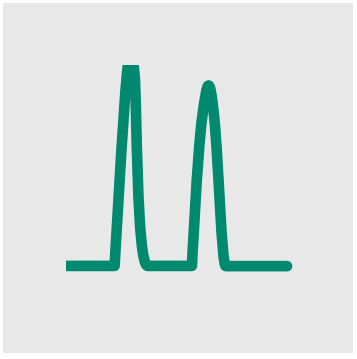
Protein purification troubleshooting guide

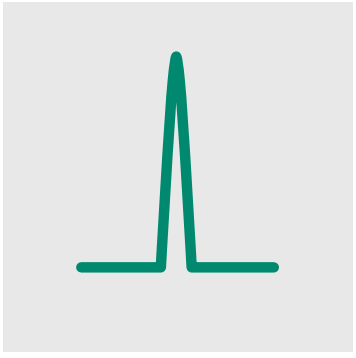
Pure protein today. Powerful results tomorrow.

Pressure and flow rate

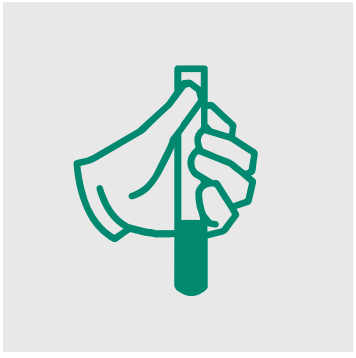
Retention time



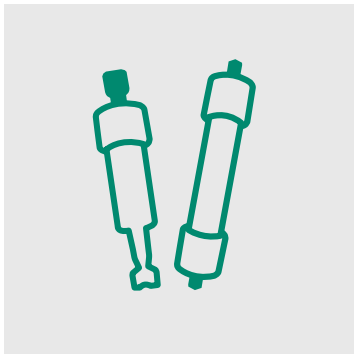
Purity and resolution



Peaks



Protein recovery and activity



The column

For ordering information, visit cytiva.com

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Pressure and flow rate

Flow is reduced or slow through column

Possible cause	Remedy
Bed compressed	• Clean column according to cleaning procedures and/or change filter • Check sample preparation. If sample is too viscous, back pressure will be high. Dilute sample or reduce flow rate. • Repack column, pack new column, or use prepacked column
Microbial growth has occurred in column	• Clean column using recommended methods • Prepare and use predefined column and system maintenance methods. Make it a habit to include these methods in a method queue. • Always filter samples and buffers. Choose low protein binding membranes such as Whatman™ regenerated cellulose. • Store in presence of 20% ethanol when not in use
Clogged end-piece, adapter, or tubing	• Remove and clean or replace if possible
Outlet closed or pumps not working	• Ensure that column outlet is open • Check pumps for signs of leakage. If using a peristaltic pump, also check tubing.
Too small system tubing i.d. for flow rate used	• Change tubing to larger inner diameter (i.d.)
Sample too viscous	• Dilute sample with buffer • Maintain protein concentration below 50 mg/mL • Reduce flow rate during sample loading using pressure-flow regulation functionality available on most modern chromatography systems
Sample not filtered properly	• Clean column, filter sample with a low protein binding filter (e.g. Whatman SPARTAN™ filter), and repeat
Clogged column filter	• If possible, replace filter or clean column with reversed flow according to cleaning procedures • Always filter samples and buffers before use. Choose low protein binding membranes such as Whatman regenerated cellulose. • Reduce flow rate during sample loading using pressure-flow regulation functionality available on most modern chromatography systems

Back pressure increases

Possible cause	Remedy
Precipitated protein in column filter and/or at top of bed	• Clean using recommended methods • Prepare and use predefined column and system maintenance methods • If possible, exchange or clean filter or use new column • If additives were used for initial sample solubilization, include them in running buffer
Clogged in-line filters	• Change inlet filter or clean, if possible. Some systems have a filter on top in the mixer.
Turbid sample	• Extend the lysis time or change lysis method • Improve sample solubility by adding ethylene glycol, urea, detergents, or organic solvents. See resin or column instructions.

Retention time

Protein elutes earlier than expected

Possible cause	Remedy
IEX*, HIC*: Column equilibration incomplete	• Repeat or prolong equilibration step until conductivity and pH are constant
IEX: Ionic strength of sample or buffer too high or pH is incorrect	• Decrease ionic strength of sample or buffer • Increase pH (anion exchanger); Decrease pH (cation exchanger)
HIC: Salt concentration of sample and buffer too low	• Increase salt in sample and buffer

Protein elutes later than expected/not at all

Possible cause	Remedy
Proteins or lipids precipitated on column or column filter	• Clean column and exchange or clean filter
Protein might be unstable or inactive in elution buffer	• Determine pH and salt stability of protein
Delivered gradient is distorted	• Air bubble caught in pump(s): Purge pumps according to user manual • Pump check valve malfunction: Flush check valves at high flow rate and/or clean with ultrasonic bath • Worn pump sealing ring: Change sealing rings

* SEC = size exclusion chromatography
IEX = ion exchange chromatography
HIC = hydrophobic interaction chromatography

SEC*:

Ionic interactions between protein and matrix	• Maintain ionic strength of buffers above 50 mM (preferably include up to 300 mM sodium chloride)
Hydrophobic interactions between protein and matrix	• Reduce salt concentration to minimize hydrophobic interaction. Increase pH. Add suitable detergent or organic solvent (e.g., 5% isopropanol).

IEX:

Incorrect buffer pH	• Check pH meter calibration. Use buffer pH closer to pI of protein.
Ionic strength too low	• Increase salt concentration in elution buffer
Hydrophobic interactions between protein and matrix	• Reduce salt concentration to minimize hydrophobic interaction. Increase pH. Add suitable detergent or organic solvent (e.g., 5% isopropanol).

HIC:

Salt concentration too high	• Decrease salt concentration in elution buffer
Hydrophobic interactions too strong	• Use resin with lower hydrophobicity or lower ligand density • Consider using an additive to reduce hydrophobic interaction

Protein elutes before void volume (SEC)

Possible cause	Remedy
Channeling in column	• Repack column using thinner slurry of resin. Avoid introduction of air bubbles.

* SEC = size exclusion chromatography
IEX = ion exchange chromatography
HIC = hydrophobic interaction chromatography

Purity and resolution

Poor resolution/purity (General)

Possible cause	Remedy
Column poorly packed	• Do a column performance test. Repack if needed • Use prepacked columns
Large mixing spaces at top of column	• Adjust top adapter to resin surface if necessary
Elution conditions not optimal (e.g., gradient too steep, flow rate too high)	• Change elution conditions (e.g., use shallower gradient, reduce flow rate)
Proteins precipitated in column	• Follow cleaning procedures in instructions • **HIC*:** Reduce salt concentration in buffer, or use existing buffer but apply aliquots of sample that has low salt concentration • **IEX*:** Modify buffer, pH, and/or salt conditions during run to maintain stability
Tubings in chromatography system too long and wide	• Decrease tubing diameter and minimize length
Separated proteins diluted between column outlet and UV flow cell	• Minimize volumes after column by decreasing tubing diameter and minimizing length • Change to injection and column valves and flow cells with smaller volumes

Poor resolution/purity (SEC*)

Possible cause	Remedy
Sample too viscous	• Dilute with buffer, but check maximum sample volume. Maintain protein concentration below 50 mg/mL
Sample contains particles	• Re-equilibrate column, filter sample with a low protein binding filter (e.g. Whatman SPARTAN filter), and repeat
Column is dirty	• Clean and re-equilibrate
Incorrect SEC resin type	• Check selectivity curve in available selection guides
Sample volume too large	• Check recommendations, and decrease sample volume loaded
Flow rate too high	• Check recommendations, and reduce flow rate
Sample diluted between injection valve and column inlet, between column outlet and UV flow cell, and/or further to fraction collector	Minimize volumes before and after column by either • Decreasing tubing diameter and minimizing length • Mounting column directly to UV cell (without column valve) • Removing all unnecessary components in flow path • Change to injection and column valves and flow cells with smaller volumes

* SEC = size exclusion chromatography
IEX = ion exchange chromatography
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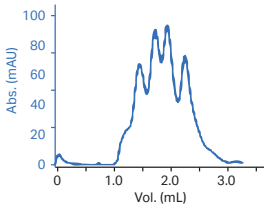
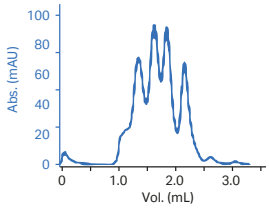
Tubing inner diameter (i.d.) affects resolution

Column: Superdex™ 200 5/150 GL;
Flow rate: 0.3 mL/min

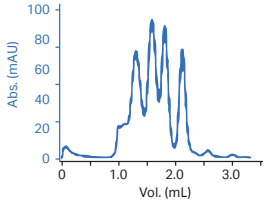
Tubing i.d., 0.75 mm



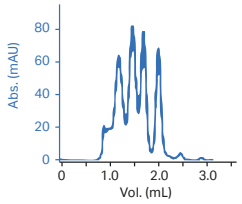
Tubing i.d., 0.50 mm



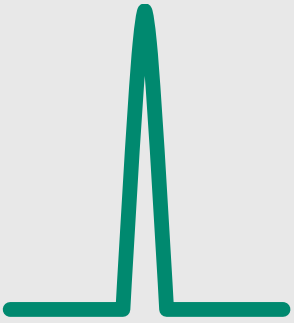
Tubing i.d., 0.25 mm



Tubing i.d., 0.15 mm



Peaks



Fronting peaks

Possible cause	Remedy
Column overloaded	• Decrease sample load and repeat
Column is "overpacked"	• Do a column performance test. Repack using lower flow rate. • Use prepacked columns
Channeling in column	• Repack column using a thinner slurry of resin. Check column packing.
Column contaminated	• Clean using recommended procedures

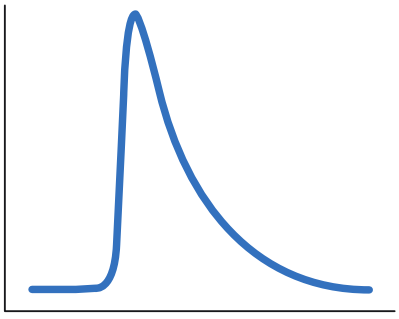
Tailing peaks

Possible cause	Remedy
Column is "underpacked"	• Do a column performance test. Repack using higher flow rate. • Use prepacked columns
Sample is not binding to column due to incorrect start buffer conditions	• Adjust pH. Check salt concentration in start buffer.
Sample too viscous	• Dilute sample in start buffer.
Column contaminated	• Clean using recommended procedures
Band broadening due to large volume in system	• Check modules, tubing, and connections for unnecessarily large volumes

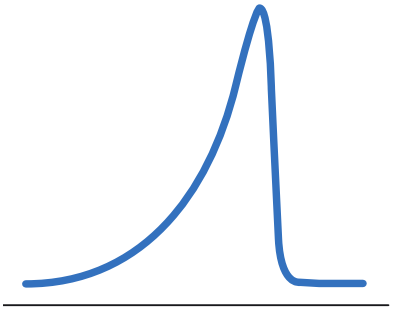
Peaks are not detected or are too small

Possible cause	Remedy
Sample absorbs poorly at chosen wavelength	• Use a different wavelength (e.g., 214 nm instead of 280 nm)
Excessive band broadening	• Check column packing. Repack if necessary or use prepacked columns.
UV baseline rises with gradient because of buffer impurities	• Use high-quality reagents

Tailing peak



Fronting peak



Protein recovery and activity

Protein recovery is higher than expected

Possible cause	Remedy
Proteins co-eluting with other substances	• Optimize running conditions to improve resolution • Check buffer conditions used for assay before and after run • Check selection of resin
Cross-contamination from a previous run on the same column	• Clean using recommended procedures • If purifying several antibodies from several sources or batches, use a column packed with MabSelect SuRe™ (NaOH CIP* can be used)

Poor binding of protein

Possible cause	Remedy
Sample has wrong pH or buffer conditions incorrect	• Use a desalting column packed with Sephadex™ G-25 to transfer sample into correct buffer
Column not equilibrated sufficiently in buffer	• Repeat or prolong equilibration step until conductivity and/or pH are constant
Microbial growth has occurred in column	• Clean according to cleaning procedures and store in 20% ethanol when not in use
Metal ion stripping from IMAC* resin	• Use a desalting column packed with Sephadex G-25 to remove metal ion stripping agents from sample or use a column packed with Ni Sepharose™ excel resin (e.g. HisTrap™ excel)
Binding capacity of resin is exceeded	• Pack a larger column • If using a HiTrap™ column, connect up to three columns in series

Activity is higher than expected

Possible cause	Remedy
Different assay conditions used before and after chromatography step	• Use same conditions for all assays
Inhibitors removed during separations	• Use a desalting column packed with Sephadex G-25/dialyze original sample before measuring activity, because cell lysates/extracts often contain low molecular weight substances that can affect activity

Protein recovery is lower than expected

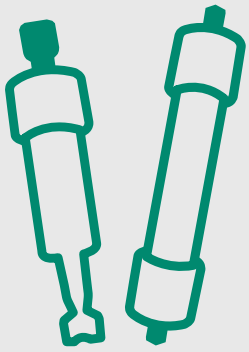
Possible cause	Remedy
Protein degraded by proteases	• Add protease inhibitors to sample and buffers to prevent proteolytic digestion • Run sample through a resin such as Benzamidine Sepharose 4 Fast Flow (high sub) to remove trypsin-like serine proteases
Protein adsorbed to filter during sample preparation	• Use another type of filter with low protein binding (e.g., Whatman SPARTAN syringe filters)
Proteins precipitated	• **HIC*:** Check salt conditions; adjust to improve solubility **IEX*:** Check pH and salt conditions; adjust to improve solubility
Hydrophobic interactions are occurring	• **IEX:** Add denaturing agents, polarity-reducing agents, or detergents. Add 10% ethylene glycol to running buffer to prevent hydrophobic interactions. **SEC*, AC*:** Use denaturing agents, polarity-reducing agents, or detergents
Nonspecific adsorption to resin	• **IEX:** Reduce salt concentration to minimize hydrophobic interaction. Add suitable detergent or organic solvent (e.g., 5% isopropanol). **SEC:** Increase salt concentration in the buffer, up to 300 mM sodium chloride
Proteins not eluting	• **HIC:** Consider use of additives to reduce hydrophobic interactions, or use a less hydrophobic resin. **AC:** If using competitive elution, increase concentration of competitor (e.g., imidazole) in elution buffer

Activity is low, but recovery is normal

Possible cause	Remedy
Protein might be unstable or inactive in buffer	• Determine pH and salt stability of protein • Include additives to stabilize protein of interest
Enzyme separated from co-factor or other necessary component	• Test by pooling aliquots from fractions and repeating assay

* SEC = size exclusion chromatography, IEX = ion exchange chromatography, HIC = hydrophobic interaction chromatography, AC = affinity chromatography, IMAC = immobilized metal ion affinity chromatography, CIP = cleaning in place

The column



Bubbles in bed

Possible cause	Remedy
Buffers not properly degassed	• Degas buffers thoroughly. Run degassed equilibration buffer through column to remove air.
Inappropriate sample loading or purification method construction	• Use air sensors to prevent air from entering system
Column hardware inappropriately assembled or mechanically damaged	• Ensure that column is correctly assembled and free from damage before packing
Blocked or partially blocked inlet filter	• Change inlet filter or clean if possible
Column packed or stored at cool temperature and then warmed up	• Remove small bubbles by passing degassed buffer through column • Take special care if buffers are used after storage in cold room or refrigerator • Do not allow column to warm up in sunshine or by heating system. If possible, repack column.
Other restrictions in flow path before pump	• Check tubing and connections on inlet side

Space between resin bed and adapter

Possible cause	Remedy
Back pressure increase or bed insufficiently packed	• Turn down adapter to resin surface. Do a column performance test. Repack if needed.
Column packing not performed according to instructions	• Repack according to recommended protocol
Flow rate too high	• Do not exceed maximum flow rate for resin or prepacked column
Resin bed compressed	• Repack using lower flow rate • Use prepacked columns • Check that system back pressure is not too high. Are there any restrictions in system?
Column operated at too high pressure	• If using recommended flow rates, clean column according to instructions • Do not exceed recommended operating pressure for resin or prepacked column • For self-packed columns, use "Column handling" functionality in UNICORN™ system control software to save a defined column with its pressure data. Then select this method to protect this column type from too high pressure.
Rapid pressure change	• Avoid an abrupt change to high flow rate • Do not turn valves during flow

Air in the column

Compressed bed

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