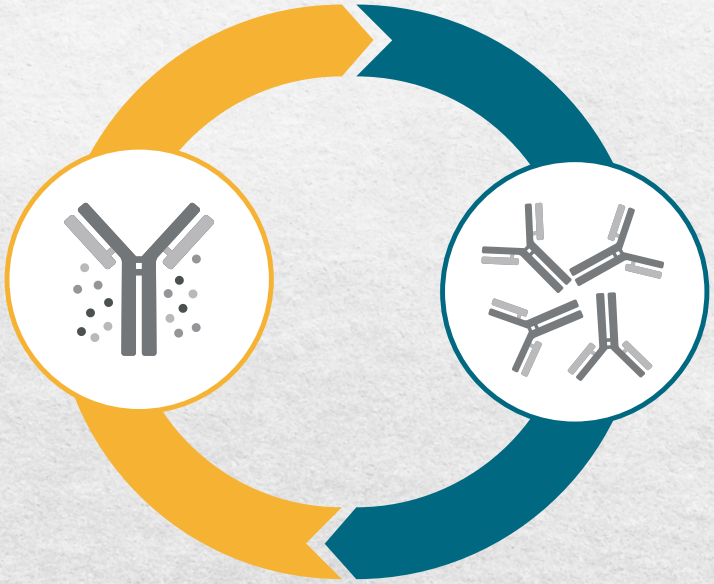
Contents

ThermoFisher SCIENTIFIC



HPLC columns and consumables

Analytical workflow solutions

to advance your **protein biotherapeutics** process development

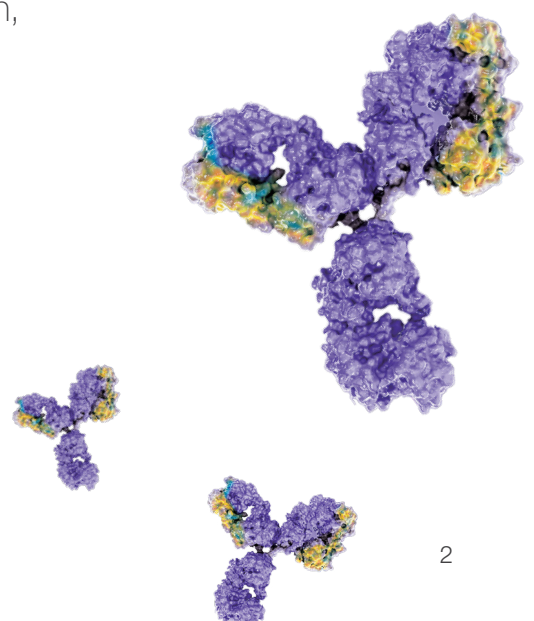
thermoscientific

Introduction

Biotherapeutic proteins are pharmaceutical drug products produced using biological systems and are often used to treat diseases like cancer, autoimmune disorders, or infectious diseases by modulating cellular signaling processes.

They encompass various biologically based drug products such as **monoclonal antibodies**, **fusion proteins**, **antibody–drug conjugates**, **bispecific antibodies**, and **cell and gene therapy products**.

They are complex and heterogeneous molecules that are subject to a variety of enzymatic and chemical modifications during their expression, purification, and long-term storage. These changes include several possible modifications, such as oxidation, deamidation, glycosylation, aggregation, misfolding, or adsorption, leading to a potential loss of therapeutic efficacy or unwanted immune reactions. Thus, in order to obtain a safe and efficacious drug, it's important to understand their higher structural order as well as primary sequence, and post-translational modifications (PTMs) to analyze variation or residues resulting from cellular production systems.



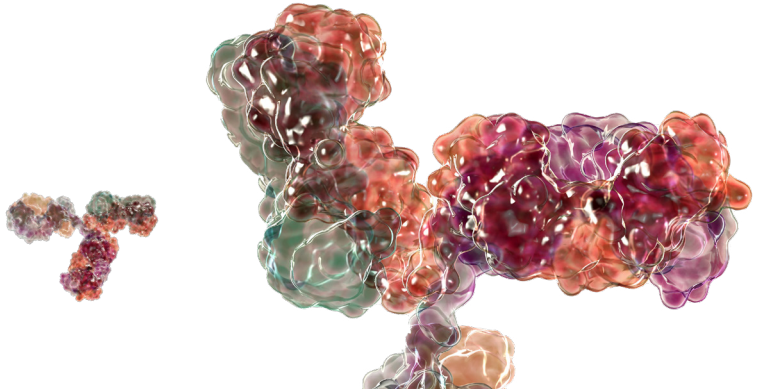
Introduction

Chromatography as the method of choice

The Thermo Scientific Vanquish UHPLC system is suitable for high resolution bioseparations and accurate measurements of biopharmaceutical monitoring with peak performance without any preconditioning or other preparation of the instrument. It is equipped with biocompatible features that are specifically selected materials that prevent undesired secondary interactions with the sample which can cause unreliable results. Its impeccable durability requires very little downtime compared to other instruments on the market – by providing you the space and time you need to run your analysis reliably every day.

Thermo Scientific instruments workflow solutions

Description	Quantity	Cat. no.
Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system	Each	IQLAAAGABHFAPUMBHV
Thermo Scientific™ Vanquish™ System Base	Each	VF-S01-A-02
Thermo Scientific™ Vanquish™ Quaternary Pumps F	Each	VF-P20-A
Thermo Scientific™ Vanquish™ Column Compartment H	Each	VH-C10-A-03
Thermo Scientific™ Vanquish™ Split Sampler FT with 25 µL sample loop	Each	VF-A10-A-02



Introduction

Sample handling: Understanding the impact of vials on your analysis

Vials have more of an impact on biotherapeutic analysis than you would think, and therefore, selecting the right vial is important.

Hydrophobic proteins and **peptides** tend to create salt adducts with glass vials over time, reducing the recovery of certain proteins or peptides. Thermo Scientific™ SureSTART™ GOLD-grade vials have an ultra-low adsorption glass surface that enables trace-level analysis for strongly adsorbing analytes like proteins. These vials deliver the highest recovery rates with trisubstituted N-atoms and tertiary amines, as well as the lowest levels of alkaline materials for less glass-wall interactions.

For biotherapeutics products, it's important to not only achieve the best sensitivity, but also assure robustness for small injection volumes. Thermo Scientific™ SureSTART™ high recovery glass snap top microvials maximize injection volumes when analyzing <2 mL samples, and with their inner V-shaped bottom, enable a residual volume of 4 µL. When you have low sample volume, such as for nano lipid particles drug delivery vehicles, these vials are designed to protect your most valuable samples and deliver consistent, reproducible results for your most demanding analyses.

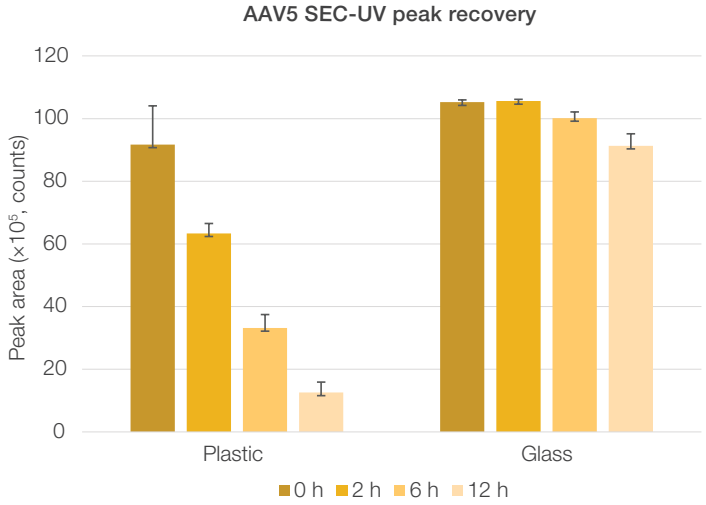
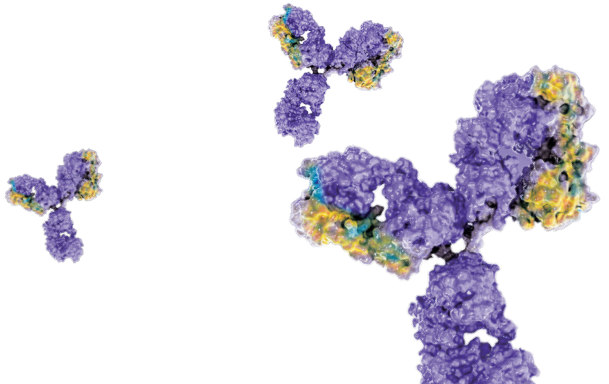


Figure 1. Bar graph plot of the peak area obtained after SEC-FLR analysis of AAV sample stored in plastic or glass vials at different time points



Charge variant analysis

Monoclonal antibodies (mAbs) are a preferred class of protein therapeutics used for the treatment of various diseases because of their ability to target specific tissues for drug delivery or the modulation of cellular activities. Cellular production and downstream manufacturing processes commonly introduce heterogeneity to the mAb structure by way of post-translational or chemical modifications that can have potential effects on product efficacy, safety, and stability. As such, thorough characterization of mAbs is required to fulfill regulatory requirements to bring new therapeutics to market.

Common modifications to the mAb structure, including lysine truncation, asparagine deamidation, and glycosylation, alter the charge of the biomolecule by addition or elimination of cationic or anionic sites resulting in increased charge heterogeneity. Charge heterogeneity analysis is critical for mAb characterization as it provides valuable information regarding product quality and stability.

Charge variant analysis uses ion-exchange chromatography (IEX) to separate proteins based on differences in charge, making it an ideal method for both qualitative and quantitative evaluation of charge heterogeneity.

Early methods use salt gradients to disrupt ionic interactions of the protein with the stationary phase and elute the analyte. A more recent method uses pH gradient to provide greatly improved results on columns with lengths as short as 50 mm, which means a five-fold increase in productivity as traditional salt gradients are run on 250 mm long columns to maintain resolution.

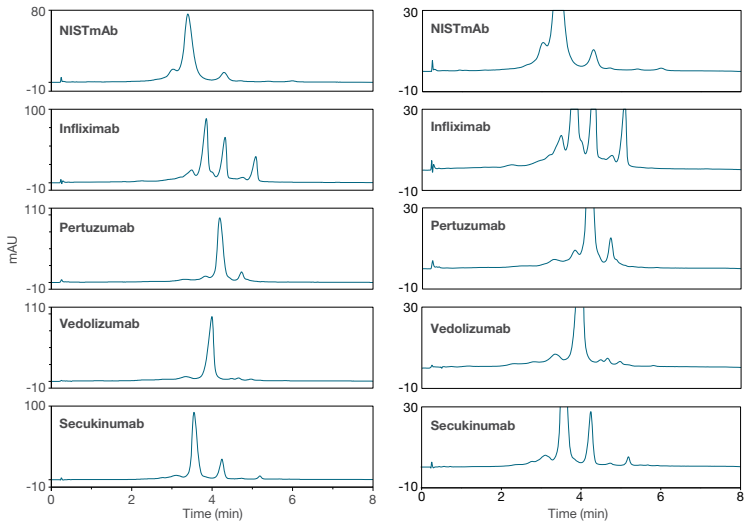
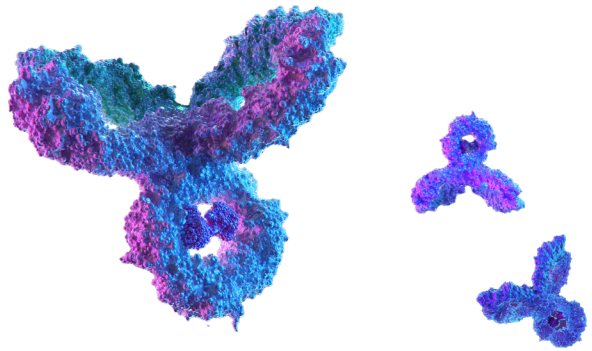


Figure 2. Separation of mAbs on a ProPac™ 3R SCX 3 µm 2 × 50 mm column. Analysis of mAbs using pH gradient, with the left chromatograms showing the full signal strength, and the right chromatograms showing the zoomed-in detailed view of the mAb variants. Read this **application note** to learn more.



Charge variant analysis (continued)

Columns and buffers

Thermo Fisher Scientific has both anion and cation exchange columns in the **Thermo Scientific™ MAbPac™** and **Thermo Scientific™ ProPac™ column families**.

Read more at thermofisher.com/chargevariantanalysis

To increase productivity the pH gradient is best performed on a cation exchange column. As it's often uncertain if the selectivity needed to separate the various species of proteins is a weak or a strong cation exchange column, it is recommended to try both options (Thermo Scientific™ ProPac™ Elite WCX column or Thermo Scientific™ ProPac™ 3R SCX column):

- ProPac Elite WCX is a high-resolution weak cation exchange column for proteins with an isoelectric point (pI) higher than 6. The column has a stable charge between pH 5 and 11.
- ProPac 3R SCX is a complementary strong ion exchange column that uses an ultra-high resolution monodisperse 3 µm resin capable of separating variants that differ by as little as one charge residue.

Salt gradients are often used for IEX analysis; however, pH gradient methods have been used as an alternative approach for separating proteins from their associated variants based on the isoelectric point (pI, pH at which the analyte charge is neutral) of each analyte. When using a pH gradient, the cationic protein is adsorbed to the stationary phase at low pH conditions followed by a gradient of increasing mobile phase pH. As the pH of the buffer increases, the charge of the protein shifts from cationic to neutral and then anionic at higher buffer pH values. The change in protein charge results in desorption from the anionic surface and elution from the column.

To easily facilitate these types of separations, Thermo Scientific™ CX-1 pH gradient buffer solutions can be used to generate highly reproducible, linear pH gradients using cation exchange chromatography. In addition to the benefit of increased productivity compared to traditional salt gradients, it is also possible to predict the pI and the expected retention of the charge variants and use a narrow pH range.

For more information on the buffers benefit see **here**.

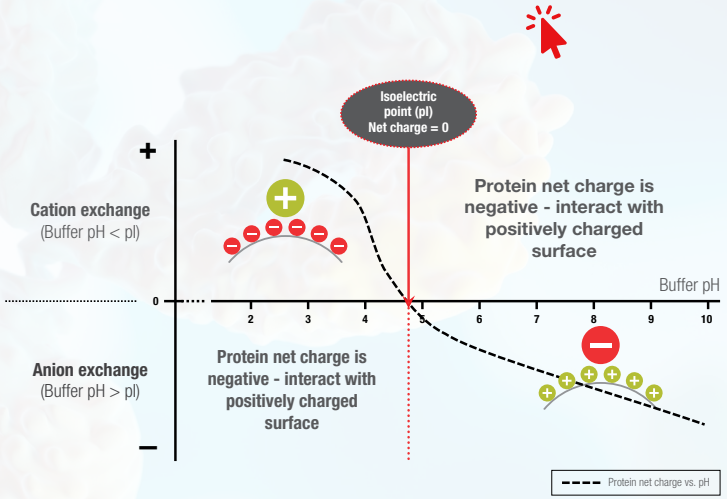


Figure 3. pH gradient based IEX

Charge variant analysis (continued)

Workflow solution

Instruments → **Columns and gradient buffers** → **Vials and caps**

Workflow solutions

Description	Quantity	Cat. no.
Thermo Scientific instruments		
Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system	Each	IQLAAAGABHFAPUMBHV
Thermo Scientific™ Vanquish™ Diode Array Detector HL	Each	VH-D10-A
Thermo Scientific™ Vanquish™ LightPipe™ 10 mm Standard Flow Cel	Each	6083.0100B
Thermo Scientific columns		
Thermo Scientific™ ProPac™ 3R SCX column	Each	43103-052068
Thermo Scientific™ ProPac™ Elite WCX column	Each	303027
Thermo Scientific gradient buffers		
Thermo Scientific CX-1 pH gradient buffer A, pH 5.6	250 mL	085346
Thermo Scientific CX-1 pH gradient buffer B, pH 10.2	250 mL	085348
Thermo Scientific vials and caps		
Thermo Scientific™ SureSTART™ 2 mL GOLD-Grade clear glass vial	100/pack	6PSV9-1PG
Thermo Scientific™ SureSTART™ 9 mm screw cap	100/pack	6PSC9TST

Conformational variance and degree of oxidation

Therapeutic proteins can be subjected to a variety of biochemical modifications during processing, delivery, and storage.

Some of these modifications have been shown to affect the safety and efficacy of these therapeutics, increasing the importance of analytical methods to detect protein variants. Among these modifications, oxidation or deamination of exposed amino acid residues is a major concern in stability studies and the adverse effect on product shelf life and bioactivity.

Oxidation of amino acid residues can alter the hydrophobic nature of the protein either by the increase in polarity of the oxidized form or result in conformational change.

Although conformational variance analysis can be done by charge variant analysis, hydrophobicity-based HPLC methods such as reversed-phase liquid chromatography (RPLC) and hydrophobic interaction liquid chromatography (HIC) has proven to be best suitable for variants with little difference in hydrophobicity, such as oxidation variants.

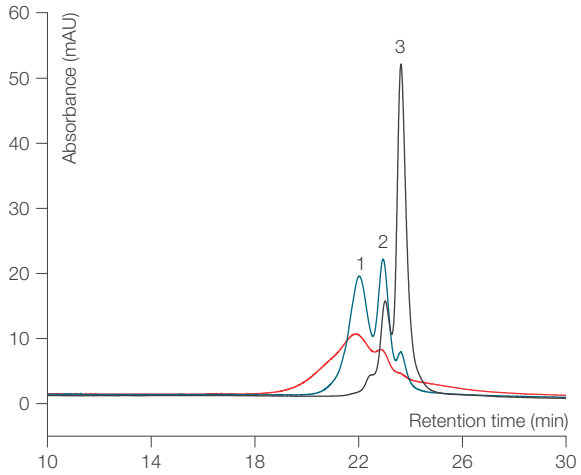


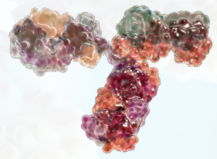
Figure 4. Separation of oxidized mAb1 on a 4.6 x 250 mm MAbPac HIC-20 column with 0.5 mL/min and 1.0–1.2 M starting salt concentration, the separation of oxidized mAb variants can be achieved without further sample processing. Read this application note to learn more.

The columns

Thermo Scientific™ MAbPac™ HIC-20 column is a high-resolution, silica-based HIC column with a unique proprietary column chemistry that provides high resolution, rugged stability, and desired selectivity for the analysis of mAbs and related variants. The advanced surface bonding technology provides excellent chemical stability. MAbPac HIC-20 is particularly good for antibody oxidation and disulfide variants.

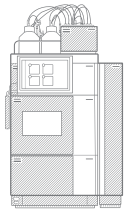
The workflow

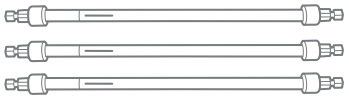
In contrast to RPLC, HIC mobile phases typically contain little or no organic solvent. The HIC mobile phase usually consists of a salting-out agent, which at high concentration retains the protein by increasing hydrophobic interaction between the protein and the stationary phase. Bound proteins are eluted in their native structure by decreasing the salt concentration, thus allowing the analysis of any conformational changes in the native protein using HIC.



Conformational variance and degree of oxidation (continued)

Workflow solution





Instruments → **Columns** → **Vials and caps**

Workflow solutions

Description	Quantity	Cat. no.
Thermo Scientific instruments		
Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system	Each	IQLAAAGABHFAPUMBHV
Thermo Scientific columns		
MAbPac HIC-20 column, 4.6 × 250 mm	Each	088554
Thermo Scientific vials and caps		
SureSTART 2 mL GOLD-Grade clear glass vial	100/pack	6PSV9-1PG
SureSTART 9 mm screw cap	100/pack	6PSC9TST

Antibody drug conjugates

Antibody-drug conjugates (ADCs) are a class of biopharmaceutical drugs designed as a targeted therapy for treating cancer and are typically composed of a mAb covalently attached to a cytotoxic drug via a chemical linker. ADCs are a hotspot for the research and development of anticancer drugs as they combine both the advantages of highly specific targeting ability and highly potent killing effect to achieve accurate and efficient elimination of cancer cells.

The conjugation of drugs often results in an ADC molecule that is heterogeneous with respect to both the distribution and loading of cytotoxic drugs on the mAb. Unconjugated mAbs have significantly lower potency, and the ADCs with high drug load are subject to rapid renal clearance. As the number of drugs attached to the mAb has been shown to directly affect the safety and the efficacy of the drug, it is therefore critical to fully characterize and monitor the heterogeneity of ADCs during development and production.

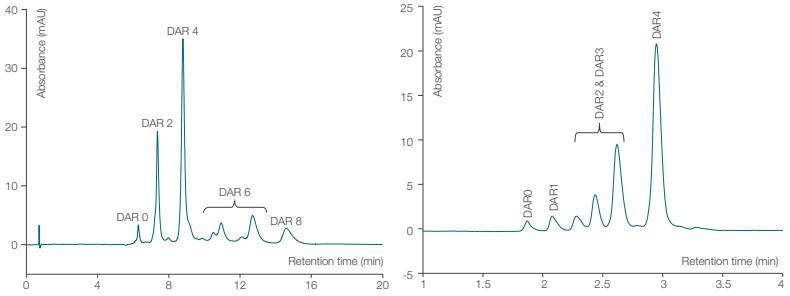


Figure 5. Separation of ADC with MAbPac HIC-Butyl and MAbPac RP – ADCs analysis by MAbPac HIC-butyl using (cysteine-conjugated ADC mimic sample) (left) and by MAbPac RP (Trastuzumab-MMAE sample) mimic (right). Read this **brochure** to learn more.

The columns

For non-mass spectrometry applications, hydrophobic interaction chromatography (HIC) is the most suitable method of choice to characterize the distribution of ADC molecules with different drug-to-antibody ratios (DARs):

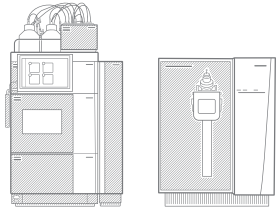
- The attachment of cytotoxin alters the hydrophobicity of the antibody so the least hydrophobic unconjugated antibody elutes first, and as the number of attached drugs increases, the elution time increases
- The most used HIC column for ADCs is the Thermo Scientific™ MAbPac™ HIC-Butyl column, packed with C4 bonded polymeric particles; the hydrophilic nature of polymer particles and the optimal density of butyl functional groups of the column lead to excellent biocompatibility, low carryover, and high resolution

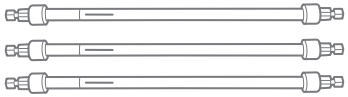
Mass spectrometry (MS) applications are becoming more popular with ADC characterization as it provides more in-depth understanding of the biomolecule and its various species. For mass spectrometry applications, it's important to have MS-suitable solvents, which rules out HIC due to its high salt content:

- Thermo Scientific™ MAbPac™ Reversed Phase (MAbPac RP) columns have a polymer particle with a significantly large pore size (1500 Å) where proteins can diffuse very efficiently. The polymer sorbent is an effective interaction source for hydrophobicity, and it also makes the column very robust, allowing for the use of stronger organic solvents and effective column cleaning reducing carryover.

Antibody drug conjugates (continued)

Workflow solution





Instruments → **Columns**

Workflow solutions

Description	Quantity	Cat. no.
Thermo Scientific instruments		
Thermo Scientific Vanquish Horizon UHPLC system	Each	IQLAAAGABHFAPUMZZZ
Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer	Each	BRE725535
Thermo Scientific columns		
MAbPac RP column, 4.0 µm, 2.1 × 50 mm	Each	088648
MAbPac HIC-Butyl column, 4.6 × 100 mm	Each	088558

Aggregate analysis

The complexity of developing and manufacturing mAb-based biotherapeutics necessitates several critical quality attributes (CQAs) that need to be measured and controlled to guarantee their safety and efficacy. The presence of **aggregates** in a formulated drug product must be assessed to avoid potential issues with immunogenicity. Aggregates are accumulated monomers of the biopharmaceutical, which stick together to form dimers, trimers, or larger order structures of antibody molecules. They are typically formed during fermentation, downstream product purification, or at storage or mishandling of the drug prior to patient administration.

Protein aggregation can cause adverse immunological reactions that result in serious safety and efficacy issues, and thus, must be monitored throughout the production process and during storage of the formulated bio-therapeutic. Size-exclusion chromatography (SEC) is the standard method for this aggregate analysis as the monomers can be differentiated from aggregates and mAb fragments by their size.

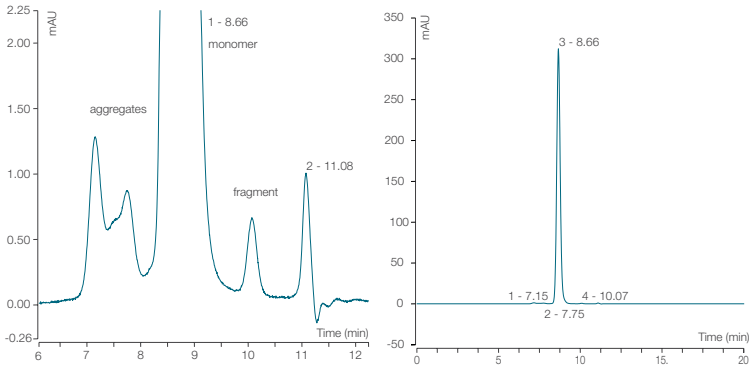


Figure 6. Separation of a mAb on a MAbPac™ SEC-1 – Rituximab SEC separation, expanded view (left), full range chromatograph (right). Read this **application note** to learn more.

The columns

The Thermo Scientific™ MabPac™ SEC-1 column is a silica-based UHPLC column covalently modified with a proprietary diol hydrophilic layer to prevent secondary interactions which can hinder the chromatography of certain proteins. SEC is one of the few chromatography methods that exhibits no 'on-column' focusing. Due to this, the pre-column dispersion on the system used is extremely important, especially at reduced flow rates on smaller i.d. columns, as there will be no focusing of broad peak volumes at the head of the column.

The MAbPac SEC-1 column separates by size. The pore size for this column (300 Å) was chosen to give a good separation in the molecular weight range of the monomer and dimers of a typical 150 kDa mAb, and thus, is ideal for mAb aggregate analysis.

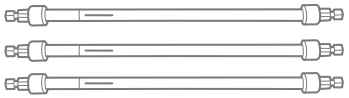
Thermo Scientific™ MabPac™ SEC-1 is compatible with UV, MALS, and also mass spectrometry, though the high salt concentrations can lead to corrosion of metal components, so a bioinert UHPLC system is recommended.

The workflow

Analysis of protein aggregation of biotherapeutic monoclonal antibodies (mAbs) run on Thermo Scientific™ MabPac™ SEC-1 columns on a low dispersion UHPLC system truly display the high-resolution ability of the column. Non-specific interactions with traditional column resins that occur during SEC analysis are eliminated when using the MAbPac SEC-1 column, and the column exhibits the required resolution for aggregate analysis. Using the MAbPac SEC-1 column allows a single globally applicable SEC chromatography method for biotherapeutic monoclonal antibodies.

Aggregate analysis (continued)

Workflow solution



Instruments → **Columns** → **Vials and caps**

Workflow solutions

Description	Quantity	Cat. no.
Thermo Scientific instruments		
Thermo Scientific Vanquish Horizon UHPLC system	Each	IQLAAAGABHFAPUMZZZ
Thermo Scientific™ Vanquish™ Diode Array Detector HL	Each	VH-D10-A
Vanquish™ LightPipe™ Flow Cells for the Vanquish™ Diode Array Detector HL	Each	6083.0100B
Thermo Scientific columns		
MAbPac SEC-1 column, 5 µm, 7.8 x 300 mm	Each	088460
Thermo Scientific vials and caps		
SureSTART 2 mL polypropylene vial	100/pack	6ESV9-1PP
SureSTART 9 mm screw cap	100/pack	6PSC9ST1

Glycan analysis

Glycosylation is the attachment of sugar moieties to proteins and is done as part of the post-translational modification (PTM) to optimize the biotherapeutics efficacy. The PTM is characterized by various linkages, including N-, O- and C-linked glycosylation, glypiation glycosylphosphatidylinositol (GPI) anchor attachment, and phosphor-glycosylation.

Biotherapeutic glycoproteins with complex glycosylation patterns have the potential to easily fall out of specification with changes in biomanufacturing processes. Glycosylation is one of the key critical quality attributes (CQAs) of mAb-based biotherapeutics, and any changes can impact a biological drug's safety, efficacy, clearance, and immunogenicity.

To meet regulatory demands such as ICH Q5E and ICH Q6B, manufacturers must carefully characterize glycosylation of proteins and its relation to the clinical activity of the medication. The complete analysis of a glycoprotein provides information on the primary structure of the oligosaccharides as well as their variation at individual glycosylation sites.

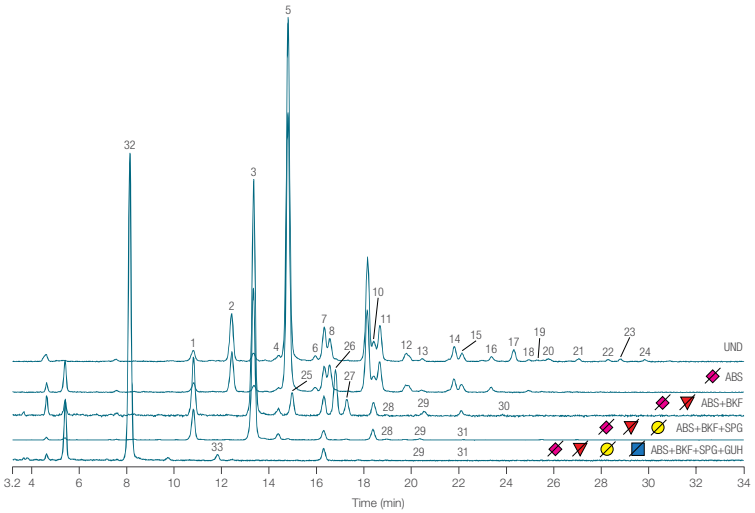


Figure 7. Glycan separation with Accucore 150 Amide HILIC column – HILIC chromatograms of the infliximab 2-AA labeled N-glycan pool (undigested, UND) and after digestion with a range of exoglycosidase enzymes. Separations were performed on an Accucore 150-Amide-HILIC 2.1 × 150 mm column. Read this application note to learn more.

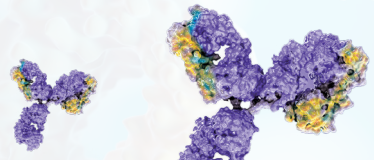
The columns

Many glycan analysis methods are run by high-performance liquid chromatography (HPLC) separation as this is a fast and robust strategy to obtain accurate data. Due to the polarity of the glycans, accurate and precise quantification of mAb-released N-glycans is best done on a Hydrophilic Interaction Chromatography (HILIC) column.

The **Thermo Scientific™ Accucore™ 150 Amide HILIC columns** are based on packing materials that offer unique selectivity, high throughput, and high efficiency in a solid core column, at a moderate column back-pressure. Their superb lot-to-lot column reproducibility and column lifetime demonstrate the consistency and robustness of the column.

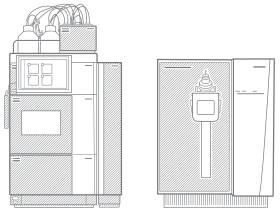
The workflow

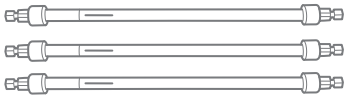
Glycan separation and characterization on an Accucore 150 Amide HILIC column coupled to a Vanquish Horizon UHPLC system provides a great platform with high efficiency and accurate fast profiling.



Glycan analysis (continued)

Workflow solution





Instruments → **Columns** → **Vials and caps**

Workflow solutions

Description	Quantity	Cat. no.
Thermo Scientific instruments		
Vanquish Flex Quaternary System	Each	IQLAAAGABHFAPUMBHV
Thermo Scientific columns		
Accucore 150 Amide HILIC column, 2.6 µm, 2.1 × 150 mm	Each	16726-152130
Thermo Scientific vials and caps		
SureSTART 2 mL GOLD-Grade clear glass vial	100/pack	6PSV9-1PG
SureSTART 9 mm screw cap	100/pack	6PSC9TST

Host cell proteins

Biotherapeutics such as mAbs and recombinant proteins are produced by DNA technology using non-human host cells such as Chinese hamster ovary cell lines. **Host cell proteins (HCPs)** are process-related impurities derived from host organism during biotherapeutics manufacturing.

Though most HCPs are removed by a number of specific chromatographic polishing steps such as protein A/G affinity chromatography, some may still be present at very low concentrations in the final product and can present potential safety risks or impact product stability and efficacy.

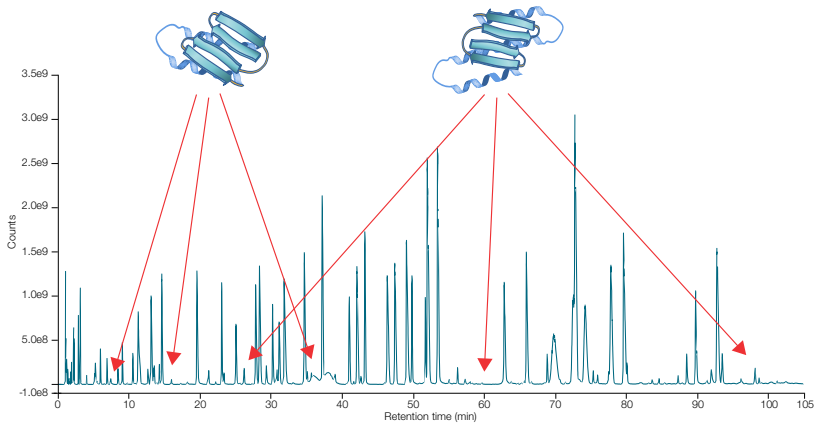
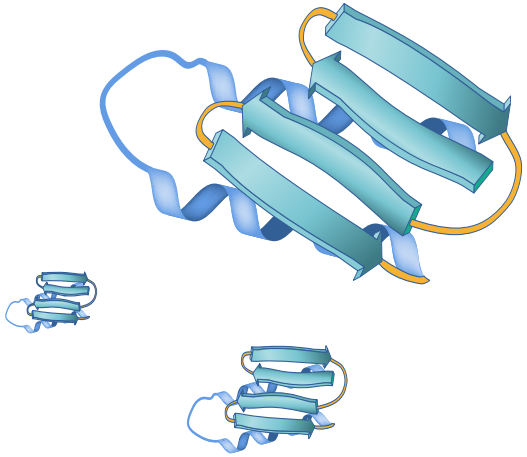


Figure 8. Deep analysis of HCPs enabled the identification of 51 proteins, including cathepsin L1. Cathepsin L1 may contribute to drug product degradation through hydrolysis at specific His-Thr sites in the upper hinge region. This emphasizes the importance of HCP monitoring to maintain product potency and stability over time. Read this **infographic** to learn more.

The detection of HCPs in biotherapeutic proteins is an important analytical critical quality attribute (CQA) requirement by regulatory agencies because HCPs can interfere with the drug product activity and/or stability, and potentially compromising patient safety.

Although the acceptable limits for HCP contamination in final product must be below 100 ppm, HCP analysis is challenging because up to six orders of magnitude of dynamic range are required to be able to detect low ppm concentrations of residual HCPs in biotherapeutics.

The most popular methods for HCP detection are ELISAs and protein gel blots. These analytical techniques use a semi-quantitative approach that targets only a small set of expected proteins and bears the risk of missing unexpected or unknown proteins that may still be present in the final drug product. An easy HCP analysis work-flow is done by optimizing a standard peptide mapping analysis of monoclonal antibodies.



Host cell proteins (continued)

The columns and sample prep

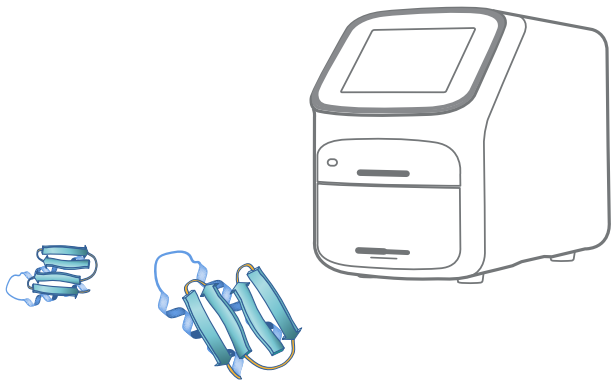
Thermo Scientific™ Acclaim™ Vanquish™ C18 UHPLC columns are highly retentive with a 2.2 µm silica particle and a pore size of 120 Å. These columns have a surface area of 300 m^2/g and 18% carbon content assuring maximum retention of complicated samples such as for peptide mapping.

Thermo Scientific™ SMART Digest™ trypsin kits provide high quality analytical results from protein digests. They provide a significant advance in sample preparation for biopharmaceutical protein research because they offer fast and simple protein digestion with high reproducibility, high sensitivity, and high levels of data quality in a format that's compatible with automation, reducing the time spent on a digest as low as 45 minutes. For automation of the digest process, use the kits with the Thermo Scientific™ KingFisher™ Duo Prime. (Click for more information on Kingfisher).

The workflow

The workflow based on a standard peptide mapping analysis of a mAb drug product with Thermo Scientific SMART Digest trypsin Magnetic bead kit for HCP and peptide mapping analysis provides high confidence results with excellent data quality.

The sample is loaded on a Thermo Scientific™ Acclaim™ Vanquish™ C18, 2.1 × 250 mm column for great reproducibility of the peptide separation.



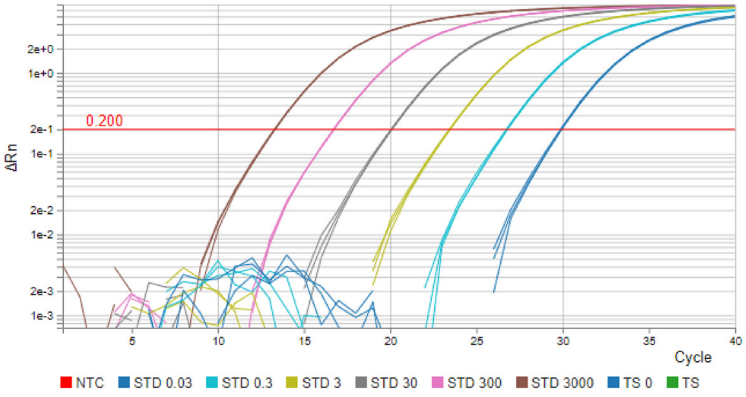
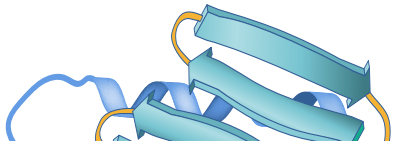
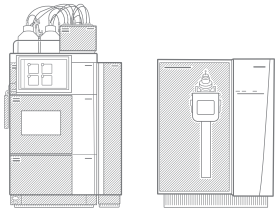


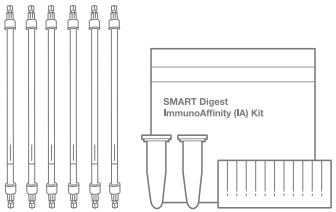
Figure 9. Host Cell DNA quantification. A complete solution with LOQ of 1.5 pg/mL



Host cell proteins (continued)

Workflow solution





Instruments → **Columns and consumables** → **Vials and caps**

Workflow solutions

Description	Quantity	Cat. no.
Thermo Scientific instruments		
Vanquish Flex Quaternary System	Each	IQLAAAGABHFAPUMBHV
Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer	Each	IQLAAEGAAPFALGMBDK
Orbitrap Exploris 480 mass spectrometer	Each	BRE725535
Thermo Scientific columns and consumables		
SMART Digest Trypsin Kit, magnetic bulk resin option	Each	60109-101-MB
Acclaim™ Vanquish C18, 2.2 µm, 2.1 × 250 mm column	Each	074812-V
Thermo Scientific vials and caps		
SureSTART 2 mL polypropylene vial	100/pack	6ESV9-1PP
SureSTART 9 mm screw cap	100/pack	6PSC9ST1

Peptide mapping

A novel approach to higher mapping sequences

Due to the inherent complexity of biotherapeutics, regulatory agencies require their **comprehensive characterization** of these drug products to ensure quality, safety, and efficacy. Primary sequence verification and the identification and relative quantitation of post-translational modifications (PTMs) of proteins is an important characterization step for therapeutic proteins and is frequently performed using peptide mapping methods.

Peptide mapping is used to measure several critical quality attributes (CQAs) required for the characterization of any biotherapeutic proteins and involves the treatment of proteins with a protease (e.g., trypsin) to produce a series of peptides which are separated, detected, and analyzed by liquid chromatography mass spectrometry (LC-MS).

LC-MS and the related multi-attribute method (MAM) analysis enables characterization and monitoring of a wide range of CQAs simultaneously.

Traditionally, a C18 reversed-phase column is used to separate the peptides based on their relevant hydrophobicity. This workflow presents a novel way to approach the more common aspects of MAM analysis by introducing not only a new protocol to protein digestion, but also a new selectivity for separation, both with impeccable results.

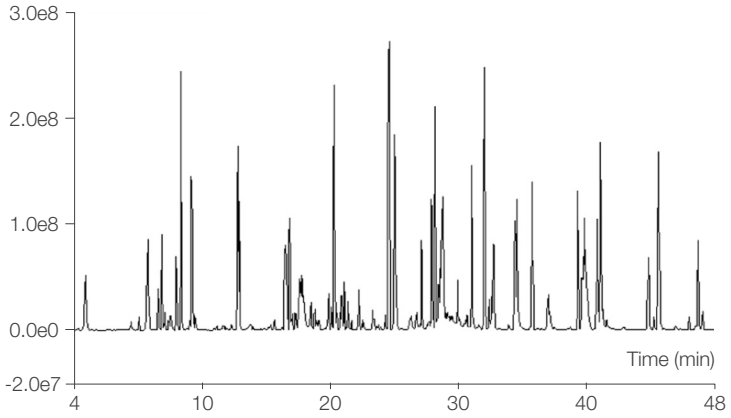
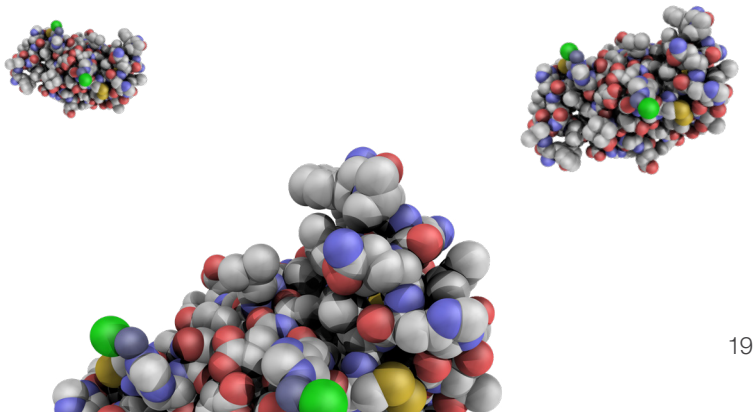


Figure 10. C4 reversed-phase separation column for peptide mapping - Hypersil GOLD C4 column performance chromatogram using commercial reference mAb (USP mAb3) digested using the two-step SMART digest workflow. Read this **publication** to learn more.



Peptide mapping (continued)

A novel approach to higher mapping sequences (continued)

The columns and sample prep

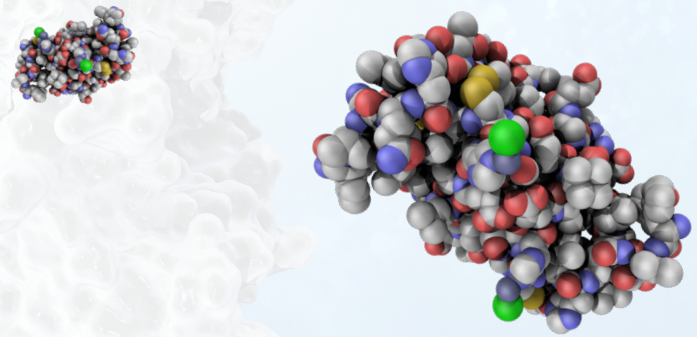
Thermo Scientific™ Hypersil™ GOLD C4 UHPLC columns have ultrapure silica particles bonded with a short butyl chain for a weak hydrophobic retention. The quality of the silica minimizes secondary interactions that could cause undesired retention such as peak tailing. Hypersil GOLD has 175 Å pore size which allows for continuous diffusion and less resistance to mass transfer and is highly beneficial for the peak shape of the peptide.

Traditional trypsin digests are labor-intensive and time-consuming processes that can take over 24 hours, involving multiple manual steps that can decrease reproducibility and sensitivity. Thermo Scientific™ SMART Digest™ Kits provide a significant advance in sample preparation for biopharmaceutical protein research because they provide fast and simple protein digestion with high reproducibility, high sensitivity, and high levels of data quality in a format that's compatible with automation, reducing the time spent on a digest to as low as 20 minutes (for a monoclonal antibody).

The workflow

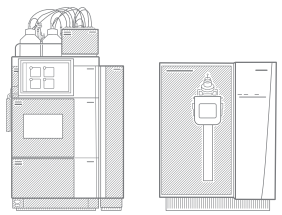
A two-step SMART digestion protocol is presented in this modified workflow, which includes a short high-temperature digestion step followed by a lower temperature digestion step, as opposed to the original one-step digestion at a high temperature. The new automatic digestion workflow significantly reduces the number of missed cleavages, obtaining a more reproducible digestion profile.

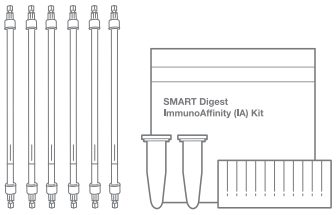
Selecting a C4 reversed-phase column, instead of the traditional C18, provides significant reduction of hydrophobic peptide bleeding in chromatographic wash steps, as well as significant reduction of hydrophobic peptide carryover to subsequent runs. Other benefits to using a C4 column include less tailing and more symmetrical peaks, and average MS/MS sequence coverages that don't change when using a C4 column chemistry.



Peptide mapping (continued)

A novel approach to higher mapping sequences workflow





Instruments → **Columns and consumables** → **Vials and caps**

Workflow solutions

Description	Quantity	Cat. no.
Thermo Scientific instruments		
Thermo Scientific Vanquish Horizon UHPLC system	Each	IQLAAAGABHFAPUMZZZ
Thermo Scientific™ Orbitrap™ Ascend Tribrid™ mass spectrometer	Each	FSN06-10000
Thermo Scientific columns and consumables		
Thermo Scientific Hypersil GOLD C4 column, 1.9 µm, 2.1 × 150 mm	Each	25502-152130
SMART Digest Kits	Each	60109-101
Thermo Scientific filter		
UHPLC Direct Connect Filter Holder	Each	27006
2.1 mm I.D. Replacement Filter Cartridge, 0.2 µm	Each	22180TS
Thermo Scientific vials and caps		
SureSTART 2 mL polypropylene vial	100/pack	6ESV9-1PP
SureSTART 9 mm screw cap	100/pack	6PSC9ST1

Easy scouting setup for new protein therapeutics

Biotherapeutic proteins will unavoidably contain some level of heterogeneity and impurities that are derived from their way of production and purification from a biological cell culture system, as well as their further storage and handling.

Exploring analytical methods for characterizing new biotherapeutics can be complex. As seen in this workflow guide thus far, characterization of biotherapeutics are commonly performed by:

- Ion exchange chromatography (IEX) that can either be cation or anion exchanger to separate charge variant analysis (CVA)
- Hydrophobic interaction chromatography (HIC) that addresses protein heterogeneity by looking into the differences in hydrophobicity between protein variants
- Size exclusion chromatography (SEC) that can monitor the extent of aggregation and higher order molecular structure

These three (IEX, HIC, and SEC) methods of separation operate under native conditions and the protein remains in its functional form. A direct consequence of this setting is that the pH needs to be carefully optimized and controlled during method development for these separation techniques. This can amount to a substantial workload, could lead to variability in the results and lack of method robustness, and a tremendous amount of time consumption.

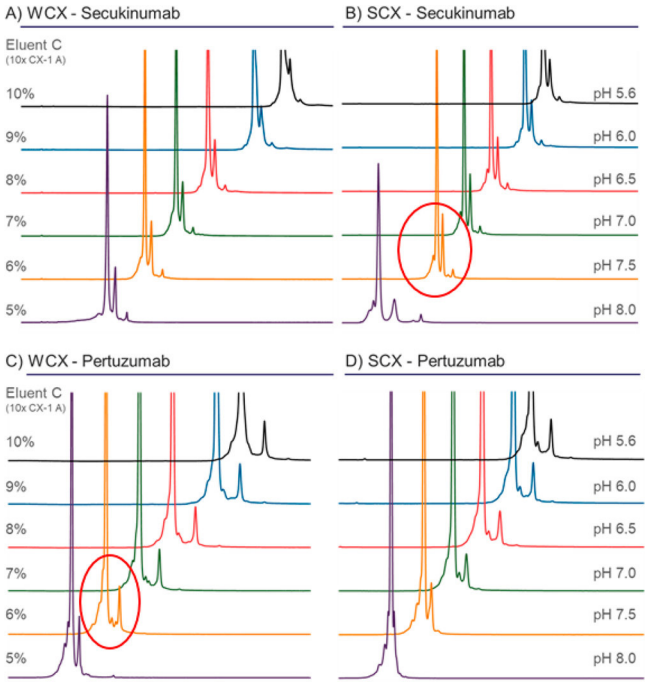
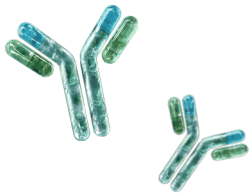


Figure 11. Effect of different pH values on salt gradient separation of charge variants from mAb. Read this publication to learn more.



Easy scouting setup for new protein therapeutics (continued)

The workflow

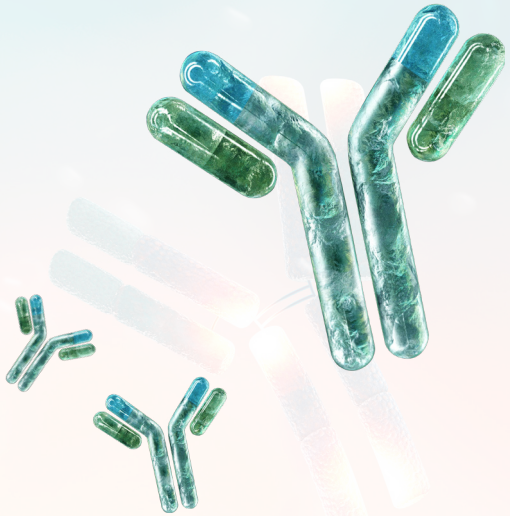
This workflow and recommended reading provide an automated method scouting approach that can help you select different methods requiring minimal manual intervention.

Importantly, a scouting setup is best performed on longer columns to maximize resolution. Hence, despite performing a pH gradient for cationic and anionic ion chromatography, respectively, the column is longer than recommended in the previous workflow. Column length can always be adjusted before moving the method into production.

Online pH and column scouting for charge variant analysis with the universal eluent system. The left panels (A, C) (see Figure 11) show the results for a ProPac Elite WCX, 5 µm particle size column and on the right (B, D) for the MAbPac SCX-10, 5 µm particle size column for secukinumab and pertuzumab, respectively. The best conditions are outlined with a red circle in the chromatograph.

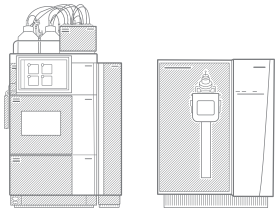
Additionally, as chromatographic methods are part of the typical purification schemes for therapeutic proteins, the efficacy of fractionation can be determined by comparing the sample chromatogram at the detector with the resulting elution profile achieved by re-analyzing the collected fractions. Higher performance fraction collectors like the Vanquish Fraction Collector enable fractionation with such high resolution and precision that the resulting elution profile matches exactly the corresponding peaks of the sample chromatogram.

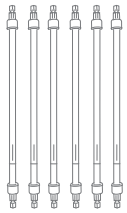
The application of pH or buffered salt gradients enables the separation on ion-exchange stationary phases, but usually hinders a direct hyphenation with mass spectrometry (MS) for further characterization of the species. Manual fraction collection and re-analysis with MS-friendly mobile phases is common practice. Two-dimensional liquid chromatography (2D-LC) (as described in this application note) provides a straightforward way to enable direct MS analysis of the first dimension ion-exchange fractions after desalting in the second dimension.



Easy scouting setup for new protein therapeutics (continued)

Workflow solution





Instruments → **Columns and gradient buffers** → **Vials and caps**

Workflow solutions

Description	Quantity	Cat. no.
Thermo Scientific instruments		
Thermo Scientific™ Vanquish™ Method Development HPLC and UHPLC System	Each	VQ-METHOD-DEV
Vanquish Analytical Purification LC system including Vanquish Fraction Collector	Each	VQ-ANALYTICAL-PUR
Thermo Scientific™ Vanquish™ Online 2D-LC Systems	Each	VQ-ONLINESPE
Thermo Scientific columns		
ProPac 3R SCX column, 4 × 100 mm	Each	43103-104068
ProPac Elite WCX column, 4 × 150 mm	Each	302972
ProPac 3R SAX column, 4 × 100 mm	Each	43203-104068
MAbPac HIC-butyl column, 4.6 × 100 mm	Each	088558
MAbPac SEC-1 column, 4 × 300 mm	Each	074696
Thermo Scientific gradient buffers		
Thermo Scientific CX-1 pH gradient buffer A	Each	303274
Thermo Scientific CX-1 pH gradient buffer B	Each	303275
Thermo Scientific vials and caps		
SureSTART 2 mL GOLD-Grade clear glass vial	100/pack	6PSV9-1PG
SureSTART 9 mm screw cap	100/pack	6PSC9TST

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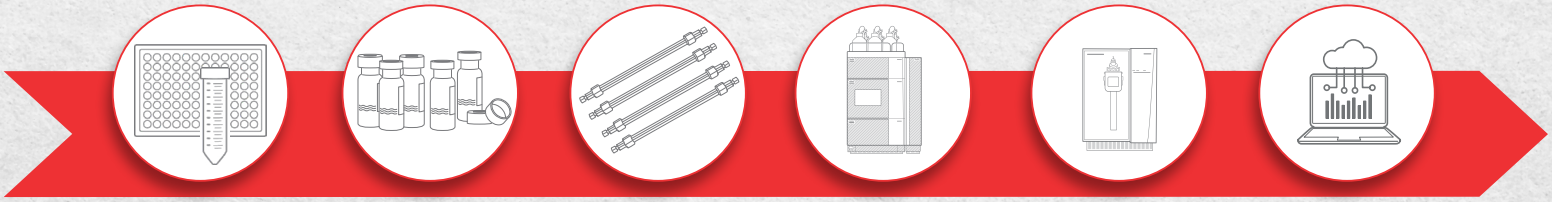

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