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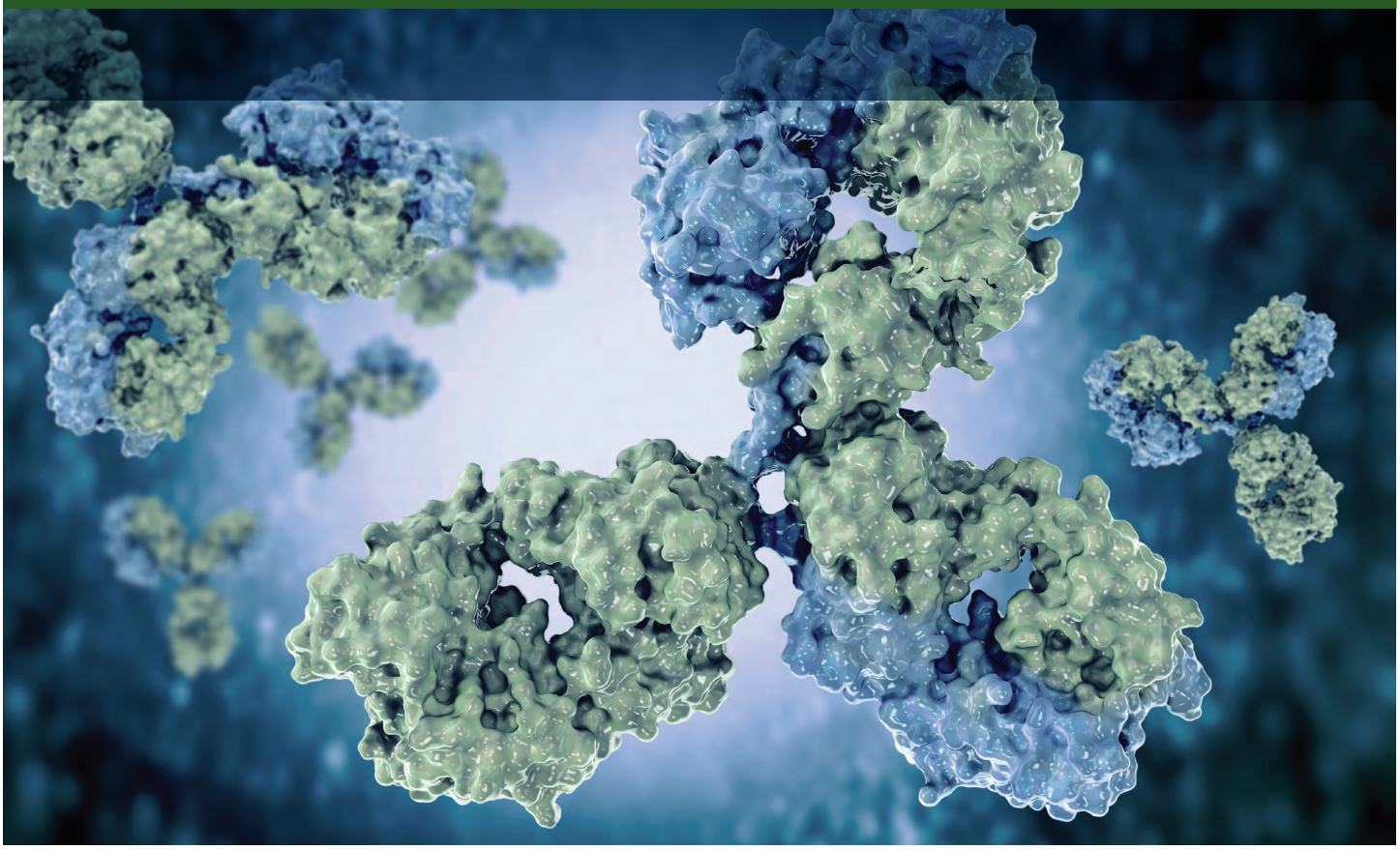
Agilent-NISTmAb

Charge Variant Analysis

Agilent BioHPLC Columns
Application Compendium



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Agilent-NISTmAb Standard (P/N 5191-5744; 5191-5745) was aliquoted from NISTmAb RM 8671 batch. Quality control (QC) testing is performed using Agilent LC-MS system. QC batch release test includes aggregate profile, charge variants and intact mass information. A certificate of analysis (CoA) can be found in each product shipment with test results.

Please note that authors used various monoclonal antibodies including Agilent-NISTmAb Standard and NISTmAb RM 8671 to demonstrate critical quality attribute workflows.

Charge Variant Analysis

Introduction

The presence of positively charged and negatively charged amino acids and negatively charged glycans (sialic acids) means that large proteins exist as multiply charged species and there are several side reactions that can result in a change in the net charge. Understanding which amino acids or glycans are involved and their specific location within a large biotherapeutic protein is of paramount importance. Ion exchange chromatography can enable the separation of some charge variants, particularly those positioned on the surface of the protein. Charge heterogeneity of mAbs comes from various modifications such as oxidation, deamidation, aspartic acid isomerization, glycan modifications, lysine truncation, and other post-translational modifications (PTMs). Variants within the antigen binding region of an antibody are likely to have a more profound effect on function. It is essential to characterize these mAb variants to understand the purity of mAbs for development and administration of a therapeutic agent.

Since most proteins contain more basic amino acids than acidic amino acids, most charge variant separations will require cation exchange. However, every protein is different and finding the conditions to deliver the best resolution will likely require considerable method optimization. Strong cation exchange columns are often easier to work with, however for monoclonal antibodies a weak cation exchange column may be an optimal choice to achieve the desired resolution. Nonetheless, separating a mAb molecule with a net charge of +50 from a variant that is +49 or +51 is still a considerable challenge. Elimination of pore structure and therefore pore diffusion by using nonporous particles goes some way to improving peak shape and gaining resolution. It is often necessary to revert to weak cation exchange columns and to perform extensive method optimization to determine the most appropriate conditions for a protein molecule. Agilent Bio Mab HPLC columns features rigid non-porous particles with hydrophilic, polymeric coating where a highly uniform densely packed, weak cation-exchange layer is chemically bonded designed ideally for mAb charge variant analysis. These columns offer superior performance promoting higher resolution and faster separations eliminating most non-specific interactions.

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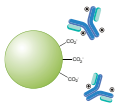

Charge Variant Analysis

Ion exchange chromatography

Enhances the accuracy and speed of biomolecule characterization

Bio MAb

Ideal for monoclonal antibodies

Attribute	Advantage
Rigid, non-porous particles	High-efficiency separations
Hydrophilic, polymeric layer	Eliminates non-specific binding
High density WCX chemistry	High ion exchange capacity ideal for MAbs

Bio IEX

Ideal for proteins and peptides

Attribute	Advantage
Rigid particles with hydrophilic coating	Eliminates non-specific binding
Strong/weak anion, cation chemistries	A column for every separation

Ion-exchange chromatography is a widely used method for separating biomolecules based on differences in ionic charge. It is a mild, non-denaturing technique that does not require organic solvents and is therefore frequently used for characterization of proteins in their native or active form, and for purification. Acidic groups include C-terminal carboxylic acids, acidic side chains of aspartic and glutamic acid, and acidic groups arising from sialic acid in glycosylated proteins; basic groups include N-terminal amines and basic side chains of arginine, lysine, and histidine. The overall charge of the molecule is therefore dependent on the pH of the surrounding solution and this in turn will affect the ion-exchange method that can be used. The isoelectric point, pI, is the pH at which the net charge of the protein is neutral (the number of positive charges is equal to the number of negative charges). If the pH is below this value, the protein will possess an overall positive charge and can be retained on a negatively charged cation-exchange sorbent; if the pH is above the pI, the protein will be negatively charged overall and can be retained on an anion-exchange sorbent.

Before beginning method development, it is crucial to determine the isoelectric point, or pI, of the target protein. If the pH of initial mobile phase conditions is too close to the pI of the protein, the protein will not be retained on the column. Depending on how widely the pI of the charge variants differs, the pH may need to be a minimum of 0.5 to 2 pH units away from the isoelectric point of the main species. Proteins may be eluted by either a salt gradient (using high ionic strength to disrupt protein adsorption to the column) or a pH gradient (proteins elute when the pH equals the pI).

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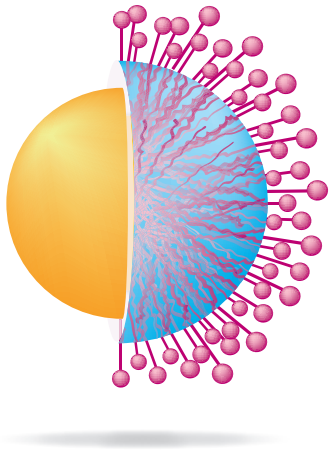
Agilent Bio MAb HPLC columns: superior performance from the inside out

- Particles, coating, and bonding are resistant to high pressures, promoting higher resolution and faster separations
- Hydrophilic coating eliminates most nonspecific interactions
- A highly uniform, densely packed, weak cation-exchange (WCX) layer chemically bonded to the hydrophilic, polymeric coating



It is worthwhile considering an instrument that allows screening of several different columns during method development. It is difficult to predict the outcome of small changes to method conditions such as ionic strength and pH; both factors will influence the net charge on the protein and, in the case of weak ion exchange columns, the net charge on the column too. A rigorous "Quality by Design" approach is recommended. Software to develop a matrix or systematic design of experiments is advisable. Agilent Buffer advisor software that can utilize the quaternary HPLC pump capabilities of an Agilent 1260 Infinity II Bio-inert LC can save considerable method development time. The featured SOP manual in this section illustrates how to use buffer advisor software to test a range of mobile phase conditions and optimized method development parameters for charge variant analysis of NISTmAb (RM 8671). When the optimum conditions for separation require very low ionic strength buffers and pH levels at the extreme limits of the buffering range then PEEK columns may also be advisable. Like size exclusion chromatography, ion exchange conditions are typically nondenaturing; the separation is conducted on the intact, native proteins. This means that the method is not MS compatible unless combined as the first dimension in a 2D-LC setup. However, quantification can be achieved by UV or fluorescence detection. In recent trend, with the use of volatile salt buffers or dual volatile pH gradients, both chromatographic separation of charge variants species and direct native mass spec detection are possible.

The technique of ion-exchange is therefore suitable for separating proteins with differing isoelectric points, but it is equally valuable in separating charged isoforms of a single protein. In the increasingly important field of biopharmaceuticals, where proteins are manufactured through bioengineering and isolated from fermentation reactions, it is important to identify charged isoforms as these indicate a difference in primary structure of the protein. A difference in primary structure could indicate a change in glycosylation, or degradation pathways such as loss of C-terminal residues or amidation/ deamidation. These protein modifications can likely result in loss in stability or activity and could potentially lead to immunologically adverse reactions. Ion-exchange is used to separate and quantify charge variants during the development process and for quality control and quality assurance during manufacture of biotherapeutics. With large molecules such as monoclonal antibodies (mAbs) it is also important to consider the size and structure of the molecule, particularly as the chromatographic interactions will only occur with surface charges.

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Quick Reference Guide

Cation Exchange Chromatography Workflow

Agilent Bio IEX HPLC Columns
Agilent Bio MAB HPLC Columns

In this document Agilent applications chemists share their recommendations for an optimum LC system and its confi guration for characterizing biomolecules. They also offer guidance on a generic method to get you started, and how this method can be further optimized to meet your specifi c separation goals. Additional application information is available at **www.agilent.com/chem/advancebio**.

Guidelines

- Basic proteins: SCX or WCX
- Consider the isoelectric point (pI) of your protein when choosing the pH of the mobile phase. If pH<pI, your protein will have a net positive charge.
- The pH of the starting buffer should be 0.5 to 1 pH unit from the pI (below pI for cation-exchange)
- If pI is unknown, start with pH 6 for cation-exchange
- Start with SCX columns, which have the widest operating range, WCX can be used to provide a difference in selectivity.
- Buffers for cation-exchange (pH 4 to7 include formate, acetate, MES, phosphate, HEPS

Column Selection

Bonded Phase
SCX (strong cation-exchange) – SO_3H
WCX (weak cation-exchange) – COOH

Samples	Column
Monoclonal antibody	Bio MAb
Peptides and proteins	Bio SCX and WCX
Globular proteins and peptides	PL-SCX 1000Å
Very large biomolecules/ high speed	PL-SCX 4000Å
Proteins, antibodies	Bio-Monolith SO3

Note: For Bio IEX and Bio MAb stainless steel HPLC columns part number, see Agilent BioHPLC Column Selection Guide, 5994-0974EN

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Agilent 1260 Infinity Bio-Inert LC System

Mobile phases

Mobile phase should contain buffer to maintain the desired operating pH, typically 20 mM.
Elution salt is typically 400 to 500 mM.

Agilent Buffer Advisor is used to develop the necessary gradient profile by mixing different proportions from the four stock solutions

Sample injection

1 to 10 µL injection for maximum resolution.
Sample must be soluble in the mobile phase.

Flow rate

Typical flow rate for 4.6 mm id columns is 0.5 to 1.0 mL/min..

Column temperature

Maximum limit 80 °C. Column lifetime is optimized when used between 10 to 50 °C.

Detection

UV, G1315D with a 10 mm bio-inert standard flow cell.

Column Selection

Description	Bio IEX HPLC Columns, PEEK Bio SCX	Bio WCX	Bio MAb HPLC Columns, PEEK
	Part No.	Part No.	Part No.
4.6 x 250 mm, 10 µm	5190-2435	5190-2455	5190-2415
4.6 x 50 mm, 10 µm	5190-2436	5190-2456	5190-2416
4.6 x 250 mm, 5 µm	5190-2427	5190-2447	5190-2407
4.6 x 50 mm, 5 µm	5190-2428	5190-2448	5190-2408
2.1 x 250 mm, 10 µm	5190-2439	5190-2459	5190-2419
2.1 x 50 mm, 10 µm	5190-2440	5190-2460	5190-2420
2.1 x 250 mm, 5 µm	5190-2431	5190-2451	5190-2411
2.1 x 50 mm, 5 µm	5190-2432	5190-2452	5190-2412

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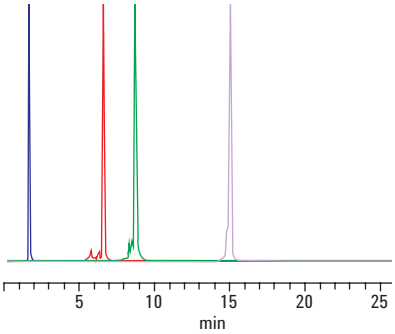
Recommended initial conditions

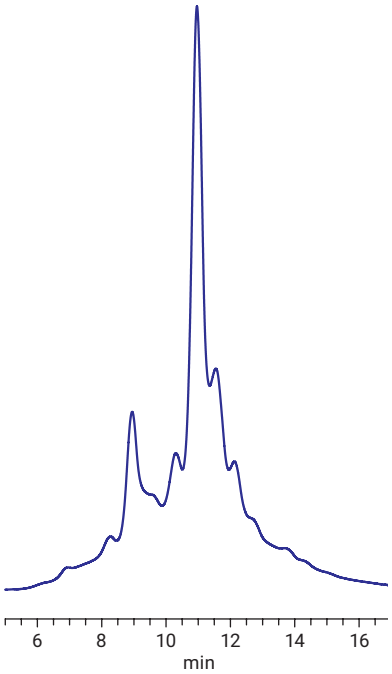
	Monoclonal antibodies		Monoclonal antibodies, Proteins and peptides
	Salt Gradient	**pH Gradient**	**Salt Gradient**
Columns	Bio WCX, 4.6 x 250 mm, 10 µm Bio WCX, 4.6 x 250 mm, 5 µm	Bio MAb, 4.6 x 250 mm, 5 µm	Bio SCX, 4.6 x 50 mm, 3 µm WCX, 4.6 x 50 mm, 3 µm Bio MAb, 4.6 x 50 mm, 3 µm
Mobile Phase	A: Water B: 1.6 M NaCl C: 40.0 mM NaH_2PO_4 D: 40.0 mM Na_2HPO_4 By combining predetermined proportions of C and D, 20 mM buffer solutions at the desired pH range are produced.	A: Water B: 1.6 M NaCl C: 40.0 mM NaH_2PO_4 D: 40.0 mM Na_2HPO_4 By combining predetermined proportions of C and D, buffer solutions at the desired pH range are produced at the selected buffer strengths.	A: 20 mM sodium phosphate, pH 5.0 for WCX or pH 6.0 for SCX B: Buffer A + 1 mM NaCl
Gradient	0 to 50% B, 0 to 20 min (constant pH, for example, pH 6.0) 50% B, 20 to 25 min 0% B, 25 to 35 min	pH 6.0 to 8.0, 0 to 20 min 0 to 800 mM NaCl, 20 to 25 min 800 mM NaCl, 25 to 30 min	1 to 100% B in 30 min for 50 mm columns, 60 min for 250 mm columns
Flow rate	1 mL/min	1 mL/min	0.5 mL/min
Temperature	Ambient	Ambient	Ambient
Injection	10 µL	10 µL	10 µL
Sample	2 mg/mL (in 20 mM sodium phosphate buffer, pH 6.0)	2 mg/mL (in 20 mM sodium phosphate buffer, pH 6.0)	
Detection	UV, 220 nm	UV, 220 nm	UV, 220 nm

Separation of protein standards at pH 7.0 using an Agilent Bio WCX, 4.6 × 250 mm, 10 µm column.

Ovalbumin (pI 4.5)
Ribonuclease (pI 9.4)
Cytochrome C (pI 9.8)
Lysozyme (pI 11)

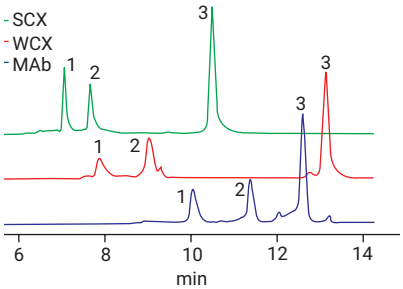


Analysis of a IgG monoclonal antibody using a pH gradient of 6.5 to 7.5 (0-20 min), 50 mM, Agilent Bio MAb, 4.6 x 50 mm, 5 µm



Separation of protein standards on Agilent 3 µm ion-exchange columns by cation-exchange chromatography

Ribonuclease (pI 9.4)
Cytochrome C (pI 9.8)
Lysozyme (pI 11)



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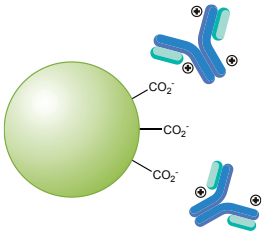
Charge Variant analysis manual for NISTmAb



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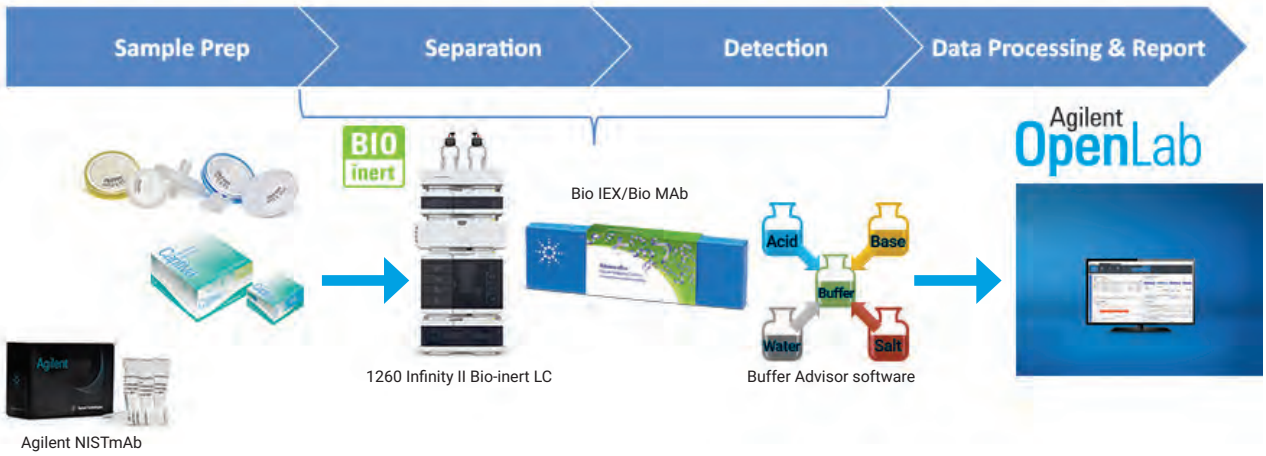
Charge Variant Analysis - Solution Description



Solution details	
Technology	Liquid Chromatography
Chromatography	Ion exchange chromatography (IEX)
Sample	Agilent-NISTmAb Standard (p/n 5191-5744)
LC	Agilent 1260 Infinity II Bio-inert LC system
Column	Agilent Bio MAb, nonporous, 2.1 × 250 mm, 5 µm, PEEK (p/n 5190-2411)
Detector:	DAD WR with Bio-inert standard flow cell
Software:	Agilent OpenLAB CDS 2.3; Buffer Advisor A.01.01 (009)

Scope

- Operationalize the charge variant analysis of NISTmAb using Ion – Exchange Chromatography using this SOP.

Background

- Charge Variants heterogeneity is considered a Critical Quality Attribute. It can impact drug stability, activity, and efficacy. Charge Variants profiles are used for regulatory drug submissions.
- Charge heterogeneity during production and purification caused by amino acid substitutions, glycosylation, phosphorylation and other post-translational or chemical modifications.
- Monitoring charge variants is a critical part of quality control and quality assurance process during the manufacturing of biotherapeutics.

Checklist:

Experimental checklist		
1260 Infinity II Bio-inert	Quaternary Pump (G5654A)	❑
	Multisampler with sample cooler (G5668A)	❑
	Multicolumn Thermostat with Bio-inert heat exchangers (G7116A)	❑
	DAD WR with bio-inert standard flow cell (G7115A)	❑
Column	Agilent Bio MAb, nonporous, 2.1 × 250 mm, 5 µm, PEEK (p/n 5190- 2411)	❑
Software	OpenLAB CDS 2.3, Buffer Advisor A.01.01 [009]	❑
Chemical	NISTmAb RM8671	❑
	Sodium hydrogen phosphate monobasic (Sigma S8282, BioXtra)	❑
	Sodium hydrogen phosphate dibasic (Sigma S9390, ACS reagent)	❑
	Sodium chloride (Sigma S7653)	❑
Additional equipment	pH meter	❑
	Milli-Q Integral system	❑
	Vacuum filtration unit with 0.2 µm	❑

Note:

- The expected results may slightly vary due to system-to-system setup. The two LC method conditions and steps (25 mins and 45 mins) are described in this protocol. The methods were optimized and best suited for the NISTmAb sample (RM 8671) IEX analysis.

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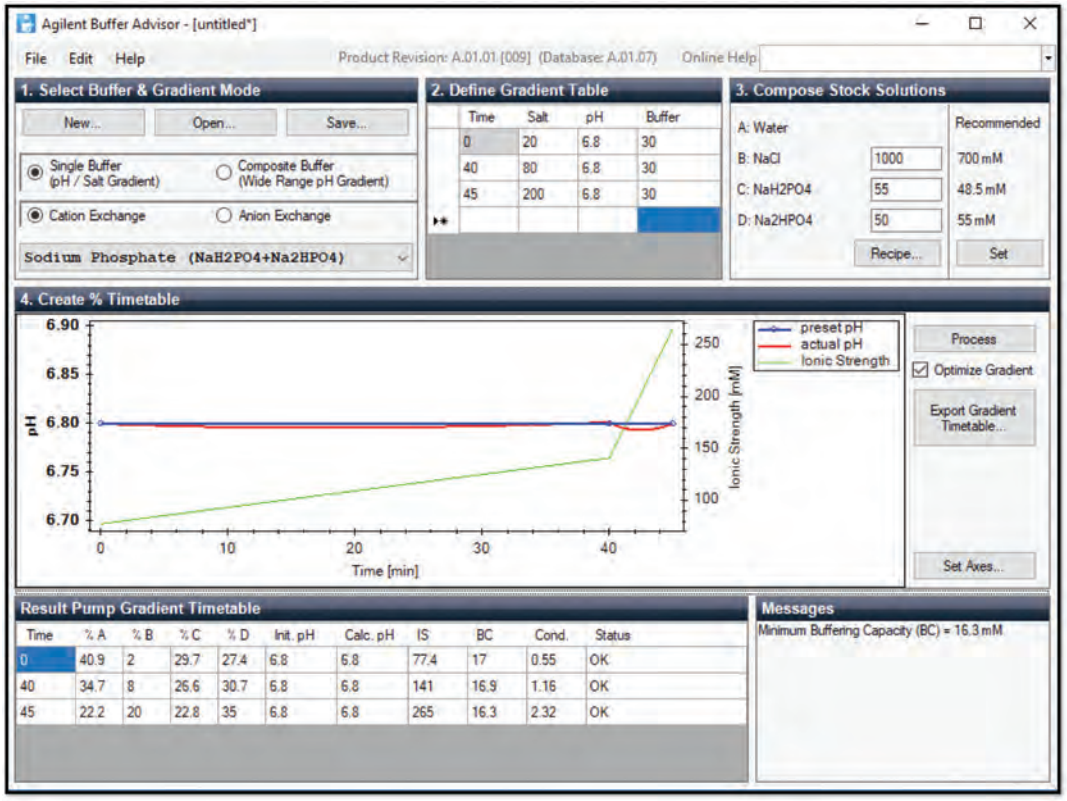
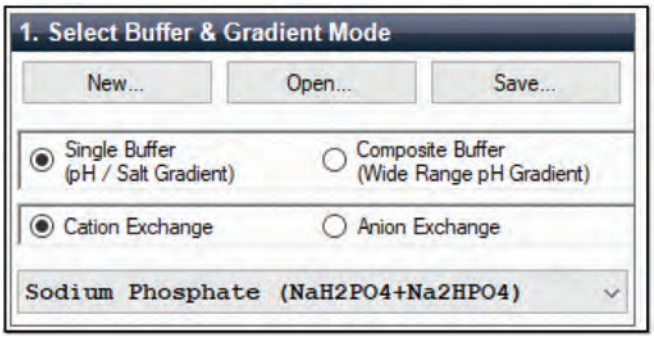
Agilent Buffer Advisor Software

Buffer Advisor Software facilitates dynamic mixing of solvents from only four stock solutions, simplifying the bioanalysis workflow and significantly reducing the time required for buffer preparation. The Buffer Advisor Software was designed to generate pH gradients for IEX separation. Below discussion illustrates buffer preparation steps for IEX separation of NISTmAb with salt gradient elution method.

Agilent Buffer Advisor software layout





⊖ **Buffer type:** select sodium phosphate buffer system (NaH_2PO_4+Na_2HPO_4)

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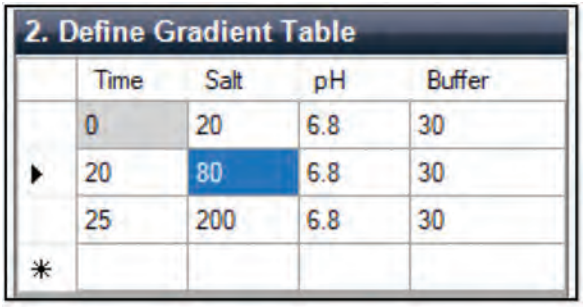
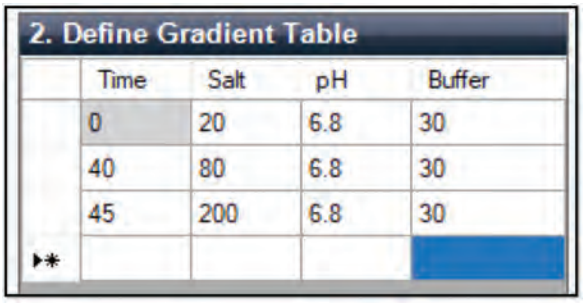
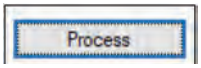
⊜ **Define Gradient:** Enter the required buffer condition (shown is for Speed and Resolution)

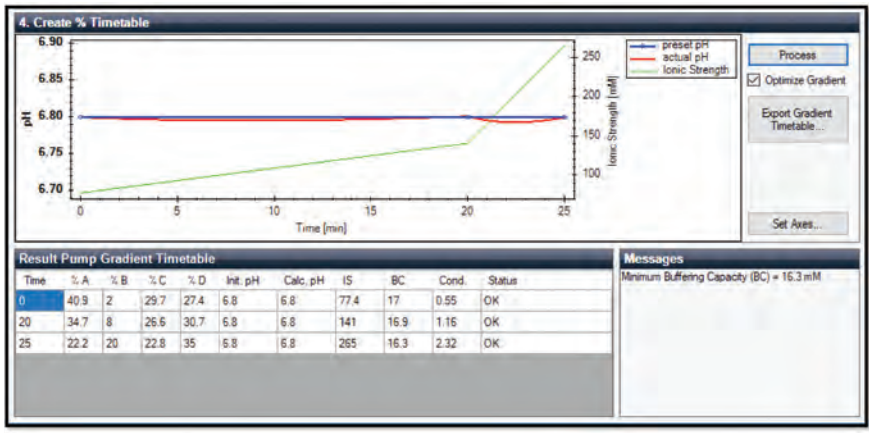




⊛ **Compose Stock Solution:** Enter the required concentrations in stock solution section

④ **Generate Gradient Timetable:** Click the "Process" tab to generate pump gradient timetable. The timetable displays mobile phase percentages to achieve set buffer condition.





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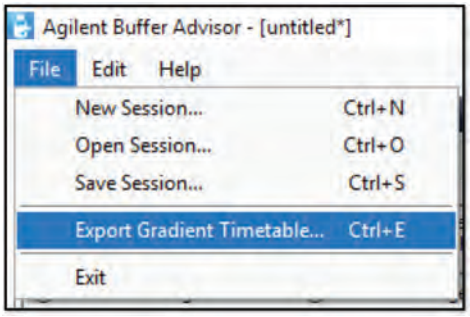
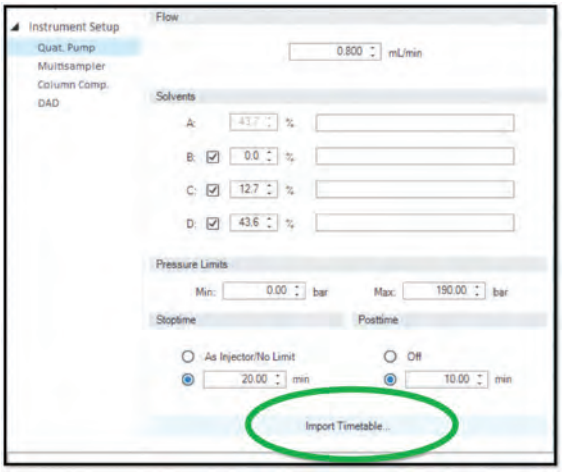
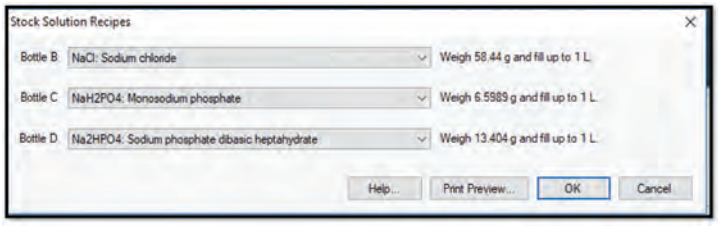
Save the timetable using Buffer Advisor “File” tab which generates “xml” file. Import this “xml” file using “Import Timetable” tab in OpenLAB CDS method editor window.





To prepare Stock Solutions: Click “Recipe” tab and it will display absolute amount of chemicals needed for 1 L buffer stock solution preparation



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Sample and Buffer Preparation

mAb sample preparation

- Agilent-NISTmAb (p/n 5191-5744) sample contains 25 µL of 10 mg/mL IgG1κ monoclonal antibody in 12.5 mmol/L L-histidine, 12.5 mmol/L L-histidine HCl (pH 6.0).
- Aliquot 25 µL of NISTmAb RM 8671 sample into LC injection vial (p/n 5188-6591) and place it in LC multisampler.

Software

- Prepare following four stock solutions in 18 megOhm-cm water (see Buffer Advisor section)

  Bottle A: Water
  Bottle B: 1000mM NaCl
  Bottle C: 55mM NaH_2PO_4
  Bottle D: 50mM Na_2HPO_4 7 H_2O

Stock Solution Recipes

- NaCl: Sodium chloride
  Recipe Bottle B: Weigh 58.44 g and fill up to 1 L .
- NaH_2PO_4: Monosodium phosphate
  Recipe Bottle C: Weigh 6.5989 g and fill up to 1 L.
- Na_2HPO_4: Sodium phosphate dibasic heptahydrate
  Recipe Bottle D: Weigh 13.404 g and fill up to 1 L.
- Filter the solution through 0.22 µm membrane filter (hydrophilic PTFE) and use immediately. Always prepare mobile phase freshly.

1260 Infinity II Bio-Inert LC Installation and Method Setup

- Follow the manual and quick guide for 1260 Infinity II Bio-inert LC installation and configuration settings (https://www.agilent.com/cs/library/usermanuals/public/G5654System.pdf)

Column

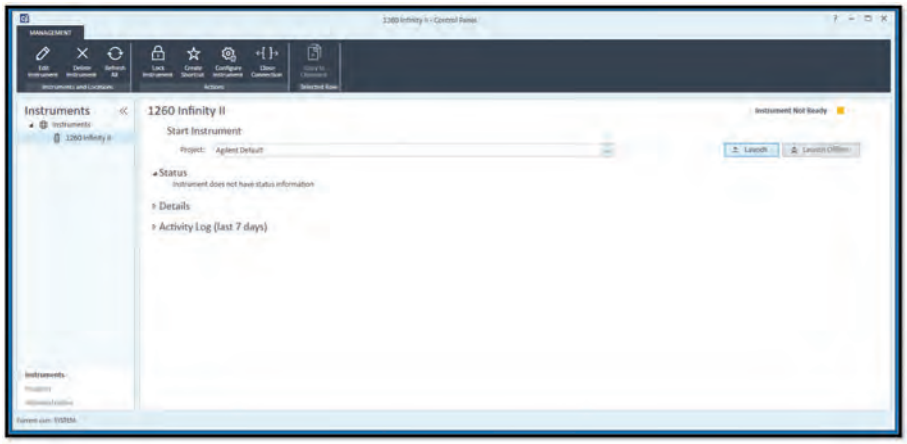
- Remove both end plugs and ensure that your system's flow direction matches the arrow on the column. Do not use the column with the flow in the reverse direction.
- Prior to applying flow over the column make tight ferrule connections.
- The columns are shipped in a 20 mM phosphate buffer, pH 6.0. Prior to first injection of the sample, purge the column with 20 column volumes of mobile phase buffer at 0.1 mL/min (starting condition) and gradually increase the flow rate (0.250 mL/min) and allow until the baseline to flatten.
- Whenever a column is not installed on the LC tightly, seal both ends of the column with the removable end plugs supplied with the column. For short term storage of less than one week, store the column in the mobile phase. For extended storage of longer than one week, flush the column with 20 mM phosphate buffer, pH 6.0 containing 0.1 % NaN_3 (sodium azide). Recommended storage temperature is 4 to 35°C.
- Further details on column maintenance can be found in the data sheet of the column (https://www.agilent.com/cs/library/datasheets/public/5973-1745.pdf)

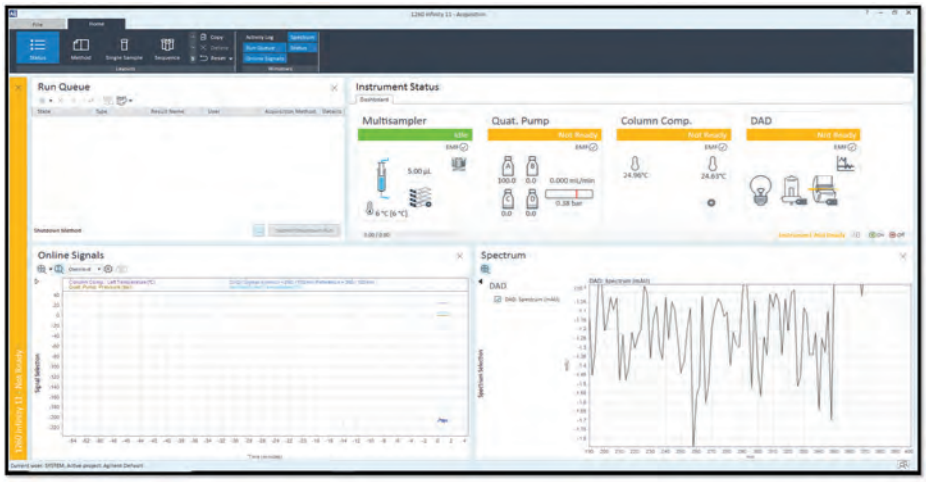
Prepare for run and method setup

Turn on the modules of the instrument. Launch the OpenLab Control panel software from the desktop.

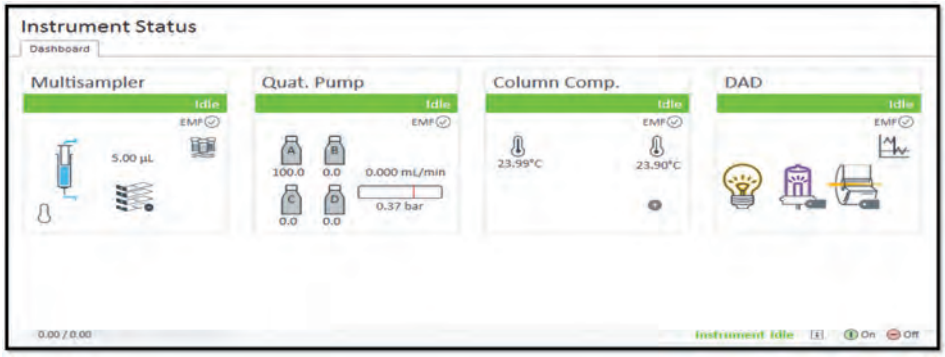
Click on the icon to launch the OpenLab Control panel.

In the opened OpenLab Control panel, click launch button to bring up the Acquisition panel.

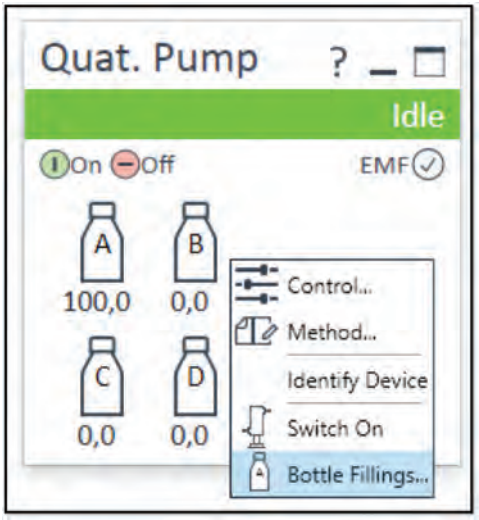


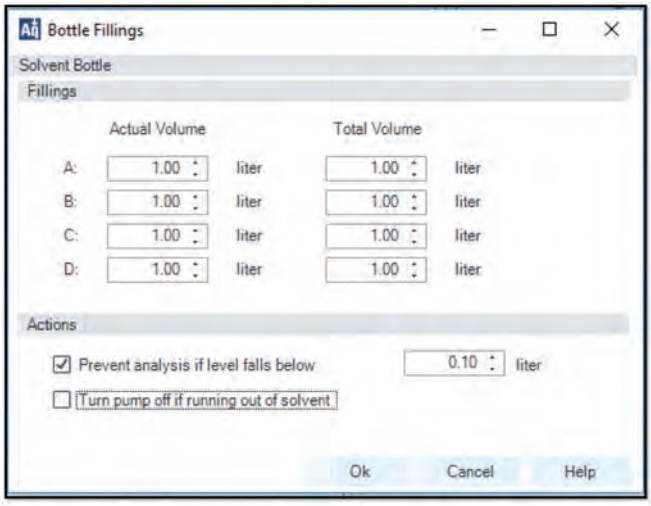


Switch on the instrument modules from the Acquisition panel



- Fill the solvent bottles with adequate buffer solutions and place it into the solvent cabinetVariation of buffer pH (± 0.2)
- Open solvent bottle filling dialog and fill in the volume of the solvents in the bottle
- Purge the pump by opening purge valve





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– Enter column information using column assignment tab

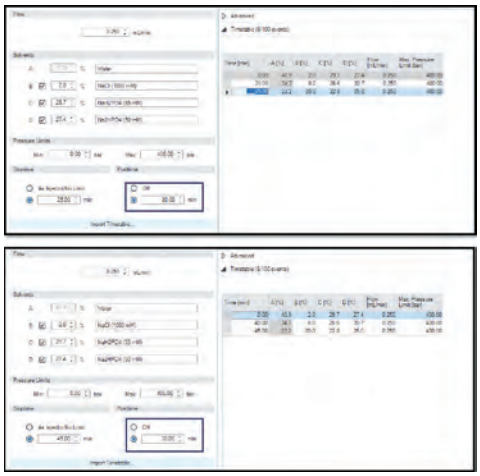
Method setup

Load the default method → Change the method for NISTmAb charge variant analysis → Save as a new method

The following screenshots shows the parameters settings for each module

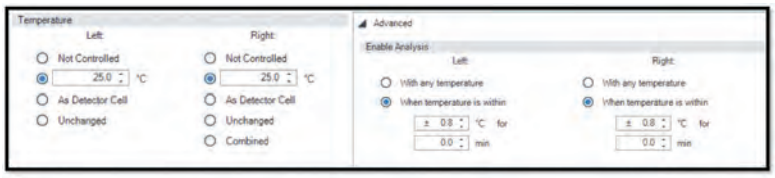
Multisampler (G5668A)

Quat. Pump (G5654A)

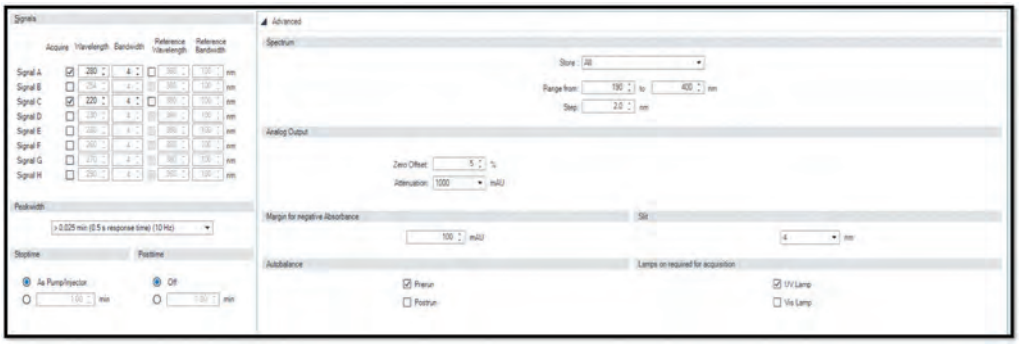


Please note the post time of 30 mins which is required to re-equilibrate the column to its initial condition. This is very important step to be included into your method. Failing to do the re-equilibration of the column leads to protein (in this case mAb) not binding to the column.

Column Comp. (G7116A)



Column Comp. (G7115A)



LC method condition summary table:

Conditions

Parameter	Settings
Column	Agilent Bio mAb, nonporous, 2.1 × 250 mm, 5 µm, PEEK (p/n 5190-2411)
Mobile phase	A: Water B: 1000mM NaCl C: 55 mM NaH_2PO_4 D: 50 mM Na_2HPO_4
TCC Temperature	25 °C
Gradient:	Fast gradient or high-resolution gradient
Run time	25 and 45 mins
Sample	NISTmAb
Injection volume	2 µL
Flow rate	0.25 mL/min
DAD	220 and 280 nm

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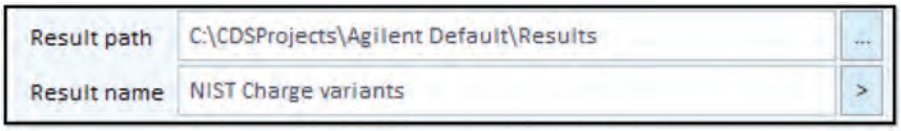
Sequence setup and sample run

– To create a sequence, navigate to sequence layout.

– In the sequence table, add lines and enter the runs as shown below and save the sequence.

		Action	Vial	Sample type	Inj/Vial	Volume	Acq. method	Injection sour	Sample name	Data file
1	☑	Inject	D1F-A1	Blank	1	Use Method	Char_VER_6.8_salt gradient 3_30mm_25...	HipAls	Blank	blank
2	☑	Inject	D1F-A1	Sample	10	Use Method	Char_VER_6.8_salt gradient 3_30mm_25...	HipAls	NISTmAb	charge variant
3	☑	Inject	D1F-D1	Blank	1	Use Method	Char_VER_6.8_salt gradient 3_30mm_25...	HipAls	Blank	blank

– Verify the sequence result path and enter a name for results file

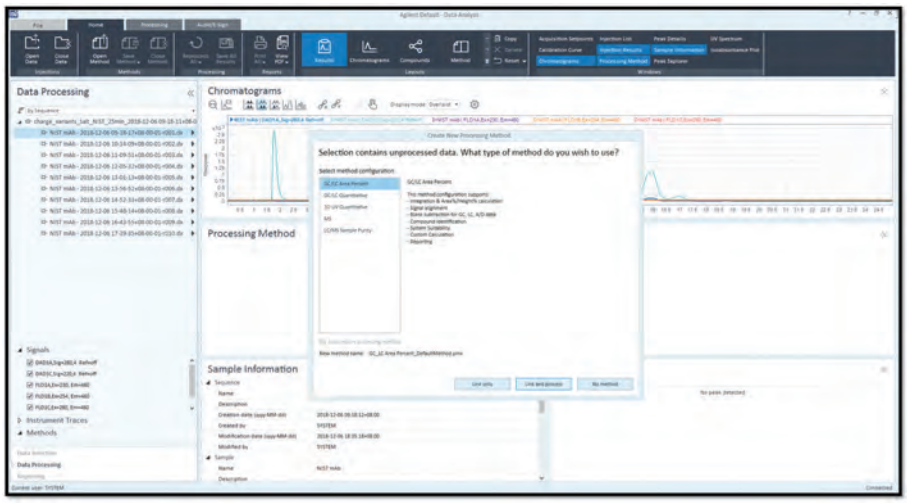


– Click run button to run the sequence

– Once the sequence is submitted, it will automatically show up in Run queue window.

Sequence setup and sample run

- In the Data Selection view, navigate to the required result set and double-click on result set.
- Navigate to the folder containing the corresponding data files, and select the required injections in the Injection List window.
- The selected result sets or injections are loaded, and the application switches to the Data Processing view.
- The workspace will be as in the following Figure:



- Use the default GC-LC processing method and link and process all the data files from the sequence. Make sure to delete the integration peaks from the injection peak manually using this button
- Export all the method attributes (retention time, area, resolution) to MS Excel and calculate the % relative standard deviations (%RSD)

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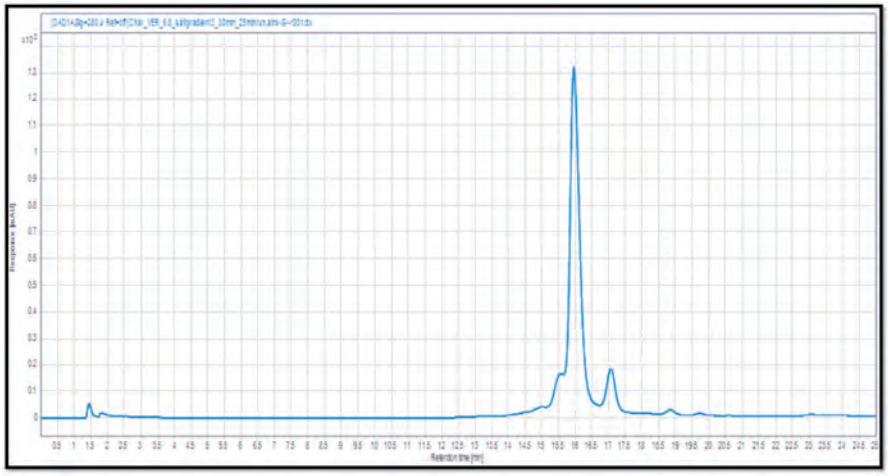
Expected results:

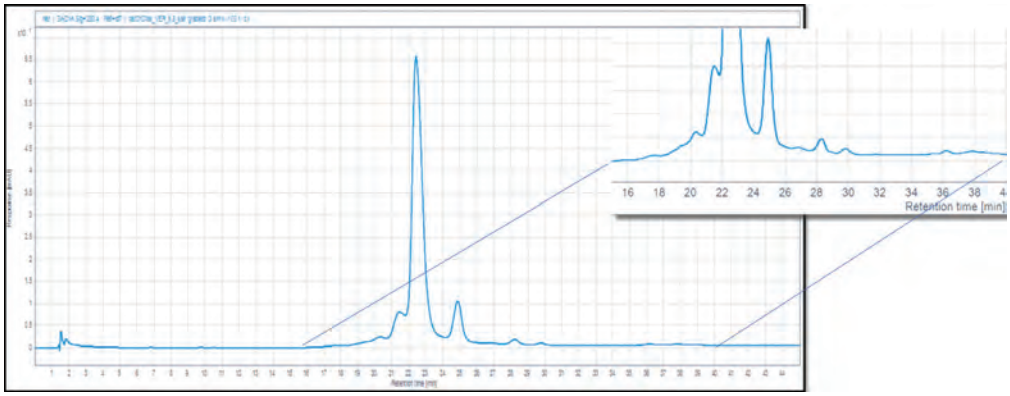
– NISTmAb RM 8671

Faster analysis



Peak id	RT (min)	Area %	Resol. USP
Acidic variants	12.09-15.65L	13.03	
Main peak	15.65-16.71	73.66	0.24 (main peak/acidic)
Basic variants	16.71-19.98	13.31	2.14 (main peak/basic)

High resolution analysis



Peak id	RT (min)	Area %	Resol. USP
Acidic variants	16.27-21.66	13.00	
Main peak	21.66-23.95	73.83	0.59 (main peak/acidic)
Basic variants	23.95-30.1	13.17	2.50 (main peak/basic)

Troubleshooting:

Condition	Reason	Fix
Poor resolution of peak of interests	Suboptimal elution conditions	Change elution conditions: use shallower gradient or reduce flow rate
	Sample is viscous	Dilute the sample with buffer to reduce viscosity of the sample
	Column overloaded	Decrease sample load
	Microbial contamination in the column	Follow cleaning procedures as recommended
Proteins does not bind to the column	Some particulates in sample	Filter the sample and re-run the experiment
	Sample condition changed during storage	Prepare fresh samples
	Column equilibration incompletes	Increase the equilibration time for the column
	Incorrect buffer pH and/or ionic strength	Prepare new solutions
Protein elutes late or does not elute from the column	Incorrect buffer pH	Prepare new solutions
	Ionic strength too low	Increase salt concentration in elution buffer
	Ionic strength of sample or buffer is too high	Decrease ionic strength of sample or buffer
Protein elutes earlier	Column equilibration incomplete	Increase the equilibration time for the column

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Application Note

Biopharmaceuticals, Biotherapeutics

Agilent

Trusted Answers

High-resolution Analysis of Charge Heterogeneity in Monoclonal Antibodies Using pH-gradient Cation Exchange Chromatography

Agilent 1260 Infinity Bio-inert Quaternary LC System with Agilent Bio Columns

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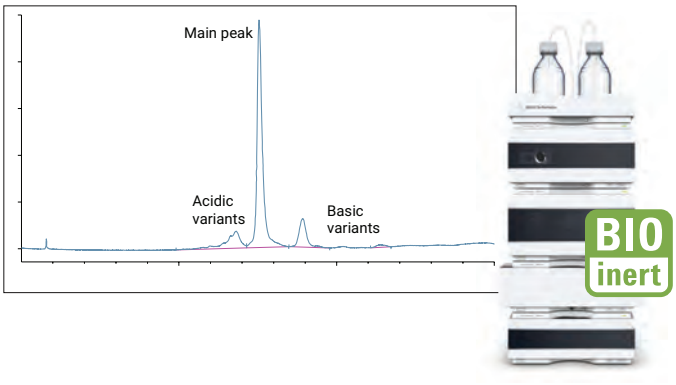
Abstract

Antibody charge variants have gained considerable attention in the biotechnology industry due to their potential influence on stability and biological activity. Subtle differences in the relative proportions of charge variants are often observed during routine manufacture or process changes and pose a challenge when demonstrating product comparability. These changes include differences in glycosylation, deamidation, oxidation, isomerization, incomplete C-terminal processing, and other post-transitional modifications that modify the isoelectric pH (pI) values. In the biotechnology industry, ion-exchange chromatography is widely used for profiling the charge heterogeneity of proteins, including monoclonal antibodies. This Application Note describes a high-resolution, pH-based separation of acidic and basic charge variants for monoclonal antibodies using the Agilent 1260 Infinity Bio-inert Quaternary LC System and an Agilent BiomAb PEEK 4.6 × 250 mm, 5 µm ion exchange column that features a unique resin specifically designed for the charge-based separation of mAbs. The robustness of the method for routine analysis was established by validation studies.

Introduction

Monoclonal antibodies (mAb) are glycoproteins of the immunoglobulin (Ig) family. MAbs have become the most rapidly growing class of biotherapeutics in the development for many different disease conditions. Novel mAb molecules are entering clinical studies at a rate of almost 40 per year, and the research pipeline includes approximately 250 therapeutic mAbs in clinical studies. There is steadily increasing need for an analytical method that can be used for high-throughput analysis of a large number of samples to support bioprocesses and formulation development. Biotherapeutics, such as mAbs, are complex molecules, and a variety of methods is required to monitor the heterogeneities associated with the mAb to ensure product quality and consistency.[1] Cation exchange chromatography is the gold standard for charge-sensitive antibody analysis. In cation exchange chromatography, method parameters often need to be optimized for each individual protein as ion exchange is dependent on the reversible adsorption of the charged protein molecules to immobilized ion exchange groups. Several authors have made significant progress in demonstrating practical separations using pH changes in the mobile phase to elute the proteins.[2] The Agilent ion exchange column family offers strong cation exchange (SCX), weak cation exchange (WCX), strong anion exchange (SAX) and weak anion exchange (WAX). The Agilent Bio MAb NP 5 (nonporous, 5 µm) PEEK, 4.6 × 250 mm, column is specifically designed to characterize the charge heterogeneity of monoclonal antibodies, including C-terminal lysine variance. The column offers even higher resolution, enabling better peak identification and accurate quantification. This Application Note describes a pH gradient based method for separating the charge variants of IgG1 using a 1260 Infinity Bio-inert Quaternary LC System and a Bio MAb NP 5 PEEK, 4.6 × 250 mm, ion exchange column. Method validation and robustness of an optimized ion exchange method are described.

Equipment



Instrumentation

A completely biocompatible Agilent 1260 Infinity Bio-inert Quaternary LC System operating up to a maximum pressure of 600 bar was used for the experiments (Table 1). The entire sample flow path is free of any metal components so that the sample does not come in contact with metal surfaces. Solvent delivery is free of any stainless steel or iron components.

Table 1. Configuration of the Agilent 1260 Infinity Bio-inert Quaternary LC System.

Description	Model number
Agilent 1260 Infinity Bio-inert Quaternary Pump	G5611A
Agilent 1260 Infinity Bio-inert High Performance Autosampler	G5667A
Agilent 1290 Infinity Thermostat (for autosampler)	G1330B
Agilent 1260 Infinity Thermostatted Column Compartment with bio-inert click-in heating elements (option 019)	G1316C
Agilent 1260 Infinity Diode Array Detector with 60-mm Max-Light high sensitivity flow cell (option 033)	G4212B

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Software

Agilent OpenLAB CDS ChemStation Edition, revision C.01.04.

Ion Exchange Chromatography Parameters

Table 2 shows the Chromatographic parameters for Ion Exchange Chromatography using Agilent 1260 Infinity Bio-inert LC System.

Reagents, samples and materials

Human monoclonal antibody IgG1 was a proprietary pharmaceutical molecule. Sodium phosphate dibasic dihydrate, sodium phosphate monobasic dihydrate, sodium chloride, sodium bicarbonate hydrochloric acid (HCl), and sodium hydroxide (NaOH) were purchased from Sigma Aldrich. All the chemicals and solvents used were HPLC grade and high purity water from Milli Q water purification system (Millipore Elix 10 model, USA) was used.

Procedures

Mobile phase A was 10 mM sodium phosphate, pH 6.0 and mobile phase B was 10 mM sodium phosphate, pH 9.5. Monoclonal antibodies were diluted to approximately 2 mg/mL in mobile phase A and elution was monitored at 220 nm and 280 nm. Area and retention time (RT) were used to calculate standard deviation (SD) and relative standard deviation (%RSD). For each elution, the column was pre-equilibrated with at least three column volumes of mobile phase A prior to sample injection. After the injection of the monoclonal antibody sample onto the column, a linear increase in the percentage of mobile phase B was delivered. The linear gradients were run from 0 to 100% B in 27 minutes at 1 mL/min flow rate. After the gradient, the mobile phase was pumped at 100% B until at least one column volume passed before the composition was returned to 100% A in preparation for the subsequent analysis. Relative percent area was used to quantify the charge variants of monoclonal antibodies.

Robustness Study

The four critical method parameters listed below were varied to validate the IEX procedure.

- Variation of injection volume (± 10%)
- Variation of buffer pH (± 0.2)
- Variation of flow rate (± 2%)
- Variation of column temperature (± 5%)

For each robustness parameter, 10 µL of IgG1 was injected six times to calculate average area and RT. The percentage deviation (% accuracy) of area and retention time (RT) was calculated from the optimized method.

Table 2. Chromatographic parameters used for IEX chromatography.

Conditions

Parameter	Settings	
Column:	Agilent Bio MAb PEEK, 4.6 × 250 mm, 5 µm column (p/n 5190-2407)	
Mobile phase A:	10 mM sodium phosphate buffer, pH 6.0	
Mobile phase B:	10 mM sodium bicarbonate buffer, pH 9.5	
Gradient:	Time (min) 0 minutes 25 minutes 27 minutes 30 minutes	Mobile phase (% B) 0% B 100% B 100% B 0% B
Injection Volume:	10 µL (needle with wash, flush port active for 7 seconds)	
Flow Rate:	1.0 mL/min	
Data acquisition:	214 and 280 nm	
Acquisition rate:	20 Hz	
Flow cell:	60-mm path	
Column Temperature:	30 °C	
Sample Throughput:	5 °C	
Post time:	5 minutes	

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Results and Discussion

Separation and detection

The Agilent Bio MAb columns are highly uniform, densely packed, weak cation exchange resin. This Application Note used the 5-µm column that features a unique resin specifically designed for high-resolution chargebased separations of monoclonal antibodies. The peaks of the ion exchange profiles were typically denoted into three distinct components[3]. Early and late-eluting peaks were called acidic and basic variants, respectively. The most abundant peak was designated as the main peak. Figure 1 shows the optimized cation exchange elution profile of IgG1 on Bio MAb PEEK column demonstrating excellent separation of IgG1 in 30 minutes into three distinct peaks: basic variants, main peak, and acidic variants. The overlay of six replicates of IgG1 shows excellent separation reproducibility (Figure 2). The high resolution separation of IgG1 facilitated the quantification of charge variants using peak areas (Figure 3). The relative peak areas for the charge variants of IgG1 are shown in Table 3. The IgG1 possessed approximately 9.97% of acidic variants, 76.92% main peak and 13.21% basic variants of the total peak area, respectively. The reproducibility of analysis was tested with six replicates.

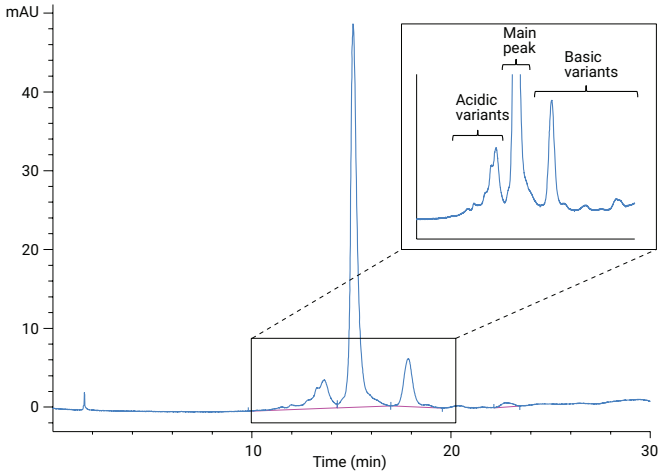

Figure 1. Elution profile of pH based separation of charge variants of IgG1 on an Agilent Bio MAb PEEK, 4.6 × 250 mm, 5-µm column. The acidic, main peak and basic variants are enlarged in the magnified view.

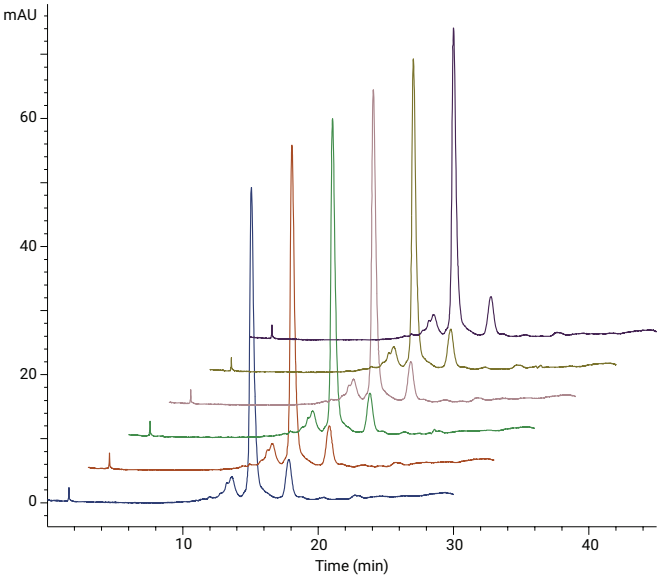

Figure 2. Overlay of six replicates of IgG1 on an Agilent 1260 Infinity Bio-inert Quaternary LC System using an Agilent Bio MAb PEEK, 4.6 × 250 mm, 5 µm column.

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Precision of Retention Time and Area

The precision of a procedure expresses the closeness of agreement between a series of measurements obtained from multiple analyses of the homogeneous sample under the prescribed conditions and often expressed as relative standard deviation (RSD). Table 4 shows the average retention times and area RSDs from six replicates of an IgG1 injection. The retention time and peak area RSDs for the main peak were 0.106% and 1.60% respectively which demonstrates excellent reproducibility of the method and thus the precision of the system.

Table 3. Charge variants quantification by area %, n = 6.

	RT (min)	Area %
Acidic variants	13.28 13.61	9.87
Main peak	15.058	76.92
Basic variants	17.82 22.69	13.21

Table 4. Retention time and area RSD (%), n = 6 for main peak.

	RT (min)	Area %
Mean (min)	15.058	1172
RSD	0.106	1.60

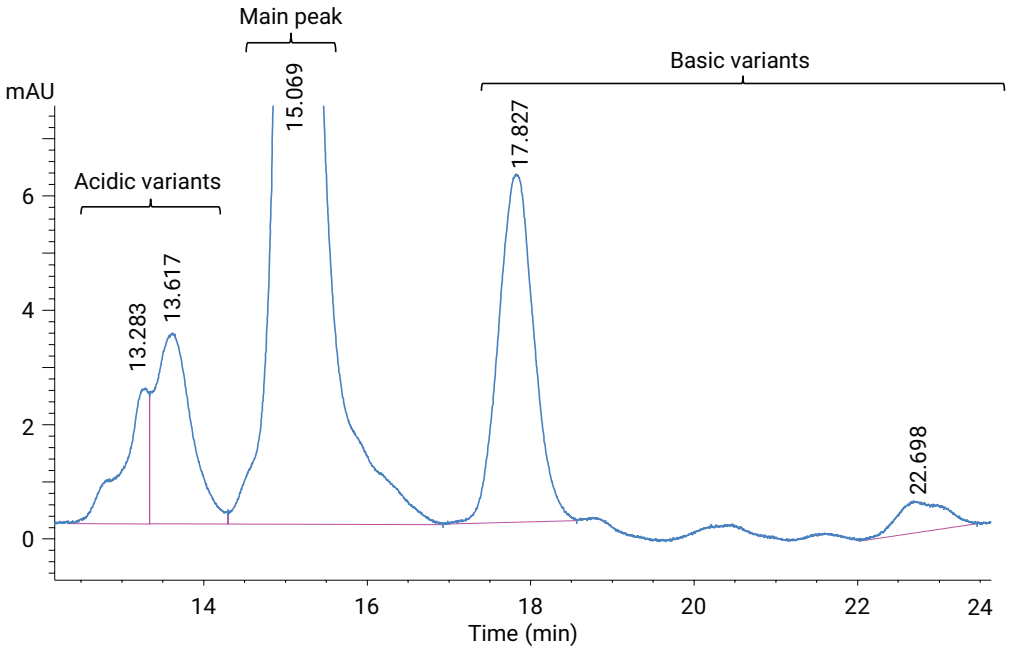

Figure 3. Expanded scale chromatogram of pH gradient-based cation exchange chormatogram of IgG1 separation using an Agilent Bio MAb PEEK, 4.6 × 250 mm, 5 µm column.

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Precision of Retention Time and Area

The The robustness of an analytical procedure is the persistence of a method under perturbations or conditions of uncertainty and provides an indication of its consistency during routine use. To evaluate the robustness of the method, four critical parameters of the optimized method were varied (Table 5). Allowed deviations for RT and area RSD were set to ± 3.0% and ± 5% respectively. The red numbers indicate where the result exceeded the allowed deviation. The impact of injection volume, column temperature, buffer pH and flow rate on RT and area RSD was within the acceptable limits.

A variation in injection volume by ± 10% compared to the actual method caused the area RSD to deviate significantly; however, this deviation is an expected due to the load on the ion exchange column. There were no further significant changes in the chromatographic pattern when deliberate variations were made in experimental conditions, thus showing the method is robust. Our results show that the method is reliable for routine QA/QC application for manufacturing and storage consistency. However, some parameters such as injection volume are critical and must be carefully controlled.

Table 5. Robustness (RT and Area % RSD) n = 6.

		RT deviation (limit: ± 3.0 %)	Area deviation (limit: ± 5.0 %)
Parameters	**Variations**	**Main peak**	
Variation in injection volume (10 µL ± 10%)	– 1 µL + 1 µL	-0.19 0	10.49 -9.89
Variation in column temperature (30 °C ± 5%)	– 5% + 5%	-1.19 0.66	2.73 2.13
Variation in buffer pH (6.0 ± 0.2)	– 0.2 + 0.2	0.199 0.99	-0.68 -0.08
Variation of flow rate (1.0 ± 2%)	– 2% + 2%	0.66 0	2.73 -1.10

Conclusion

Cation exchange chromatography has been widely used for separating charge heterogeneity of monoclonal antibodies. This Application Note shows how the Agilent 1260 Infinity Bio-Inert LC System and an Agilent Bio MAb PEEK, 4.6 × 250 mm, 5 µm column were used to perform reproducible and high resolution analysis of charge variants in monoclonal antibodies for biopharmaceutical process development and process monitoring. A simple pH gradient-based cation exchange method for separation and quantification of charge variants was developed. Area, RT precision, and robustness of the method were excellent and show the reliability of the method. There were no significant changes in the chromatographic profile when the modifications were made in experimental conditions, thus showing the method to be robust. The bio-inertness and corrosion resistance of the instrument coupled with simple and reproducible method make this solution particularly suitable for the QA/QC analysis of monoclonal antibodies in the biopharmaceutical industry.

Reference

1. Yan He *et al., J. Sep. Sci.,* **2011**, 34, 548–555
2. Dell Farnan, *Anal. Chem.,* **2009**, 81, 8846–8857
3. Jennifer C. Rea, *Journal of Pharmaceutical and Biomedical Analysis,* 54 (**2011**) 317–323

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