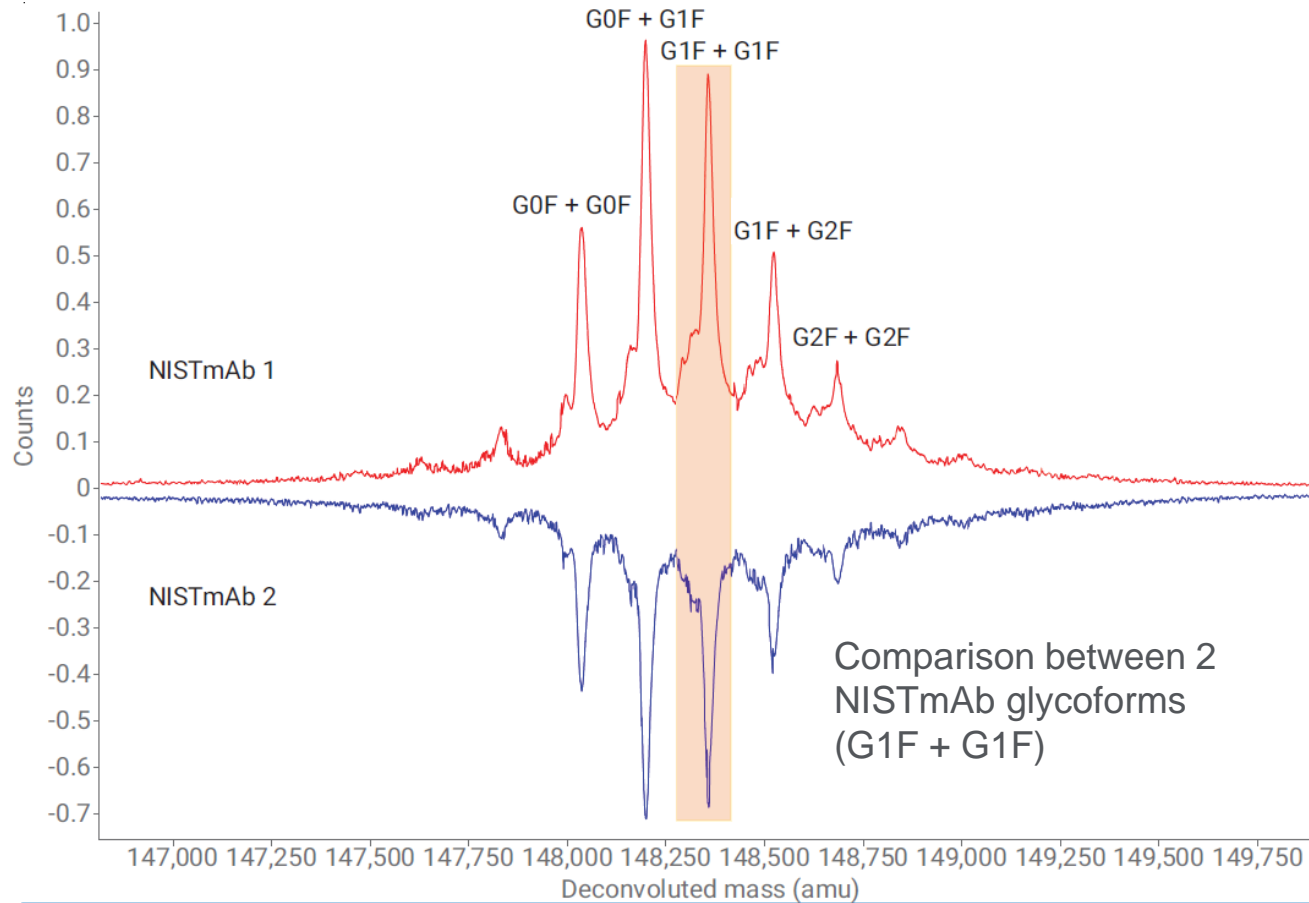
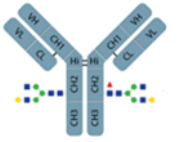
1. Schiel et al.; *State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization Volume 2. ... ACS Symposium Series; American Chemical Society: Washington, DC, 2015.*

Reproducible Mass Accuracy even at Low Levels

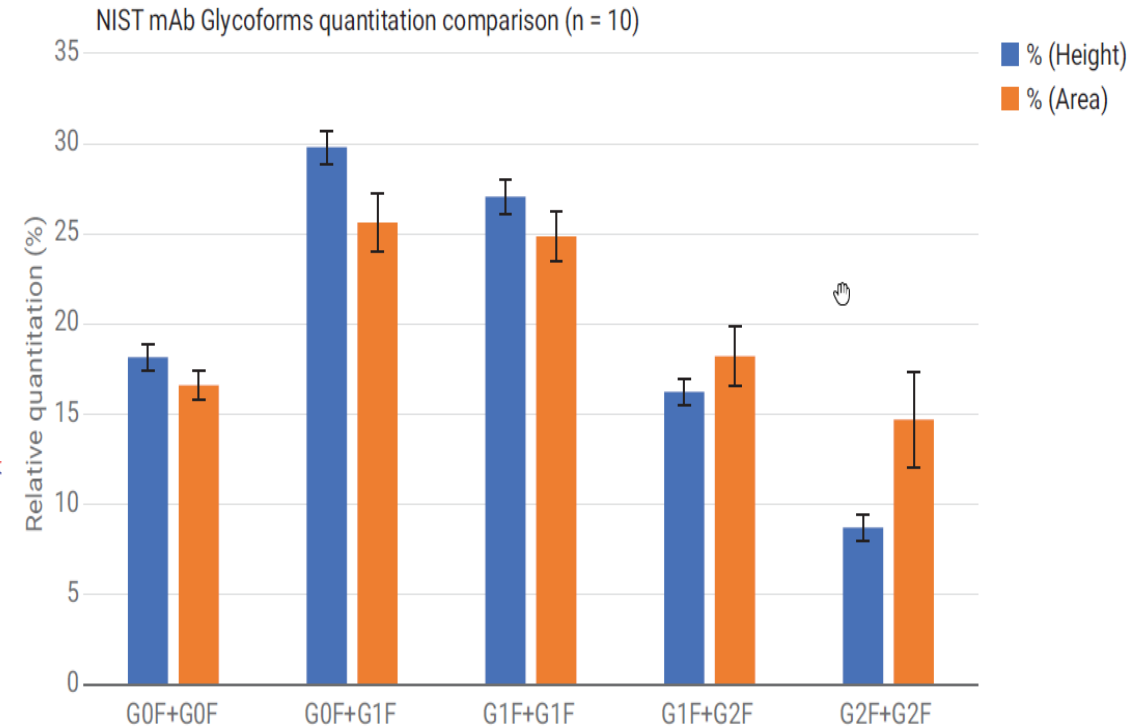


Histogram of mass accuracies for 100 replicate 10ng injections of trastuzumab. The top two glycoforms for every injection were recorded

Agilent 6545XT AdvanceBio Q-TOF Quantitation Comparison of Five Glycoforms



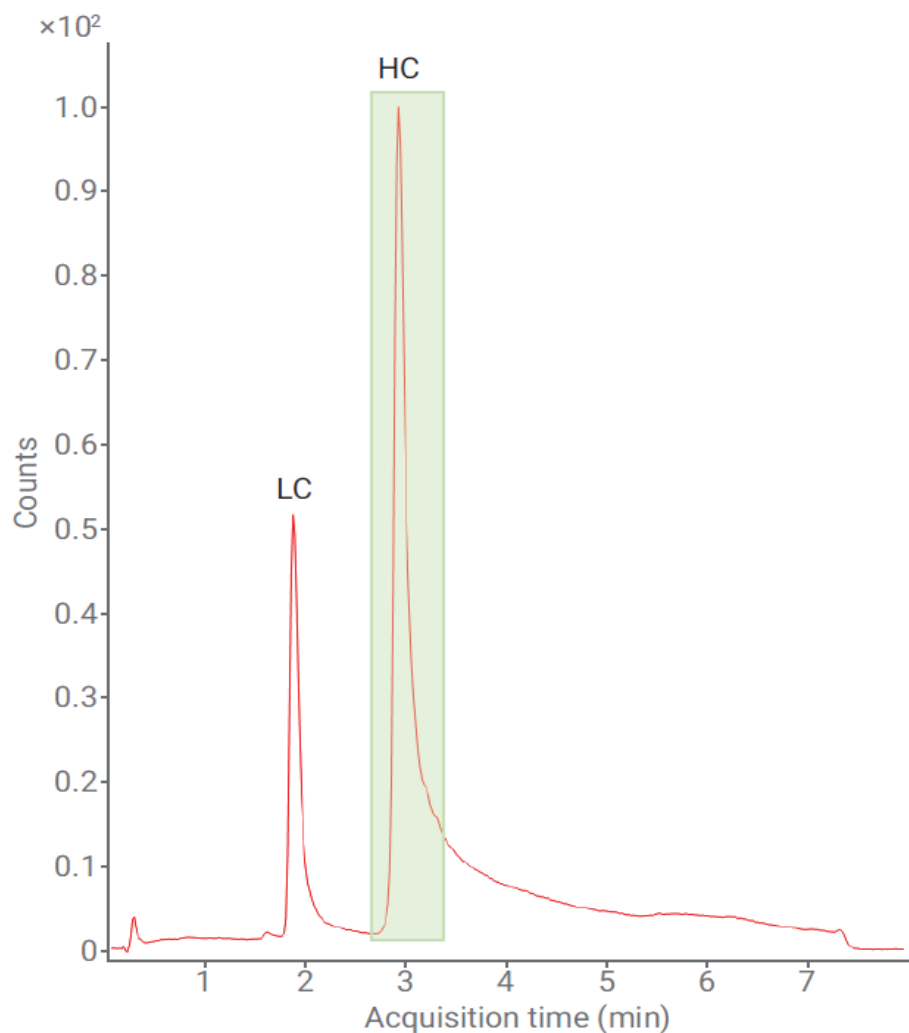
General				% Quantitation				Sequence Match
Mass	RT	File	Use for %Quant	Height (MS)	%Quant (Height)	Area (MS)	%Quant (Area)	Pred Mods
148363.0381	2.229	NIST mAb_1.d	<input checked="" type="checkbox"/>	8387	28.43	6527163	27.71	2*G1F(1607.5013)
148362.5884	2.261	NIST mAb_2.d	<input checked="" type="checkbox"/>	6268	29.5	4936695	28	2*G1F(1607.5013)



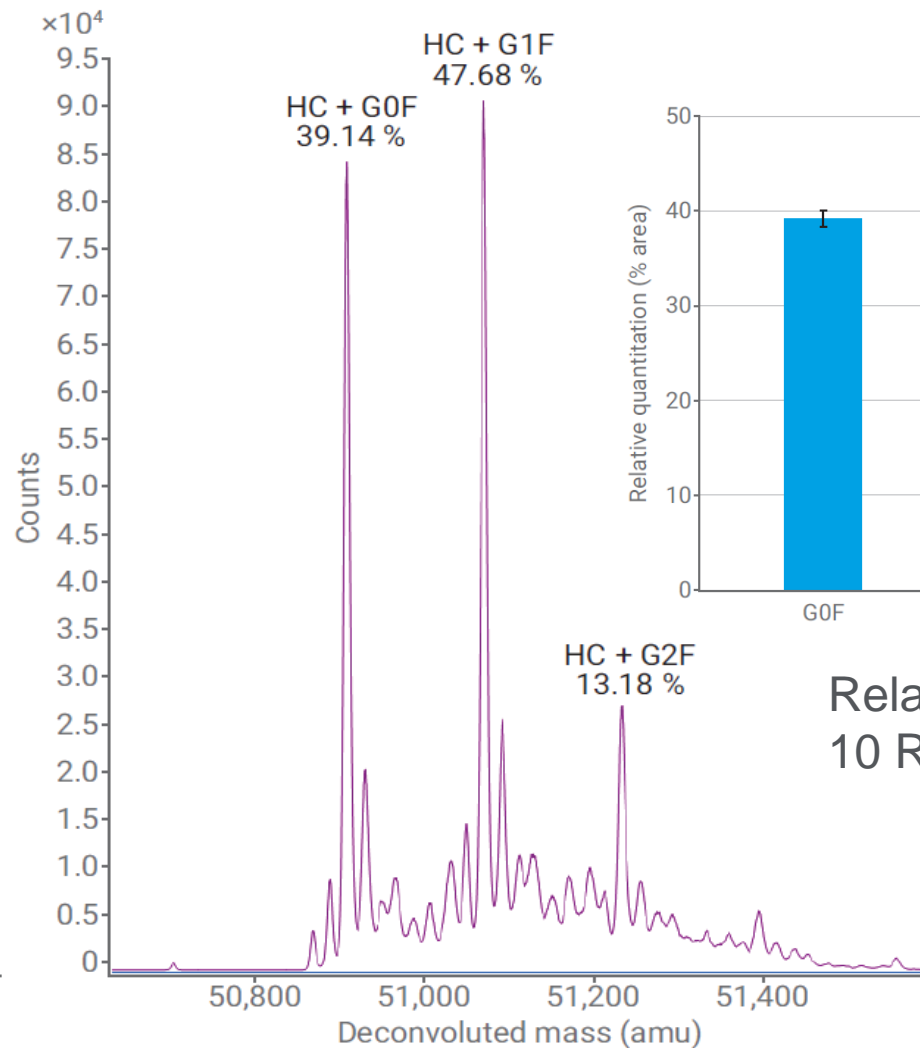
Quantitation results from intact NIST mAb glycoforms – 10 replicates

Agilent Pub # [5991-8796EN](#)

Accurate Quantitation of NISTmAb Sub-units Ten Replicates



Total Ion Chromatogram (TIC)



% Quant Value of MS Deconvolution of HC

Relative Quant % of Sub-units
10 Replicates SD < 0.24%

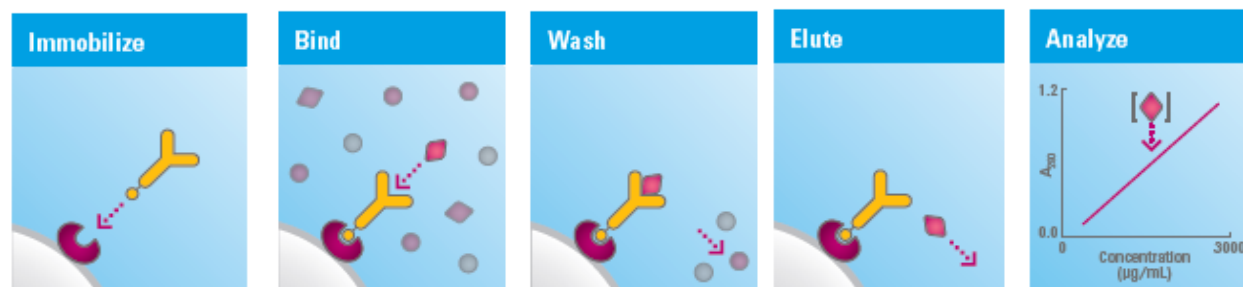
Pub# [5991-8796EN](#)

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- Live demonstration of 6545XT AdvanceBio Q-TOF

Ultra-Sensitive Intact Monoclonal Antibody Quantification

- Monoclonal antibody drugs (trastuzumab and rituximab) calibration curve (0.02 to 10 $\mu\text{g/mL}$) and quality control (4 levels) samples were prepared in mouse plasma.
- All sample preparation steps were performed by using the AssayMAP Bravo platform.
- First, 2.5 μg of biotinylated anti-human Fc antibody was immobilized on streptavidin cartridges at 3 $\mu\text{L/min}$ and washed twice with 100 μL PBS buffer at 20 $\mu\text{L/min}$.
- Then, 50 μL of plasma aliquots fortified with different concentration of mAb were loaded onto each cartridge at 3 $\mu\text{L/min}$. After loading, the cartridges were washed two times with 150 μL PBS buffer at 20 $\mu\text{L/min}$.
- The final elution step was carried out by loading 25 μL of 0.1% trifluoroacetic acid to the cartridge at 2 $\mu\text{L/min}$. 5 μL of 50mM ammonium bicarbonate buffer was added to the final elution and 15 μL was injected into 6545XT AdvanceBio LC/Q-TOF system for intact protein analysis.



Immobilization of capture antibody on streptavidin cartridge

Immobilization: Using AssayMAP v1.0

Application Settings

Number of Full Columns of Cartridges:

Step	Conduct Step?	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Initial Syringe Wash	<input checked="" type="checkbox"/>			<input type="text" value="3"/>
Prime	<input checked="" type="checkbox"/>	<input type="text" value="100"/>	<input type="text" value="300"/>	<input type="text" value="1"/>
Equilibrate	<input checked="" type="checkbox"/>	<input type="text" value="100"/>	<input type="text" value="5"/>	<input type="text" value="1"/>
Load Sample	<input type="checkbox"/>	<input type="text" value="100"/>	<input type="text" value="5"/>	<input type="text" value="3"/>
Collect Flow Through	<input type="checkbox"/>			
Cup Wash 1	<input checked="" type="checkbox"/>	<input type="text" value="25"/>		<input type="text" value="1"/>
Internal Cartridge Wash 1	<input checked="" type="checkbox"/>	<input type="text" value="50"/>	<input type="text" value="10"/>	<input type="text" value="3"/>
Collect Flow Through	<input type="checkbox"/>			
Load Blocking Reagent	<input checked="" type="checkbox"/>	<input type="text" value="100"/>	<input type="text" value="5"/>	<input type="text" value="3"/>
Collect Flow Through	<input type="checkbox"/>			
Cup Wash 2	<input checked="" type="checkbox"/>	<input type="text" value="25"/>		<input type="text" value="1"/>
Internal Cartridge Wash 2	<input checked="" type="checkbox"/>	<input type="text" value="50"/>	<input type="text" value="10"/>	<input type="text" value="3"/>
Collect Flow Through	<input type="checkbox"/>			
Stringent Syringe Wash	<input type="checkbox"/>	<input type="text" value="50"/>		<input type="text" value="1"/>
Re-Equilibrate	<input type="checkbox"/>	<input type="text" value="50"/>	<input type="text" value="10"/>	<input type="text" value="1"/>
Final Syringe Wash	<input type="checkbox"/>			<input type="text" value="3"/>

Deck Layout

1. Wash Station	2. Cartridges	3. Priming & Equilibration Buffer
4. Sample	5. Cartridge Wash Buffer 1	6. Cartridge Wash Buffer 2
7. Flow Through Collection	8. Stringent Syringe Wash Buffer	9. Blocking Reagent

Labware Table

Deck Location	Labware Type
1	96AM Wash Station
2	96AM Cartridge & Tip Seating Station
3	12 Column, Low Profile Reservoir, Natural PP
4	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
5	12 Column, Low Profile Reservoir, Natural PP
6	12 Column, Low Profile Reservoir, Natural PP
7	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
8	12 Column, Low Profile Reservoir, Natural PP
9	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro



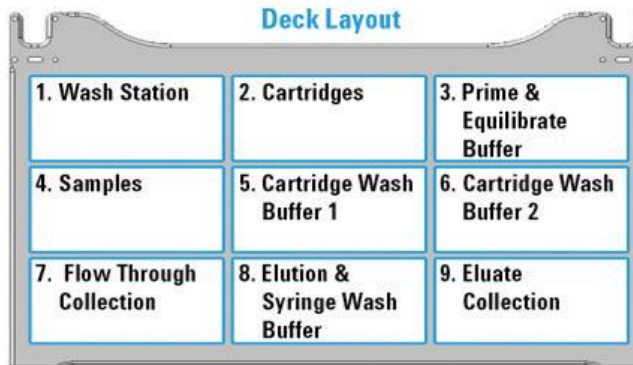
Immunoaffinity purification of monoclonal antibody from matrix

Affinity Purification: Using AssayMAP v1.0

Application Settings

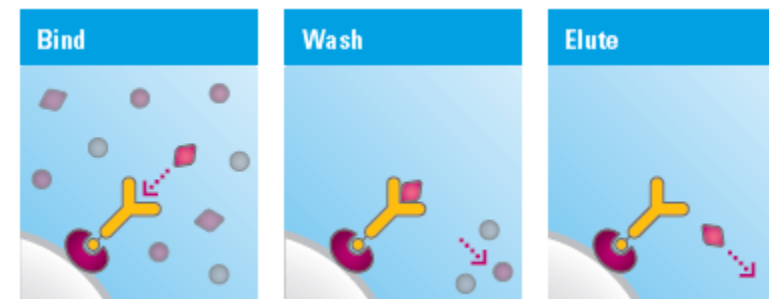
Step	Conduct Step?	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Number of Full Columns of Cartridges <input type="text" value="4"/>				
Initial Syringe Wash	<input checked="" type="checkbox"/>			<input type="text" value="3"/>
Prime	<input type="checkbox"/>	<input type="text" value="100"/>	<input type="text" value="300"/>	<input type="text" value="1"/>
Equilibrate	<input type="checkbox"/>	<input type="text" value="50"/>	<input type="text" value="10"/>	<input type="text" value="1"/>
Load Sample	<input checked="" type="checkbox"/>	<input type="text" value="100"/>	<input type="text" value="3"/>	<input type="text" value="3"/>
Collect Flow Through	<input checked="" type="checkbox"/>			
Cup Wash 1	<input checked="" type="checkbox"/>	<input type="text" value="25"/>		<input type="text" value="1"/>
Internal Cartridge Wash 1	<input checked="" type="checkbox"/>	<input type="text" value="150"/>	<input type="text" value="10"/>	<input type="text" value="3"/>
Collect Flow Through	<input type="checkbox"/>			
Cup Wash 2	<input type="checkbox"/>	<input type="text" value="25"/>		<input type="text" value="1"/>
Internal Cartridge Wash 2	<input type="checkbox"/>	<input type="text" value="50"/>	<input type="text" value="10"/>	<input type="text" value="3"/>
Collect Flow Through	<input type="checkbox"/>			
Stringent Syringe Wash	<input type="checkbox"/>	<input type="text" value="50"/>		<input type="text" value="1"/>
Elute	<input type="checkbox"/>	<input type="text" value="25"/>	<input type="text" value="5"/>	<input type="text" value="1"/>
Eluate Discard	<input type="checkbox"/>	<input type="text" value="0"/>		
Add to Flow Through	<input type="checkbox"/>			
Existing Collection Volume		<input type="text" value="0"/>		
Final Syringe Wash	<input checked="" type="checkbox"/>			<input type="text" value="3"/>

Deck Layout



Labware Table

Deck Location	Labware Type
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2	96AM Cartridge & Tip Seating Station
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4	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
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6	12 Column, Low Profile Reservoir, Natural PP
7	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
8	12 Column, Low Profile Reservoir, Natural PP
9	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro



1290 UHPLC Conditions

UHPLC: Agilent 1290 II binary pump, multisampler, thermostatic column compartment, DAD detector



LC Conditions		
Column:	PLRP-S 1000 Å, 2.1x50 mm, 5 µm	
Column Temperature:	80°C	
Injection Volume:	15 µL	
Multisampler Temp:	4°C	
Mobile Phase:	A: Water + 0.1% formic acid; B: Acetonitrile + 0.1% formic acid	
Flow Rate:	0.4 mL/min	
Gradient:	Time	%B
	0	5
	1.0	20
	6.0	50
	7.0	70
	7.1	90
	8.0	90
	8.1	5
Stop time:	10 min	

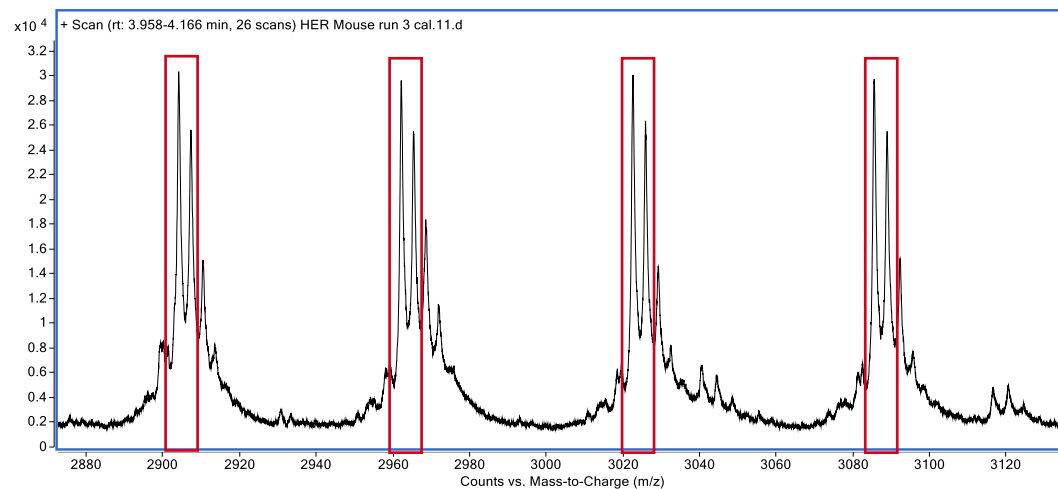
6545XT Q-TOF Method



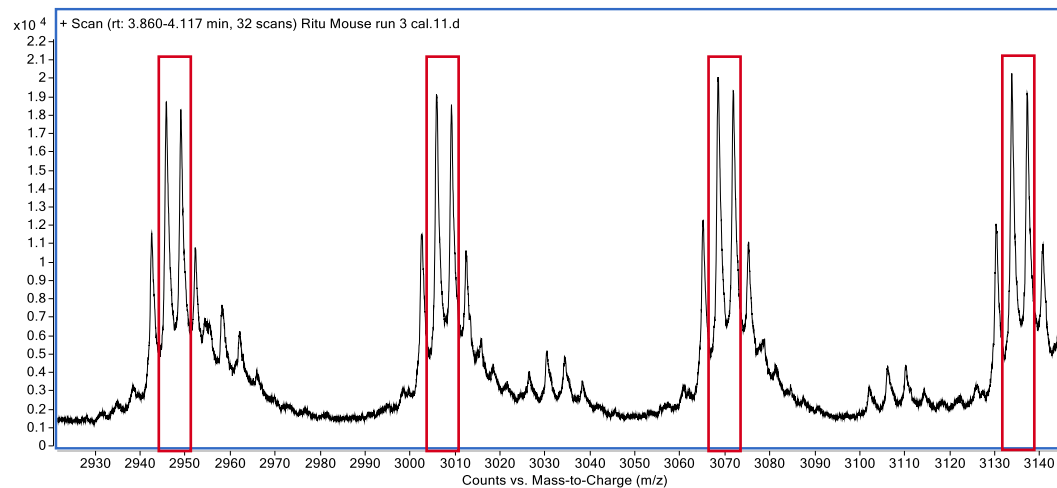
AdvanceBio 6545XT Q-TOF parameters

Ion Mode:	Positive
Source:	DAJS
Gas Temperature:	380 °C
Drying Gas Flow:	13 L/min
Nebulizer gas:	45 psig
Capillary/nozzle Voltage:	5500 V/2000 V
Fragmentor/skimmer:	380 V/140 V
Mass Range:	2000-3200 m/z
Acq. Rate:	2 spectrum/sec

Raw spectrum of trastuzumab and rituximab



trastuzumab



rituximab

Automatic peak summation for intact protein quant

Method Table

Time Segment: <All> Compound: test Reset Table View

Sample					
Name	Data File	Type	Level	Acq. Method File	Acq. Date-Time
HER cal.10	HER Mouse run...	Cal		ASMS intact_Ion...	5/8/2019 3:47 P...

Quantifier								
Name	TS	Scan	Type	MZ	Uncertainty	Extract Left m/z	Extract Right m/z	
test		2	Scan	Target	2904.1779	Relative	100.0000	100.0000 PPM

Qualifier						
MZ	Rel. Resp.	Uncertainty	Area Sum	MZ Extraction Window Units	Extract Left m/z	Extract Right m/z
3022.6194		20.0	<input checked="" type="checkbox"/>	PPM	100.0000	100.0000
2962.1881		20.0	<input checked="" type="checkbox"/>		100.0000	100.0000
2848.2966		20.0	<input checked="" type="checkbox"/>		100.0000	100.0000
2965.4243		20.0	<input checked="" type="checkbox"/>		100.0000	100.0000
3025.9258		20.0	<input checked="" type="checkbox"/>		100.0000	100.0000
2849.0716		20.0	<input checked="" type="checkbox"/>		100.0000	100.0000
2907.3015		20.0	<input checked="" type="checkbox"/>		100.0000	100.0000

Intact Protein Method Setup

Please enter Mass Range for the selected compound

2840 to 3030

Number Of Qualifiers: 3

OK Cancel

Sample Information

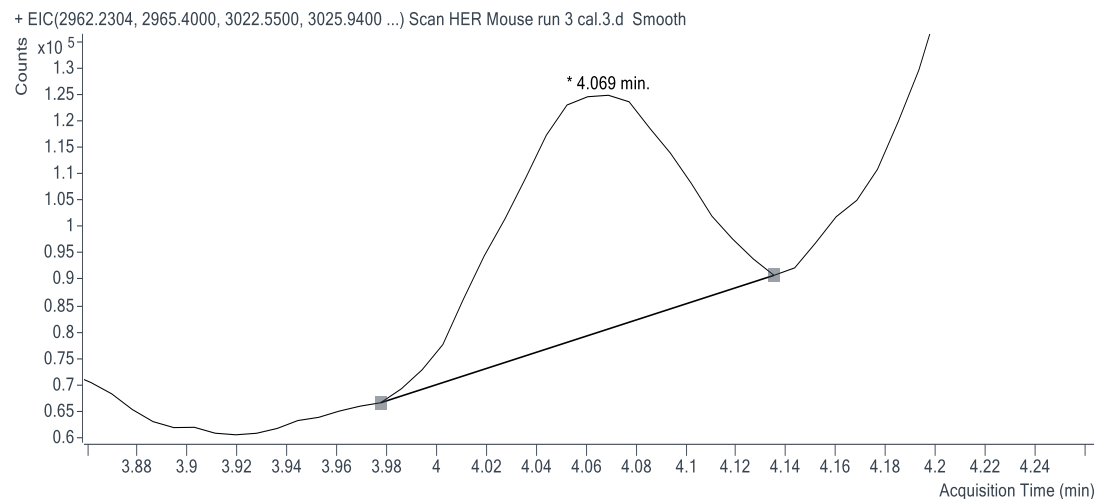
TIC Scan HER Mouse run 3 cal.10.d

Scan (rt: 3.961-4.086 min, 16 scans) HER Mouse run 3 cal.10.d

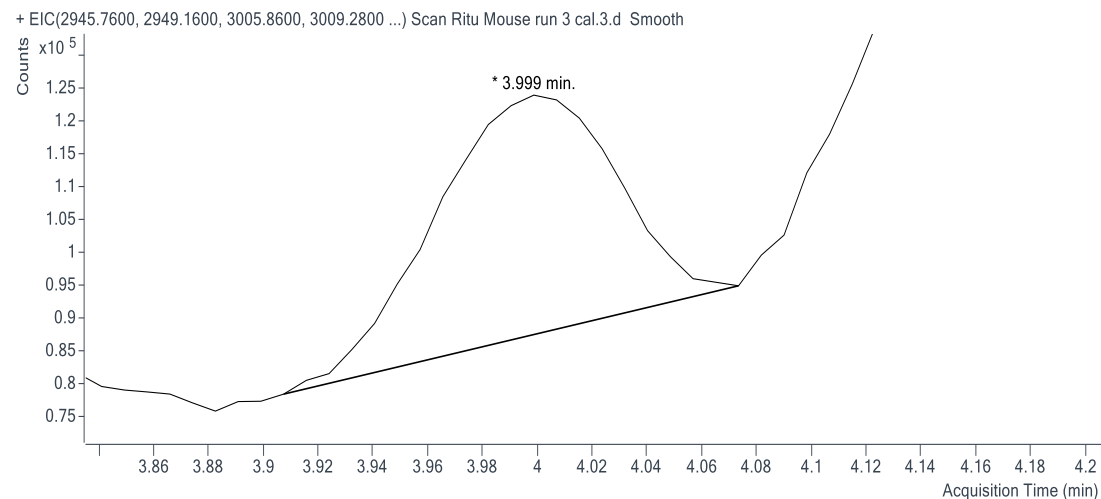
2833.3947 2848.3045 2904.0503 2962.2333 3022.6489 3040.6116

Compound Information

Extracted ion chromatogram of lowest calibration point of trastuzumab and rituximab in mouse plasma

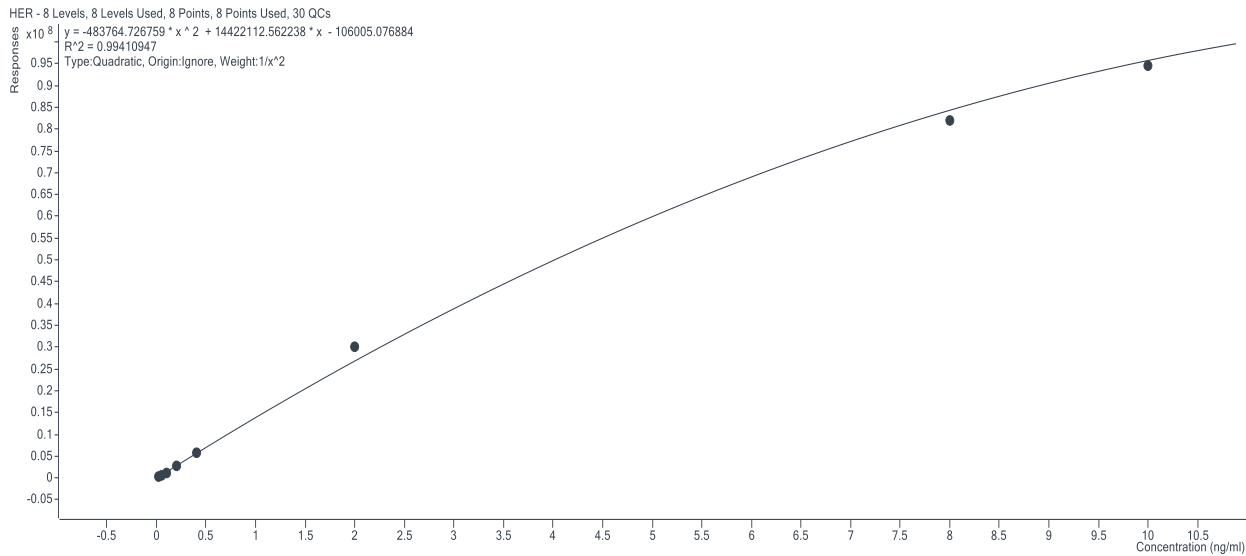


trastuzumab

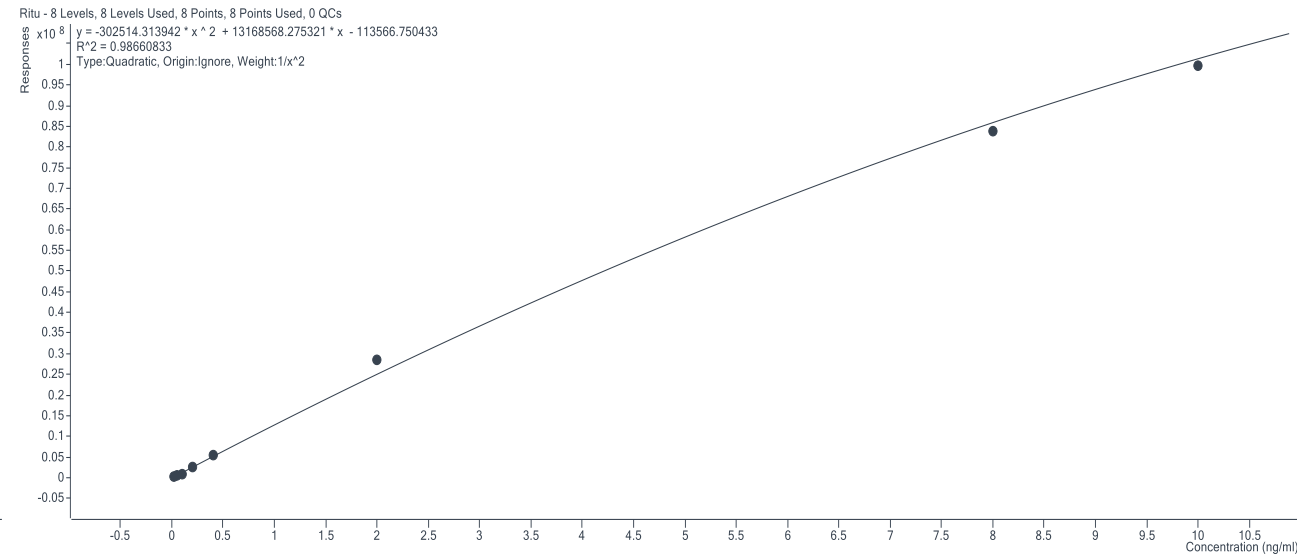


rituximab

Calibration curve of trastuzumab and rituximab from 0.02 to 10 µg/mL in mouse plasma.



trastuzumab



rituximab

Precision and accuracy of quality control samples in mouse plasma (n=6)

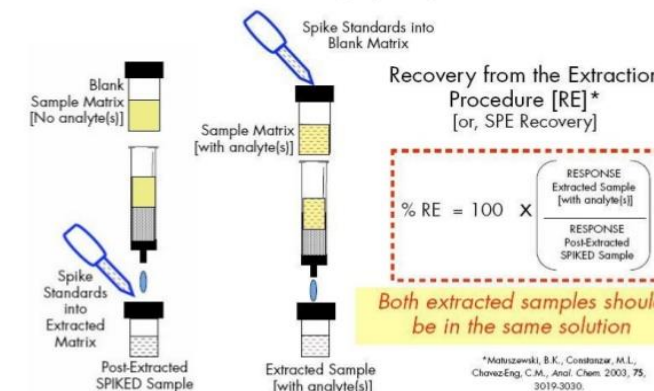
Day	Statistic	LLOQ QC (0.02 µg/mL)		Low QC (0.06 µg/mL)		Mid QC (1.0 µg/mL)		High QC (7.5 µg/mL)	
		Trastuzumab	Rituximab	Trastuzumab	Rituximab	Trastuzumab	Rituximab	Trastuzumab	Rituximab
Day 1	Mean	0.0234	0.0240	0.0519	0.0508	1.08	1.11	8.08	7.70
	% bias	17.0	20.0	-13.5	-15.3	8.0	11.0	7.8	2.7
	% CV	11.3	3.1	9.7	4.9	5.0	5.4	5.9	5.1
Day 2	Mean	0.018	0.0199	0.054	0.0568	1.19	1.19	7.85	6.97
	% bias	-10.0	-0.5	-10.0	-5.3	18.5	19	4.6	-7.1
	% CV	6.7	9.3	11.7	13.3	3.3	5	8.0	2.8
Day 3	Mean	0.0176	0.0207	0.0494	0.0540	1.03	1.18	7.42	7.78
	% bias	-12.0	3.5	-17.7	-10.0	3.4	18	-1.1	3.7
	% CV	10.2	6.5	6.2	5.3	2.4	4.3	6.4	5.2
Inter-day	Mean	0.0197	0.0215	0.0518	0.0539	1.10	1.16	7.78	7.48
	% bias	-1.7	7.7	-13.7	-10.2	10.0	16.0	3.8	-0.2
	% CV	16.5	10.1	4.4	5.6	7.0	3.8	4.3	6.0

Recovery of quality control samples in mouse plasma (n=4)

1

	Mid QC	High QC	Average
Trastuzumab	60.1	69.6	64.9
Rituximab	51.4	56.0	53.7

Extraction Recovery (RE)



The workflow achieved lower limit of quantification of 20 ng/mL by using 50 µL sample for two mAb drugs in biological matrix.

The final on column quantification limit was about 0.3 ng for both drugs after counting recovery, which is lower than current published literatures.

Summary

- The AssayMAP bravo automation platform and 6545XT AdvanceBio LC/Q-TOF system are ideal for intact protein quantification.
- The workflow demonstrated here achieved lower limit of quantification of 20 ng/mL by using 50 μ L of mouse plasma samples for two monoclonal antibody drugs.
- In three qualification runs, the intra-day and inter-day precision and accuracy of quality control samples all met regulatory acceptance criteria demonstrating excellent assay performance and reproducibility.



Contents

- 6545XT AdvanceBio Q-TOF: Designed for Biopharma
- 6545XT AdvanceBio Q-TOF: Features
- Intact Protein Analysis Performance on 6545XT AdvanceBio Q-TOF
- Intact protein quantification in biological matrix by 6545XT AdvanceBio Q-TOF
- Live demonstration of 6545XT AdvanceBio Q-TOF

Thank you

