

Analytical LC-MS Platform Methodologies to Support Upstream Bioprocess Optimization

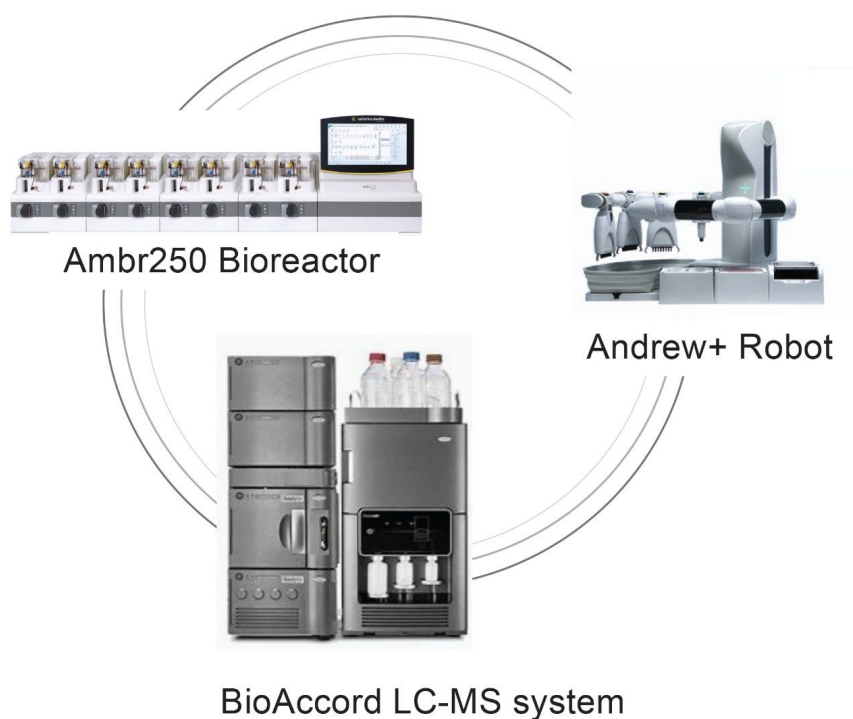
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Introduction

Effective process monitoring and product critical quality attribute analysis are critical components for a given upstream process optimization operation. It is desirable that these analyses can be carried out by multi-disciplined scientists who can easily perform the analysis in the absence of deep discipline-based understanding. For liquid chromatography-mass spectrometry (LC-MS) analysis, high throughput is expected to ensure quick turn-around time, such that the results can be fed back to the cell culture design of experiment (DOE) model for a fast design-make-test cycle.



At Waters Immerse Delaware (WID) laboratory, cross-disciplinary scientists are assembled to include cell culture engineers and traditional LC-MS analytical scientists. In proof-of-concept studies, the team conducts upstream bioprocess optimization experiments for a monoclonal antibody (mAb) producing CHO cell line using an Ambr® 250 eight modular bioreactor system. The resulting harvested cell culture fluid (HCCF) samples are subject to LC and LC-MS analysis for process and product critical attribute measurements. These include titer, aggregation and impurities determination, cell culture media components and metabolite analysis, intact protein mass determination and major glycosylation profiling, reduced protein to heavy chain for major glycosylation profiling, and released glycan analysis, among others. All these analyses are carried out using ACQUITY™ LC and/or BioAccord™ LC-MS systems. BioAccord is a high-resolution mass spectrometer system with small footprints and user-friendly instrument control and operation. This allows the instrument to fit in bioprocess labs easily and enables scientists who have minimal training in LC-MS technology to carry out analysis quickly. The WID lab is also equipped with an Andrew+™ Robot with intuitive OneLab™ software for writing automation protocols; this allows for the automation of both routine and complicated sample preparations, such as ProA purification or released glycan analysis. A summary of major instruments in WID laboratory that are involved in the present study are shown on the title page.

This application note is a compilation of optimized automation and analytical methods for ease of reference. Method optimization resulted in both reduced analysis time and enhanced operational efficiency. These include sharing mobile phases among multiple analyses and using commercially available mobile phases for minimizing preparations. Assays included are summarized in the table below:

Assay	Chromatography	Run time (min)	Mobile phases
Titer determination	Protein A affinity	3	A: DPBS B: H ₂ O/0.1%FA
Aggregation/fragmentation	Size-exclusion	4	A: DPBS
Cell culture media and metabolites analysis	Reversed-phase	9	A: H ₂ O/0.1%FA B: 90% ACN/ 10% IPA/ 0.1% FA
Intact protein analysis	Reversed-phase	5	A: H ₂ O/0.1%FA B: 90% ACN/ 10% IPA/ 0.1% FA
Reduced protein analysis	Reversed-phase	5	A: H ₂ O/0.1%FA B: 90% ACN/ 10% IPA/ 0.1% FA
Released glycan analysis	HILIC	10	A: 50 mM ammonium formate B: ACN

Table 1. summary of assays included in this application note.

Experimental

I. Sample Collection and Distribution

- In a typical DOE designed experiment in upstream process optimization, HCCF could be collected daily for a 14-day fed-batch experiment. After centrifugation and filtration, the clarified samples would then be stored in 1.4 mL tubes in 96 well format at -80 °C.
- These clarified samples are subject to analysis such as protein titer, amino acids and metabolites, and aggregation screening. For product critical attributes such as intact protein, reduced protein, released glycan and other assays, the product protein is purified based on Protein A affinity purification using Andrew+ Robots.

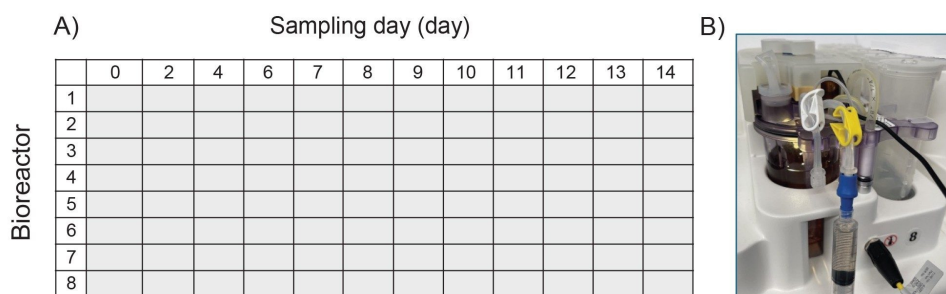


Figure 2. (A) Representative sample placement for a 14-day fed-batch experiment using Ambr 250 in a 96 well plate format. Samples are arrayed by day of harvest on the x-axis, and by bioreactor vessel on the y-axis. (B) HCCF sampling from an Ambr 250 modular system.

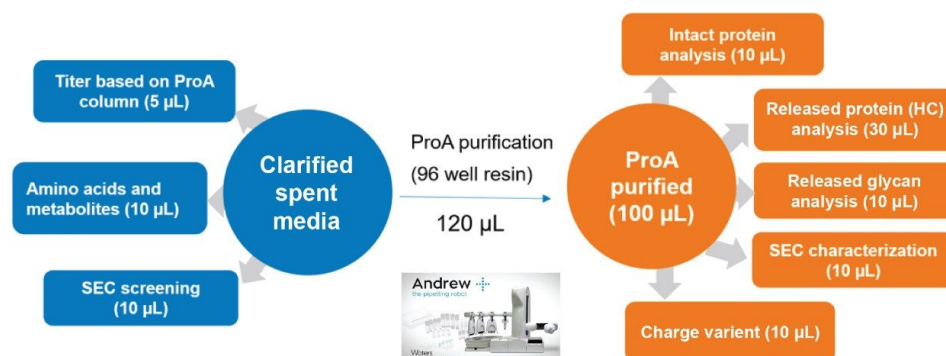


Figure 3. HCCF sample distribution volume consumed for various process and product attributes measurements. A total of 150 µL of HCCF is needed.

II. Titer Determination using Protein A Affinity Chromatography

- Protein titer measurement is based on Protein A affinity chromatography on an ACQUITY Premier LC-UV System. The run time is 3 minutes. Samples are centrifuge- and filter-clarified HCCF solutions without further preparation.

LC Method Conditions I

System:	ACQUITY Premier BSM LC-UV system	
LC conditions	BioResolve Protein A Affinity Column, MaxPeak Premier, 3.5 µm, 2.1 × 20 mm (p/n 186011369)	
	Mobile phase	(A) 1:10 dilution of DPBS (Sigma-Aldrich D1408) (B) 0.1% Formic acid
	Column temperature	Ambient temperature
	Sample temperature	6 °C
	Injection volume	1–2 µL
	UV wavelength	280 nm
	Sampling rate	80 points/sec
	Run time (gradient)	3 min
Samples	Clarified HCCF sample	
Software	waters_connect	

Gradient Table

Time (min)	Flow rate (mL/min)	Composition A(%)	Composition B(%)	Curve
0.00	0.75	100	0	initial
0.50	0.75	100	0	6
0.51	0.75	0	100	6
1.00	0.75	0	100	6
1.01	0.75	100	0	6
1.20	0.75	100	0	6
1.21	0.75	0	100	6
1.40	0.75	0	100	6
1.41	0.75	100	0	6
1.60	0.75	100	0	6
1.61	0.75	0	100	6
1.80	0.75	0	100	6
1.81	0.75	100	0	6
3.00	0.75	100	0	6

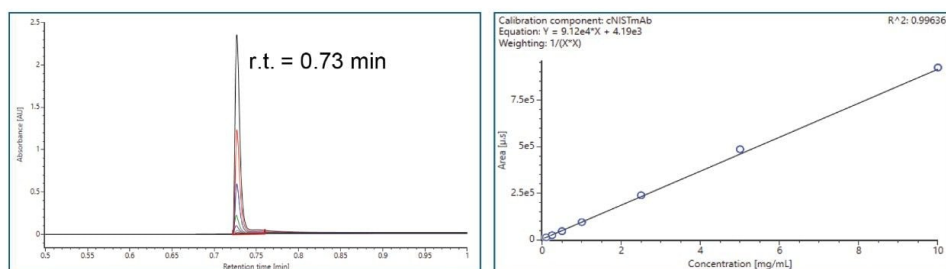


Figure 4. Overlaid chromatograms and calibration curve of NISTmAb standard solution with concentration ranging from 0.1 mg/mL to 10 mg/mL at 1 μ L injection volume, collected with LC-UV using Waters BioResolve Protein A Affinity Column.

Peak characteristics	Calibration		
Width at 50%	0.36 s	Linear range at 1 μ L inj. V.	0.1–10 g/L
Width at 10%	0.69 s	Load-on-column	0.1–10 μ g
Width at 4.4%	0.84 s	LOQ	0.1 μ g
Asymmetry at 10%	2.28	R_2	0.996
Asymmetry at 4.4%	2.59		

Table 2. Calibration range and peak characteristics of NISTmAb at 10 mg/mL (g/L).

III. Cell Culture Media and Metabolites Analysis

- Spent culture media and metabolites analyses are based on reversed-phase chromatography using Waters ACQUITY Premier HSS T3 Column on BioAccord System. Samples are centrifuge- and filter-clarified HCCF solution, diluted 20 to greater than 400 times using 0.1% formic acid (FA). Run time is 9 minutes.
- The method includes 220+ compounds library and guided workflow for ease of data processing/reporting.

LC-MS Method Conditions I

System:	BioAccord Premier LC-MS with BSM	
LC conditions	ACQUITY Premier HSS T3 1.8 μ m 2.1 $\times$ 100 mm (p/n 186009468)	
	Mobile phase	(A) H ₂ O/ 0.1% formic acid (B) 90%ACN/10%IPA/0.1% formic acid
	Column temperature	40 °C
	Sample temperature	6 °C
	Injection volume	2 μ L
	Run time (gradient)	9 min
MS Conditions	Mass range	Small molecule (50-800 <i>m/z</i>)
	Scan rate	5 Hz
	Desolvation temperature	550 °C
	Capillary voltage	1 kV (positive scan), 0.8 kV (negative scan)
	Cone voltage	20 V (positive scan), 15 V (negative scan)
Samples	Clarified HCCF sample, diluted with 0.1% FA (20 to greater than 400 times)	
Amino acid standards	Amino Acid Cell Culture Standard Kit (p/n 186009300) Calibration solution concentration: 0.05-5 μ M	
Internal standard	Positive: 5-methyltryptophan (0.1 μ M in 0.1%FA) Negative: 4-hydroxybenzoic acid (0.5 μ M in 0.1% FA)	
Salt matrix for standard preparation	Earl's Balanced Salt solution (Millipore Sigma E2888), 1000x dilution using 0.1% FA	
Software	waters_connect	

Gradient Table

Time (min)	Flow rate (mL/min)	Composition A(%)	Composition B(%)	Curve
0.0	0.35	100	0	initial
1.0	0.35	100	0	6
2.5	0.35	95	5	6
4.5	0.35	60	40	6
7.0	0.35	5	95	6
8.0	0.35	5	95	6
8.1	0.35	100	0	6
10.0	0.35	100	0	6

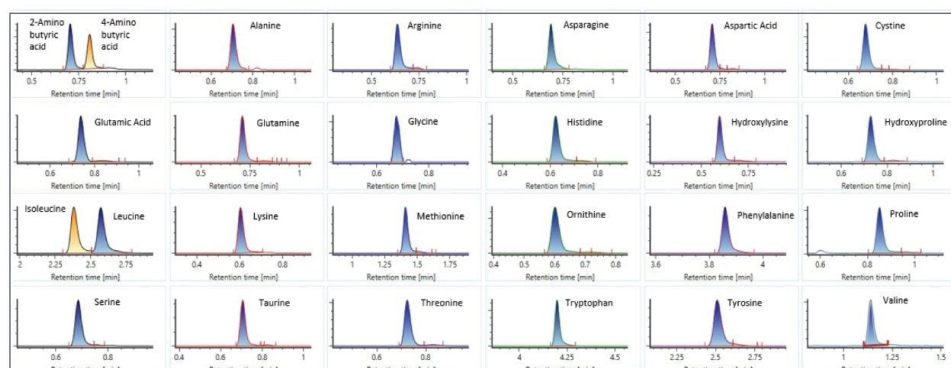


Figure 5. Extracted ion chromatogram (XIC) of 26 compounds in the amino acid cell culture standard kits. Two isobaric compound pairs, isoleucine/leucine and 2-amino/4-amino butyric acid are baseline separated.

IV. Aggregation/Fragmentation Measurement Based on SEC Column

Two methods are available for aggregate and fragment measurement using size-exclusion chromatography (SEC) on ACQUITY Premier LC-UV System.

- The screening method is used for rapid throughput with 4 minutes run time.
- The characterization method uses a longer column for purified final product when separation of fragment impurities is important.

LC-UV Conditions - Screening

System:	ACQUITY Premier BSM LC-UV system	
LC conditions	ACQUITY Premier Protein SEC, 250 Å, 1.7 µm, 4.6 × 100 mm (p/n 186011018)	
	Mobile phase (isocratic)	1:10 dilution of DPBS (Sigma-Aldrich D1408)
	Flow rate	0.5 mL/min
	Column temperature	25 °C
	Sample temperature	6 °C
	Injection volume	1 µL
	UV wavelength	280 nm
	Sampling rate	10 points/sec
	Run time	4 min
Samples	Clarified HCCF sample	
Software	waters_connect or Empower	

LC-UV Conditions - Characterization

System:	ACQUITY Premier BSM LC-UV system	
LC conditions	ACQUITY Premier Protein SEC Column, 250 Å, 1.7 µm, 4.6 × 300 mm (p/n 186009964)	
	Mobile phase (isocratic)	1:10 dilution of DPBS (Sigma-Aldrich D1408)
	Flow rate	0.3 mL/min
	Column temperature	25 °C
	Sample temperature	6 °C
	Injection volume	1 µL
	UV wavelength	280 nm
	Sampling rate	20 points /sec
	Run time	15 min
Samples	Protein A affinity purified sample	
Software	waters_connect	

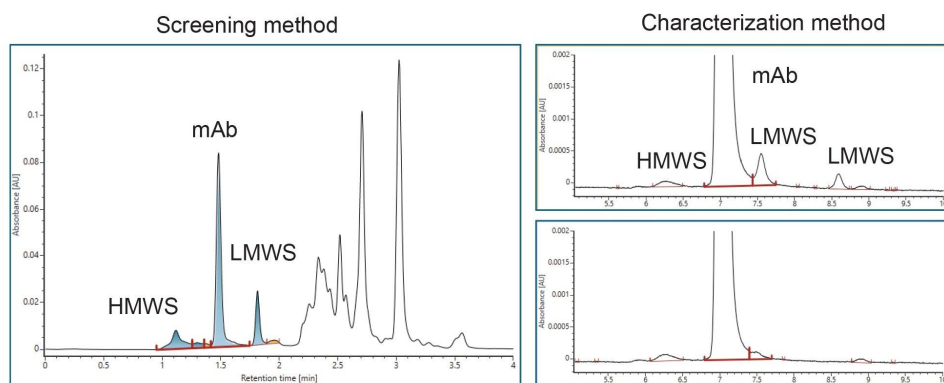


Figure 6. (A) Raw spent media sample from mAb producing CHO-cell line using screening method. (B) (Top) mAb size variant standard (p/n 186009429), (bottom) NISTmAb (RM8671 ext. NIST.gov) using characterization method.

V. Intact Protein Analysis

- Intact protein analysis uses ACQUITY Premier Protein BEH C4 Column on BioAccord HRMS System. Run time is 5 minutes.
- Clarified HCCF media samples or Protein A purified samples using Andrew+ Pipetting Robot.

LC-MS Method Conditions II (Protein Analysis)

System:	BioAccord Premier BSM HRMS system	
LC conditions	ACQUITY Premier Protein BEH C ₄ 300 Å 1.7 µm, 2.1 × 50 mm (p/n 186010326)	
	Mobile Phase	(A) H ₂ O/0.1% formic acid (B) 90%ACN/10%IPA/0.1% formic acid
	Column Temperature	80 °C
	Sample Temperature	6 °C
	Injection volume	5 µL
	Run time (gradient)	5 min
MS conditions	Mass range	High (400–7000 <i>m/z</i>), Positive scan
	Scan rate	2 Hz
	Desolvation temperature	550 °C
	Capillary voltage	1.5 kV
	Cone voltage	70 V
Samples	(A) Protein A purified sample is preferred, with 1:10 dilution of sample containing IgG at ~1.5 g/L concentration. (B) Non-purified, clarified HCCF sample can also be analyzed.	
Software	waters_connect, Intact Mass	

Gradient Table

Time (min)	Flow rate (mL/min)	Composition A(%)	Composition B(%)	Curve
0.0	0.4	95	5	initial
0.5	0.4	95	5	6
1.0	0.4	85	15	6
3.5	0.4	15	85	6
3.7	0.4	5	95	6
4.3	0.4	5	95	6
4.5	0.4	95	5	6
5.0	0.4	95	5	6

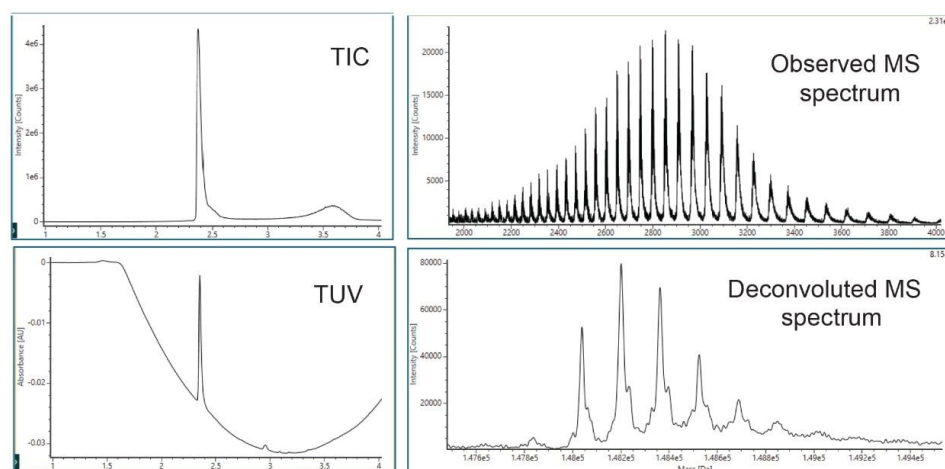


Figure 7. (Left) Total ion chromatogram and TUV trace of NISTmAb as 1 mg/ mL solution with 2 μ L injection volume. (Right) Observed and deconvoluted MS spectra.

VI. Reduced Protein Analysis via LC and HC (DTT Reduction)

- Reduced protein analysis uses ACQUITY Premier Protein BEH C4 Column on BioAccord System. Run time is 5 minutes.
- Sample is prepared using guanidine denaturation and DTT reduction to produce light chain (LC) and heavy chain (HC).

LC-MS Method Conditions III (DTT Reduction)

System:	BioAccord Premier BSM HRMS system	
LC conditions	ACQUITY Premier Protein BEH C ₄ 300 Å 1.7 µm, 2.1 × 50 mm (p/n 186010326)	
	Mobile phase:	(A) H ₂ O/0.1% formic acid (B) 90%ACN/10%IPA/0.1% formic acid
	Column temperature	60 °C
	Sample temperature	6 °C
	Injection volume	2 µL
	TUV	260 nm
	Run time (gradient)	5 min
MS conditions	Mass range	High (400–7000 <i>m/z</i>), Positive scan
	Scan rate	2 Hz
	Desolvation temperature	450 °C
	Capillary voltage	1.2 kV
	Cone voltage	30 V
Samples	Combine DTT, Guanidine HCl, and mAb, incubate at 60 °C for 30 min.	
Software	waters_connect, Intact Mass	

Gradient Table

Time (min)	Flow rate (mL/min)	Composition A(%)	Composition B(%)	Curve
0.0	0.4	95	5	initial
0.5	0.4	95	5	6
1.0	0.4	75	25	6
3.5	0.4	65	35	6
3.7	0.4	10	90	6
4.3	0.4	10	90	6
4.5	0.4	95	5	6
5.0	0.4	95	5	6

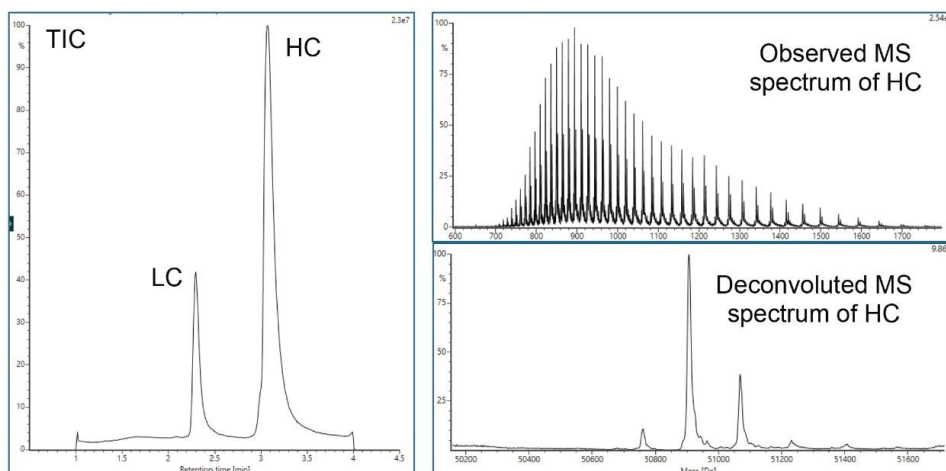


Figure 8. (Left) Total ion chromatogram of NISTmAb after DTT reduction to form heavy chain (HC) and light chain (LC). (Right) observed and deconvoluted spectra of heavy chain.

VII Released Glycan Analysis

- Released glycan analysis is based on HILIC chromatography using BioAccord System. Run time is 10 minutes. Samples are prepared using Waters GlycoWorks™ RapiFluor-MS™ released N-Glycan Kits using Andrew+ Pipetting Robots.

LC-MS Method Conditions IV (Glycan Analysis)

System:	BioAccord Premier BSM HRMS system	
LC conditions	ACQUITY Premier Glycan BEH Amide 130 Å 1.7 µm 2.1 × 50 mm (p/n 186009522)	
	Mobile phase	(A) Ammonium formate solution pH 4.4- glycan analysis (p/n 186007081) (B) ACN (Acetonitrile)
	Column temperature	60 °C
	Sample temperature	6 °C
	Injection volume	1 µL
	TUV	265 nm
	Run time (gradient)	10 min
MS conditions	Mass range	Low (50–2000 <i>m/z</i>), Positive scan
	Scan rate	5 Hz
	Desolvation temperature	550 °C
	Capillary voltage	1.2 kV
	Cone voltage	35 V
Samples	Protein A purification followed by glycan release using GlycoWorks <i>Rapi</i> Fluor-MS N-Glycan Eco Starter Kit-96 Samples (p/n 176005285)	
Software	waters_connect software	

Gradient Table

Time (min)	Flow rate (mL/min)	Composition A(%)	Composition B(%)	Curve
0.0	0.7	25	75	initial
7.0	0.7	40	60	6
7.1	0.7	60	40	6
7.5	0.7	60	40	6
7.6	0.7	25	75	6
10.0	0.7	25	75	6

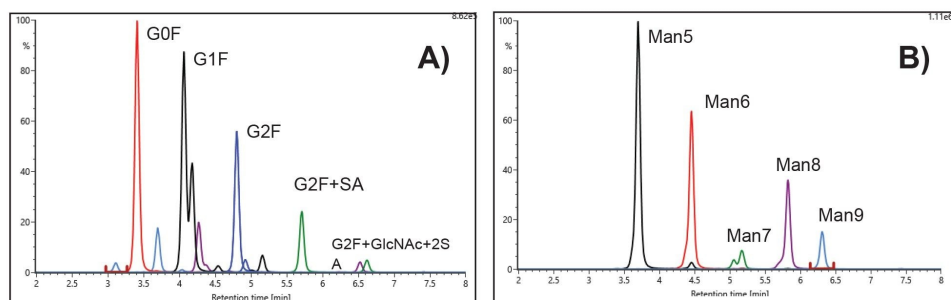


Figure 9. (A) Extracted ion chromatogram of RapiFluor-MS Glycan Performance Test Standard (p/n 186007983). (B) Extracted ion chromatogram of RapiFluor-MS high Mannose Standard (p/n 186008317).

VIII. Protein A Purification using Andrew+ Robot Supply Guide

- Dominos, supplies, and consumables used in Protein A purification on Andrew+ Robot is summarized in tables below. More detailed information on the purification can be found in Waters application note "Analytical Scale 96-well Protein A Affinity Resin-Based Purification using Andrew+ Automation Robot Supporting Upstream Bioprocessing".

List of equipment, dominos and consumables	Quantity	Description	Part number
Equipment and Andrew+ Domino			
Extraction+	1	Applies vacuum to filter plate	176005201
Plate Manifold Spacers	2	One 15mm and one 17mm spacer to raise Quan Recovery plate to the filter plate	186010527
Tip insertion system Domino	2	At least 136 tips (300 µL) distributed between two dominos arrayed in columns	186009612
Deepwell microplate Domino	1	Holds the 12-channel reservoir	186009597
Storage plate Domino	1	Holds the QuanRecovery plate	186009596
Microplate Domino	1	Holds the 96-round well collection plate	186009600
Microplate gripper	1	Moves the QuanRecovery plate	186009776
Pipette 8 ch-300 µL	1	Pipettor which pipettes into one 8-well column at a time	186009607
Eppendorf thermomixer C	1	ProA resin mixing	Eppendorf 5382000023
Eppendorf SmartBlock for microplates and deepwell plates	1	Holds filter plate	Eppendorf 5363000039
Consumables			
Axygen 12-channel trough	1	Holds the five reagents listed in table below	Corning RES-MW12-HP
AcroPrep advanced 350 µL 96-well filter plate 10K membrane	1	For ProA affinity capturing	Pall or Cytiva 8019
Waters QuanRecovery 700 µL 96 well plate	1	Receives samples for released mAb from ProA resin	186009184
300 µL pipette tips	2 boxes	For use by 8-channel pipettor	700013297 refill
Waters 350 µL 96-round well collection plate	1	Holds 96 HCCF samples containing IgG mAb to be purified	186002643

Table 3. Dominos, supplies, and consumables used in Protein A purification on Andrew+ Robot.

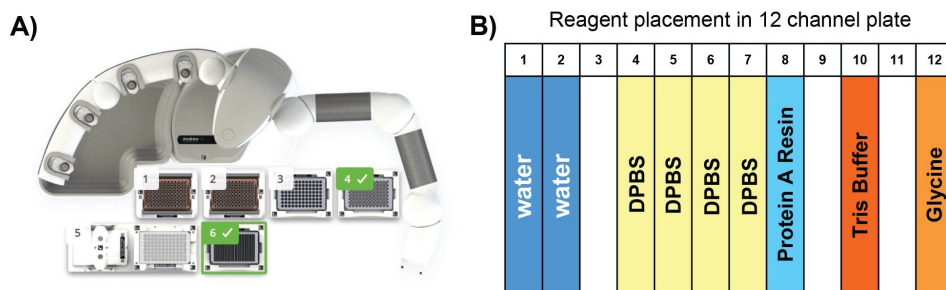


Figure 10. (A) Placement of Andrew+ dominos for the ProA affinity purification of 96 samples. Position 1&2, 300 μ L pipette tips; 3, Quanrecovery plate; 4, HCCF sample; 5, Extraction+ with filter plate; and 6, reagent trough. Users should follow OneLab screen instruction for the final placement. (B) Dialog box for entering number of samples upon execution of the protocol.

Reagents for 96 samples	Well location	Volume need for 96 samples	Description
Water	A1-A2	2 $\times$ 16 mL	Resin washing
Binding/wash buffer (1 $\times$ PBS)	A4-A7	4 $\times$ 16 mL	Resin conditioning (Sigma-Aldrich DPBS p/n D1408)
ProA Resin	A8	7 mL	mAb affinity capture (Cytiva, p/n 17519901)
Neutralization buffer	A10	2 mL	1M Tris buffer, pH 7.5 (Invitrogen, p/n 15567-027)
Elution Buffer	A12	10 mL	100 mM glycine, pH 3.0 (Sigma-Aldrich, p/n G7126)

Table 4. Reagents and their placement on Andrew+ Robot for Protein A purification.

Reagents and their preparation for Protein A purification on Andrew+ Robot.

1. Binding/wash buffer: Source Millipore Sigma 10x DPBS, dilute to 1x DPBS using H₂O.
2. ProA resin: The resin comes as 50% resin in 20% ethanol: Follow Cytiva's instruction, centrifuge at 1000 g for 3 minutes, replace supernatant with 400 mM NaCl in 20% ethanol to 50% resin level. Further dilute with 1xDPBS to 25% resin as working solution. Thoroughly mix prior to transfer to reagent trough. After

each use, mark the liquid level on the tube. In next use, if liquid level is reduced due to evaporation, fill to mark with 20% ethanol.

3. Elution buffer: 100 mM glycine, pH adjusted to 3.0

4. Neutralization buffer: 1M Tris-HCl, pH 7.5

IX. JMP Add-in for Data Visualization and Analysis

- In JMP marketplace place, there is a Waters add-in available for download, free-of-charge. The add-in enables data import, organize and display based on analyte class or biotransformation pathways. One example is shown below displaying overlaid plot of amino acids in 8 bioreactors over 14 days of incubation.

Waters

Bioprocess Monitor

A tool for Bioprocess Engineers to visualize and analyse in-process analytics data in upstream process development with Waters Bioprocess Walk-up Solutions

Data Analysis, Data Visualization

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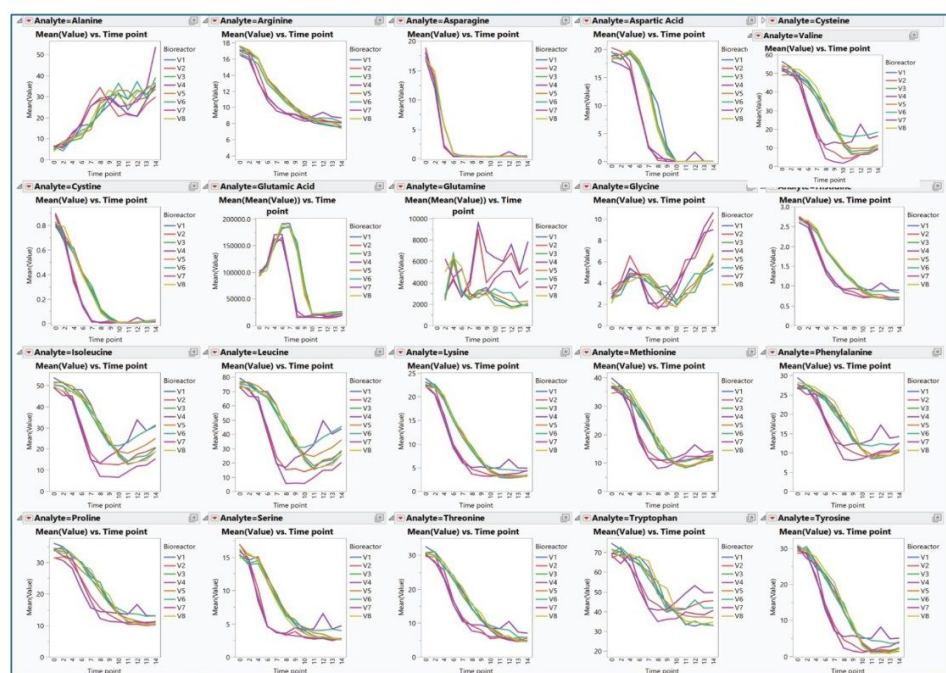


Figure 11. Cell culture media and metabolite analysis of an incubation using NISTCHO cell line (RM8675 NIST.gov) showing trending plot of amino acid concentration changes overtime during a 14-day, eight bioreactor Ambr 250 experiment.

References

1. Titer determination based on Protein A affinity chromatography
 - BioResolve Protein A Affinity Columns Care & Use Manual (p/n: 720008817)
 - Lowering Quantitation Limits for mAb Titer Measurements Using Small Volume 3.5 µm Particle-Size Protein-A Affinity Columns (p/n: 720008775)
2. Aggregation/fragmentation analysis
 - ACQUITY Premier Protein SEC 250 A, 1.7 µm columns Use and Care Manual (p/n: 720007477)
3. Cell culture and metabolites analysis
 - Monitoring Nutrients and Metabolites in Spent Cell Culture Media for Bioprocess Development Using the BioAccord LC-MS System with ACQUITY Premier (p/n: 720007359)
 - Introducing a Rapid Throughput LC-MS Method for Cell Culture Media Nutrient and Metabolite Analysis Supporting Upstream Bioprocessing (p/n: 720008170)
4. Intact protein analysis
 - ACQUITY UPLC and ACQUITY Premier Protein BEH C4, 300 Å Columns Care and Use Manual (p/n: 715001870)
5. Released glycan analysis
 - GlycoWorks RapiFluo-MS N-Glycan Kit—24 Sample Care and Use Manual (p/n: 715004903)
 - Rapid Preparation of Released N Glycans for HILIC Analysis Using a Labeling Reagent that Facilitates Sensitive Fluorescence and ESI-MS Detection, Anal. Chem. 2015, 87, 5401–5409 DOI: 10.1021/acs.analchem.5b00758
6. Protein A purification
 - Analytical Scale 96-well Protein A Affinity Resin-Based Purification using Andrew+ Automation Robot Supporting Upstream Bioprocessing, Waters application note 720009002
 - Automated High-Throughput Analytical-Scale Monoclonal Antibody Purification Using Production-Scale Protein A Affinity Chromatography Resin, Waters application note 720007861.
7. JMP data visualization, MVA analysis
 - Transforming BioAccord LC-MS Quality Attribute & Cell Culture Analysis with JMP Workflows (in layout)

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ACQUITY Premier System <

<https://www.waters.com/nextgen/global/products/chromatography/chromatography-systems/acquity-premier-system.html>>

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waters_connect for Quantitation <https://www.waters.com/nextgen/global/products/informatics-and-software/waters_connect-for-quantitation.html>

Empower Chromatography Data System (CDS) <<https://www.waters.com/nextgen/global/products/informatics-and-software/chromatography-software/empower-software-solutions/empower-cds.html>>

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