HiFliQ® Ni Advance FPLC Columns

User Guide

Protein Ark's HiFliQ® Ni Advance FPLC columns designed for rapid one-step purification, and ideal for preparative purification and contaminant removal. HiFliQ® Ni Advance FPLC columns are supplied pre-packed and ready to use with pre-charged Fastback Ni Advance Agarose resin for affinity purification of poly-histidine tagged recombinant proteins by immobilized metal ion affinity chromatography (IMAC).

The Ni Advance Agarose resin provides high binding capacity with negligible Ni^{2+} ion leakage for high stability, chemical compatibility and reuse. Compatible with all common liquid chromatography instruments (including ÄKTA™ FPLC's), peristaltic pumps and syringes.

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Features of the HiFliQ® Ni Advance FPLC columns:

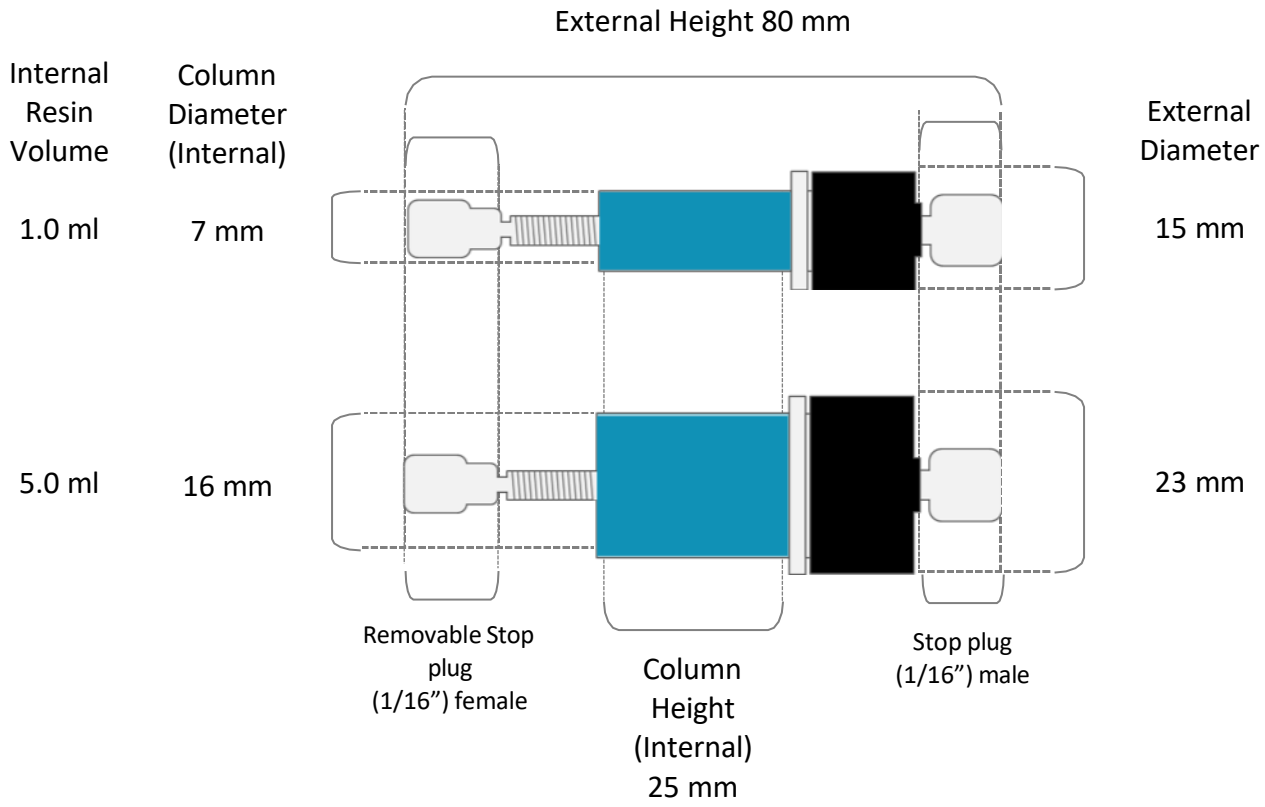
- Fast and reliable affinity purification.
- Pre-packed 1 ml and 5 ml columns with Fastback Ni Advance Agarose resin.
- Nickel ions are carefully loaded onto a 6% highly cross-linked agarose matrix via chelating coupled ligand to obtain a stable affinity matrix with a high binding capacity for histidine residues.
- High protein binding capacity combined with negligible Ni^{2+} ion leakage.
- Keep your buffer in its preferred conditions as Fastback Ni Advance resin is resistant to EDTA (up to 20mM) and DTT (up to 20mM) for IMAC chromatography – Ni ions remain bound to ligand
- Biocompatible polypropylene casing.
- Universal 10.32 (1/16'') UNF threads (Inlet Female/Outlet Male) compatible with all common chromatography instruments (including ÄKTA™ FPLC's and Bio-Rad NGC's).
- 10-32 finger tight connector supplied with all HiFliQ®s.
- Compatible with low pressure pumps (requires a 1/16'' male connector) and syringes (requires a Luer Female – 1/16'' male connector).
- Connect in series for increased capacity.

ÄKTA™ is a registered trademark of GE Healthcare Limited.

Specification:

Item:	HiFliQ®1-Ni-Adv	HiFliQ®5-Ni-Adv
Column Volume:	1 ml	5 ml
Resin:	Fastback Ni Advance	Fastback Ni Advance
Base Matrix:	6% cross-linked Agarose	6% cross-linked Agarose
Coupled ligand:	Chelating ligand	Chelating ligand
Metal ion capacity:	>75 µmol/ml	>75 µmol/ml
Typical Binding Capacity:	80 mg/ml	400 mg/ml
Mean Bead Size:	90 µm	90 µm
Recommended flow rate:	1 ml/min	1-5 ml/min
Max. operating pressure:	0.5 MPa (72 psi)	0.5 MPa (72 psi)
External Dimensions:	15 mm D. x 80 mm H.	23 mm D. x 80 mm H.
Column Dimensions (internal):	7 mm D. x 25 mm H.	16 mm D. x 25 mm H.
Column Construction:	Polypropylene	Polypropylene
Inlet Port:	10-32 (1/16") Female	10-32 (1/16") Female
Outlet Port:	10-32 (1/16") Male	10-32 (1/16") Male
Storage (2-8°C):	20% Ethanol	20% Ethanol

HiFliQ® FPLC column schematic:



NOTE:	HiFliQ® FPLC columns are supplied with a moulded removable (1/16") female stop plug attached to the (1/16") male outlet port. Remove prior to use, reverse and use to seal the column for storage.
NOTE:	HiFliQ® FPLC columns cannot be opened or repacked.

Chemical compatibility:

Buffer compatibility	
Phosphate buffers:	Up to 100mM phosphate buffer recommended
Tris, HEPES & MOPS:	Up to 100 mM (secondary or tertiary amines may reduce the metal ions)
pH range:	3.0-12.0
Chelating agents	
*EDTA:	Up to 20 mM
Sulfhydril reagents	
β-mercaptoethanol:	Up to 20 mM (can cause some reduction of metal ions)
**DTT:	Up to 20 mM
TCEP:	Up to 20 mM
Denaturants	
Urea:	8 M
Guanidinium hydrochloride:	6 M
Detergents	
DM (n-Decyl-β-Dmaltopyranoside):	1.00%
DDM (n-Dodecyl-β-Dmaltoside):	1.00%
NM (n-Nonyl-β-Dglucopyranoside):	1.00%
OG (n-Octyl-β-Dglucopyranoside):	1.50%
TDM (n-Tetradecyl-β-Dmaltopyranoside):	0.005%
Triton®:	2.00%
Tween®:	2.00%
NP-40:	2.00%
Cymal 6:	1.00%
Fos-Choline 16:	0.05%
CHAPS:	up to 1%
Other additives	
Imidazole:	500 mM
NaCl:	2.0 M (recommended concentration 300 mM)
$MgCl_2$:	Up to 4 M
$CaCl_2$:	Up to 5 mM
Glycerol:	Up to 50%
Methanol:	100%
Ethanol:	100%
Acetonitrile:	30% (v/v)

*NOTE: Stability measured after 24 hours incubation with 20 mM EDTA results in no decay in binding capacity.
**NOTE: Stability measured after 24 hours incubation with 20 mM DTT results in no decay in binding capacity.

Principles of metal chelate chromatography:

Immobilized metal ion affinity chromatography (IMAC) technology was introduced by Porath *et al* (1975). Certain amino acids such as histidine, tryptophan, cysteine and tyrosine can act as electron donors on the surface of the protein and bind reversibly to a transition metal ion. IMAC purification utilizes this metal ion binding by coupling a chelating group (such as Nitrilotriacetic Acid (NTA) or Iminodiacetic Acid (IDA)) to a stable chromatographic resin (such as Agarose). Transition metal ions such as Ni^{2+}, Co^{2+}, Cu^{2+}, or Zn^{2+} (see Porath and Olin,

1983; Porath, 1988; Sulkowski, 1989) can then be loaded and immobilised onto the chelating group enabling high affinity binding, in the majority of cases this is via an 6-8x polyhistidine-tag engineered at the N- or C-terminus of a recombinant protein (K_d-10^{-13} at pH 8.0). Nickel and Cobalt are the most widely used metal ions as they confer the highest affinity (Ni^{2+}>Co^{2+}) with the highest specificity (Co^{2+}>Ni^{2+}) for the IMAC tags.

The simplicity of IMAC technology is extremely attractive as it lends itself to a bind-wash-elute mode of operation if the appropriate buffer formulations are selected. IMAC purification can also be achieved using samples without any prior treatment (e.g. buffer exchange steps). The use of metal chelate affinity is widespread for the selective adsorption of engineered recombinant proteins and has largely superseded non-affinity methods of chromatography for purifying recombinant proteins.

Often a researcher needs to choose the correct base matrix, select the optimal activation chemistry and couple a suitable ligand to develop an affinity column to purify their target protein. Protein Ark's HiFliQ® columns remove the guesswork associated with optimizing the resin chemistry by offering high quality 1 and 5 ml FPLC columns pre-packed and pre-charged with high capacity Fastback Ni Advance resin that satisfied stringent quality control to guarantee reproducible purification performance.

Fastback Ni Advance resin:

Nickel ions are carefully loaded onto an agarose matrix via chelating coupled ligand to obtain a stable affinity matrix with a high binding capacity for histidine residues. The resin is charged with Ni^{2+} to give a marine blue appearance. Fastback Ni Advance is very stable and can resist the following conditions in most situations: Buffers at pH 4-9, 100% methanol, 100% ethanol, 8 M urea, 6 M guanidinium hydrochloride, 30% (v/v) acetonitrile, 20 mM DTT, 20 mM EDTA. Fastback Ni Advance is also stable at pH >9.0 and can be regenerated by alkaline solutions, such as sodium hydroxide.

Application drivers for Metal Chelate chromatography:

Conditioned media secreting target proteins
Screening expression clones for high levels of His-tagged proteins.
Purification of recombinant proteins for raising antibodies.
Purification of recombinant proteins for activity and/or structural studies.

General considerations for selecting optimal binding conditions for the IMAC resin:

This resin exploits the polyhistidine sequence that permits efficient purification of the expressed protein from a broad host such as: bacterial cells, yeast cells, insect cells, or mammalian cells. Lysis conditions are dependent upon the type of expression system and nature of the recombinant protein. Mammalian or Baculovirus-infected insect cells can be lysed by sonication at +4°C with either freeze/thaw cycles or the addition of up to 1 % non-

ionic detergents. While, cell lysis of *E.coli* bacteria is usually achieved by sonication on ice or homogenization either with or without lysozyme treatment.

The culture pellet containing the expressed protein is harvested and resuspended in lysis buffer at a pH close to pH 7.4-8.0 using a similar concentration of buffer, imidazole, and salt to that of a pre-equilibration buffer used for the IMAC column. Typically, a protease inhibitor cocktail, such as Boehringer "Complete EDTA-free", along with other additives like DNase I (5-50 µg/ml), Benzonase® and 5 mM TCEP are added to the lysis buffer. The inclusion of β-mercaptoethanol to the lysis, binding, wash and elution buffers is optional and depends upon whether the His-tagged protein elutes with any additional contaminants which may be linked via disulphide bonds.

If the binding efficiency is found to be poor and the lysis buffer differs significantly from the pre-equilibration buffer, it is recommended that the lysate be dialysed, titrated with a concentrated stock solution, or buffer exchanged using an ultrafiltration device with a more appropriate pre-equilibration buffer.

It is imperative that the lysate is completely clear prior to loading on the column as any particulate matter (e.g. cell debris) may partially foul and clog-up the resin resulting in an increased back pressure and reduced flow rates. This will significantly increase the binding, washing and elution times and effect the final purify of the eluted protein. It is recommended that the cleared lysate be filter just prior to loading even if it has been previously filtered several days before. Ideally samples should be processed rapidly and, if the need arises, the protein purified at 4°C. It is also recommended that number of freeze/thaw cycles be minimised during storage to reduce the amount of aggregation/precipitation of the proteins.

Optimal buffer conditions for binding the target molecule to a resin are critical for successful separation of the protein. If the binding conditions are not optimal with respect to pH, salt concentration, presence of metal ions or chelating agents, flow rates, residence time ...etc, purification can be adversely affected.

Choosing the correct buffer conditions for IMAC separations:

We recommend sodium phosphate buffers as buffers with secondary or tertiary amines e.g. Tris buffers can significantly reduce the Nickel and Cobalt ions. pH levels of 7-8 works well for most immobilized Ni^{2+} applications and 0.15-0.5 M NaCl is often added to the buffers to prevent non-specific ionic interactions occurring and to stabilize the proteins in solution.

When a recombinant protein is expressed at high levels in *E.coli*, the protein can often elute as insoluble aggregates called inclusion bodies. Non-ionic detergents such as 8 M urea and 6 M guanidinium hydrochloride do not interfere with metal chelate affinity separations. These denaturants completely unfold the target protein making the His-tag much more accessible for interaction with the immobilized Ni^{2+} matrix (see the section 'protein purification under denaturing conditions' for further details).

Protein purification under native conditions:

This protocol describes the purification of recombinant His-tagged proteins from an *E. coli* cell pellet under native conditions using Protein Ark HiFliQ® Ni Advance FPLC Columns. Reagent amounts given apply to IPTG-induced bacterial culture of a well-expressed protein (approximately 10-50 mg/l). Cells are lysed with lysozyme because it is an inexpensive and efficient method for cells that have been frozen. However, lysis methods based on physical disruption (e.g., sonication or homogenization) or detergents (e.g., CHAPS) can also be used. The His-tagged protein is then purified from the cleared lysate under native conditions in a bind-wash-elute procedure. This method is most efficient when the target protein is present at low concentrations or the His-tag is not fully accessible. It is recommended that the bind-wash-elute conditions be tested and optimised in order to achieve the optimum conditions for purification. All volumes are given in column bed volume (CV).

Example: Buffers for NATIVE purifications using imidazole elution.

Lysis buffer:

50 mM Sodium phosphate buffer, 0.3 M NaCl, 10 mM imidazole, pH 8.0

Wash buffer:

50 mM Sodium phosphate buffer, 0.3 M NaCl, 20 mM imidazole, pH 8.0

Elution buffer:

50 mM Sodium phosphate buffer, 0.3 M NaCl, 500 mM imidazole, pH 8.0

NOTE:	Imidazole concentrations required for lysis, wash and elution are protein-specific. Higher or lower concentrations may be required. We recommend a concentration gradient using the elution buffer to identify the minimum imidazole concentration required for protein elution from the column.
NOTE:	The precise buffer conditions for your target protein may require optimizing as there are several factors which may affect protein stability, accessibility of the His-tag, and the protein behaviour under native conditions during IMAC purification.

Procedure

1. Thaw the *E. coli* cell pellet on ice.

NOTE: Freezing the cell pellet at –20°C for 30 min prior to incubation at room temperature improves lysis by lysozyme.

2. Resuspend the cell pellet in 'Lysis buffer' (50 ml / litre cell media) supplemented with 1 mg/ml Lysozyme.

NOTE: Benzonase® is recommended to reduce the viscosity caused by the nucleic acid (3 U/ml bacterial culture) if mechanical lysis is not used.
NOTE: Add 1 protease inhibitor table to reduce protease activity and protein cleavage

3. Incubate at room temperature for >30 min (or > 1 hour at 4°C) on an end-over-end shaker.

NOTE: Mechanical lysis can also be used by repeated freeze/thaw, vortexing, homogenization, sonication or french press if required

4. Centrifuge the lysate for 30 min at room temperature and 10,000 x g. Collect the supernatant.

NOTE: If the supernatant remains cloudy then repeat step 4.

5. Connect the HiFliQ® Ni Advance column to an FPLC/syringe/pump and wash with 3-5 CV's of distilled water to remove the 20% ethanol before equilibrating with 5 CV's of 'Lysis buffer'.

6. Filter the cleared lysate through a 0.2 µm syringe filter directly before loading onto the column at the recommended flow rate.

NOTE: For optimal binding reduce the flow rate over the column to maximise the interaction time with the resin.

7. After loading wash the column with 'Wash buffer' until the measured absorbance (OD=280nm) reaches a stable baseline.

NOTE: This will typically take 10-15 CV's.
NOTE: Keep the wash fractions for SDS-PAGE analysis if required.

8. Elute the His-tagged protein with a one-step, multiple steps or a linear gradient using the 'Elution buffer'.

NOTE: A longer elution gradient maybe required to separate proteins of similar binding affinities.
NOTE: Collect the eluate in separate tubes for SDS PAGE analysis and protein concentration determination.

9. Analyze all fractions by SDS-PAGE.

NOTE: Western Blot experiment using an anti-His antibody can be performed if required.

NOTE: Under normal conditions Ni^{2+} leakage is low and the column can be washed with 5 CV's of distilled water before storing in 20% ethanol. The column is then ready for re-use.
NOTE: It is recommended that the column is cleaned, prior to use with a different recombinant protein to avoid cross-contamination.

Protein purification under denaturing conditions:

This protocol describes the purification of recombinant His-tagged proteins from an *E. coli* cell pellet under denaturing conditions using Protein Ark HiFliQ® Ni Advance FPLC Columns. Reagent amounts given apply to IPTG-induced bacterial culture of a well-expressed protein (approximately 10-50 mg/l). Cells are lysed with a high concentration of urea, which also aids to dissolve insoluble protein aggregates. The His-tagged protein is then purified from the cleared lysate under denaturing conditions in a bind-wash-elute procedure. This method is most efficient when the target protein is present at low concentrations or the His-tag is not fully accessible. Two examples of different buffer conditions are given using imidazole (example 1) and acidic pH (example 2). It is recommended that the bind-wash-elute conditions be tested and optimised in order to achieve the optimum conditions for purification. All volumes are given in column bed volume (CV).

Example 1: Buffers for DENATURING purifications using imidazole elution.

Denaturing lysis buffer:

50 mM Sodium phosphate buffer, 0.3 M NaCl, 8 M urea (or 6 M guanidinium hydrochloride), 10 mM imidazole, pH 8.0

Denaturing wash buffer:

50 mM Sodium phosphate buffer, 0.3 M NaCl, 8 M urea (or 6 M guanidinium hydrochloride), 20 mM imidazole, pH 8.0

Denaturing elution buffer:

50 mM Sodium phosphate buffer, 0.3 M NaCl, 8 M urea (or 6 M guanidinium hydrochloride), 500 mM imidazole, pH 8.0

Example 2: Buffers for DENATURING purifications using low pH elution.

Denaturing lysis buffer:

100 mM Sodium phosphate buffer, 10 mM tris, 8 M urea, pH 8.0

Denaturing wash buffer:

100 mM Sodium phosphate buffer, 10 mM tris, 8 M urea, pH 6.3

Denaturing elution buffer:

100 mM Sodium phosphate buffer, 10 mM tris, 8 M urea, pH 4.5

NOTE:	Pre-filter all buffers through a 0.2 µm filter prior to use
NOTE:	Reducing agent such DTT or TCEP can be added to the buffers prior to use in order to reduce the disulphide bonds if required.
NOTE:	0.5-1 M NaCl can be added to the buffers and samples to eliminate ion-exchange effects that may affect separation.

Procedure

1. Thaw the E. coli cell pellet on ice.

2. Resuspend the cell pellet in 'Denaturing lysis buffer' (50 ml / litre cell media).

NOTE: Benzonase® is recommended to reduce the viscosity caused by the nucleic acid (3 U/ml bacterial culture) if mechanical lysis is not used.

3. Incubate at room temperature for >30 min on an end-over-end shaker.

NOTE: Mechanical lysis can also be used by repeated freeze/thaw, vortexing, homogenization, sonication or french press if required.

4. Centrifuge the lysate for 30 min at room temperature and 10,000 x g. Collect the supernatant

NOTE: If the supernatant remains cloudy then repeat step 4.

1. Connect the HiFliQ® Ni Advance column to an FPLC/syringe/pump and wash with 3-5 CV's of distilled water to remove the 20% ethanol before equilibrating with 5 CV's of 'Denaturing lysis buffer'.

6. Filter the cleared lysate through a 0.2 µm syringe filter directly before loading onto the column at the recommended flow rate.

NOTE: For optimal binding reduce the flow rate over the column to maximise the interaction time with the resin.

7. After loading wash the column with 'Denaturing wash buffer' until the measured absorbance (OD=280nm) reaches a stable baseline.

NOTE: This will typically take 10-15 CV's.
NOTE: Keep the wash fractions for SDS-PAGE analysis if required.

8. Elute the His-tagged protein with a one-step, multiple steps or a linear gradient using the 'Denaturing elution buffer'.

NOTE: A longer elution gradient maybe required to separate proteins of similar binding affinities.
NOTE: Collect the eluate in separate tubes for SDS PAGE analysis and protein concentration determination.

9. Analyze all fractions by SDS-PAGE.

NOTE: Optional Western Blot experiment using an anti-His antibody can be performed if required.

NOTE: Under normal conditions Ni^{2+} leakage is low and the column can be washed with 5 CV's of distilled water before storing in 20% ethanol. The column is then ready for re-use.
NOTE: It is recommended that the column is cleaned, prior to use with a different recombinant protein to avoid cross-contamination.

Desalting and concentrating the purified protein:

Imidazole, EDTA, or acid pH solutions do not need to be removed for your downstream application since the Fastback Ni Advance resin is resistant to EDTA (up to 20mM) and DTT (up to 20mM) for IMAC chromatography – Ni ions remain bound to ligand.

Protein refolding:

Attempts can be made to renature the target protein by dialyzing it sequentially against binding buffers containing decreasing levels of urea or by passing decreasing levels of urea in binding buffer over the washed protein bound to the HiFliQ® Ni Advance FPLC column and eluting the refolded protein with 300 mM imidazole (between pH 7 and pH 8). Alternatively, denatured proteins can be diluted into a large volume of buffer lacking denaturant. The dispersive effect dilutes out the denaturant resulting in the re-folding of the protein.

Column washing and regeneration conditions:

Protein Ark HiFliQ® Ni Advance FPLC columns should be carefully washed after each run, and regenerated after 2-5 runs (though we recommend to regenerate the resin after each run if used under reducing conditions). This protocol describes the washing and regenerating procedures for 1 and 5 ml HiFliQ® Ni Advance FPLC columns. Do not strip the Ni ions with EDTA. All volumes are given in column bed volume (CV).

Wash procedure: recommended after each run.

1. Connect the HiFliQ® Ni Advance column to an FPLC/syringe/pump and wash with 10 CV's of distilled water.

2. Wash the column with 10 CV's 500 mM NaOH

3. Wash the column again with 10 CV's distilled water

4. Wash the column with 10 CV's Neutralization buffer (150 mM sodium chloride; 200 mM Na2HPO4, pH 7.0)

5. Wash the column again with 10 CV's distilled water
6. Wash the column with 10 CV's 20% (v/v) Ethanol, 10 mM sodium acetate, pH 6.50 for storage

Side note:

The ligand cannot be stripped with EDTA. The Nickel Ions bind strongly to it. Therefore this protocol does not contain a "reload" with new nickel ions as it the case with Ni-NTA regeneration protocol

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Storage conditions:

Item:	HiFliQ®1-Ni-Adv	HiFliQ®5-Ni-Adv
Shipping:	20 % Ethanol at room temperature	20 % Ethanol at room temperature
Short-term storage:	Equilibration buffer	Equilibration buffer
Long-term storage:	20% Ethanol at 4°C	20% Ethanol at 4°C

NOTE:	Do not freeze or store the column in buffer or water for long periods. Each column is stored in 20 % ethanol and reusable for up to 2 years from the date of resin manufacture. Please see the label for the recorded expiry date.

Questions and answers:

1. *What is the shelf-life of the HiFliQ® Ni Advance FLPC Column?*
   The resin is guaranteed for 2 years after the date of manufacture provided they are stored at 2-8°C.

2. *Do I need to filter the buffers prepared in my laboratory?*
   It is good laboratory practice to filter all buffers.

3. *How should I prepare my sample for metal chelate separation?*
   Many chromatographic procedures demand that the sample is pre-conditioned prior to loading. We recommend that all samples are filtered to at least 0.45 µm (preferably 0.20 µm) pore size. High viscosity is mostly attributed to contaminating DNA or RNA. The intrinsic viscosity of a lysate can be reduced by either drawing it through a syringe needle several times or by adding appropriate amounts of DNase and/or RNase (5-10 µg/ml) to the lysis buffer and incubating the mix on ice for 15 mins.

4. *Should I add β-mercaptoethanol (or DTT / TCEP) to the lysis buffer?*
Reducing agents are compatible up to 20 mM DTT and 20 mM EDTA with this resin.

5. *How can I regenerate the metal chelate resin?*
We recommend that you wash the resin with elution buffer and then re-equilibrate the resin with binding buffer. Proceed to the pre-equilibration step if resin is to be re-used immediately. After regeneration, the resin can also be stored in a screw-capped bottle containing 0.1 % sodium azide (made up in distilled water) at 2-8 °C until further use.

6. *How can I ensure that levels of contaminants in the final eluate remain low?*
We recommend that the binding buffer contains minimum 10 mM imidazole and the wash buffer contains minimum 20-30 mM imidazole.

7. *Should I be concerned if the column partially dries out during the chromatographic steps?*
The resin is robust. Partially dried resin rehydrates rapidly. There are no adverse effects upon the performance of the resin.

8. *Should I remove imidazole after the final elution step?*
You should always remove imidazole if the protein is going to be stored. Otherwise, the protein may precipitate out of solution at -20°C or -80°C.

9. *Can I load purified protein immediately on to an SDS-gel?*
Proteins purified under native conditions can be loaded on to an SDS-polyacrylamide gel. Those proteins purified under denaturing conditions in 6-8 M urea can also be loaded directly on to a denaturing SDS-polyacrylamide gel. However, proteins purified in the presence of 4-6 M guanidine hydrochloride should be buffer exchanged using an ultrafiltration device into a buffer lacking the denaturant prior to loading on to a denaturing SDS-PAGE.

10. *Do I need to remove the His-tag from the recombinant protein after purification?*
For most applications it is not necessary to remove the terminal His-tag. However, it can affect the activity, stability, or structure determination. If required a protease cleavage site (e.g. Factor Xa Protease, TEV, or enterokinase) can be engineered between the His-tag and the target protein. The tag can then be cleaved off and the protein re-purified by passing it back through the IMAC column in order to remove the digested tag and undigested His-tagged protein.

11. *Under what circumstances can I re-use the column?*
The HiFliQ® Ni Advance FPLC columns are designed for re-use. We recommend regular cleaning between purifications in order to maintain performance.

Troubleshooting assistance:

Bubbles or cracks appear in the resin bed:

- The resin has been stored at a cool temperature and then rapidly warmed up. The resin should be warmed slowly to room temperature before use.

The sample does not flow easily through the column:

- The resin is clogged with particulates. Pre-filter the sample just before loading it on to the metal chelate resin.
- If the column is not stored at 2-8°C, or it has been used more than once and stored in the absence of a bacteriostat, microbial growth may restrict flow through the column.

No elution of the target protein is observed from the column:

- The elution conditions are too mild to desorb the target protein. Use a higher concentration of imidazole or lower the elution pH further!
- Ensure that the resin is blue in appearance. Otherwise the expressed protein will not bind effectively to the resin.
- Ensure that there are no chelators or reductants in the sample which will interfere with binding of the target protein to the resin.
- The protein may have precipitated in the column. Try denaturing conditions!
- The cell disruption method may have liberated proteolytic activities. Purify the protein under denaturing conditions if you do not need to purify an active protein.

The recovery of target protein is low:

- The His-tag maybe inaccessible. Try increasing the length of the spacer between the His-tag and protein, move the affinity tag to the other end of the protein or perform the purification under denaturing conditions.
- Ensure that the column binding capacity is proportionate to the level of expressed His-tagged protein. The target protein may pass through into the sample wash if the capacity of the column is insufficient for the level of expressed protein. If necessary connect up multiple columns in series to increase the capacity.
- Confirm levels of target protein by immunoassay. This will help determine if your cell disruption methods have been successful.
- The target protein may contain hydrophobic stretches which could have been toxic to the host bacterium, *E.coli*.
- Ensure that the protein is not insoluble i.e. exists in inclusion bodies and resides in the pellet. Solubilize the insoluble protein using 6-8 M urea or 4-6 M guanidine hydrochloride.
- Add further protease inhibitors to the buffers as the full-length protein may have been degraded by hydrolytic enzymes. Alternatively, reduce the time of expression, lower the temperature at which the protein is exposed or use special *E.coli* strains devoid of proteases.

Poor resolution of the target protein:

- The sample volume or concentration may be too large for the capacity of the column. In this case, reduce the sample load or sample volume.
- The sample may need to be filtered carefully.

The target protein elutes at an unexpected position:

- There may be an ionic interaction between the protein and metal chelate resin. You should maintain the ionic strength above 0.1 M.
- There may be hydrophobic interactions between the sample and the resin. In this instance, reduce the salt concentration and add suitable detergents or organic solvents.
- Co-purification of contaminants may occur if both the expressed protein and the contaminant have similar affinities for the matrix. In this case, a further chromatographic method such as gel filtration or ion exchange chromatography is recommended.

The elution profile cannot be reproduced:

- The nature of the sample may have altered and so it may be important to prepare a fresh sample. The His-tag may have been removed by proteases. Work at 2-8°C and add a protease inhibitor cocktail to the lysis buffer.
- Accessibility of the His-tag may have altered. If the His-tag becomes buried in the protein, the binding capacity of any metal chelate resin for this target protein will be significantly reduced under native conditions. In this instance, the purification needs to be performed under denaturing conditions.
- The sample load may be different from the original sample load. It is advisable to keep all these parameters constant.
- Proteins or lipids may have precipitated within the column. Use elution conditions, which stabilize the sample.
- The buffer pH and ionic strength are incorrect and new buffers will need to be prepared.

Glossary:

affinity chromatography - chromatographic separation based on a specific interaction between an immobilized ligand and a binding site on a macromolecule.

baculovirus – a virus vector for expression of recombinant proteins in insect cells.

chaotropic agent - a molecule which interferes with hydro-phobic interactions by disrupting the ordered structure of water molecules. Examples include urea and guanidine hydrochloride.

chelating agent – a compound such as EDTA or EGTA that is able to combine with a metal ion to form a structure with one or more rings.

cleared lysate – the soluble cell extract after the cell debris and other particulates have been removed by centrifugation.

column bed volume (CV) - the total volume occupied by the chromatographic packed bed. It is also referred to as the column volume or CV.

DL-Dithiothreitol (DTT) – reducing agent used to break disulphide bonds.

expression vector – a cloning vector intended for the foreign gene to be expressed in the host organism.

french pressure cell – a device that uses high shear forces to rupture microbial cells. The suspension is poured into a chamber, which is closed at one end by a needle valve and at the other end by a piston. Pressures of up to 16,000 lb/in2 are applied by a hydraulic press against a closed needle valve. When the desired pressure is attained, the needle valve is fractionally opened to marginally relieve the pressure. The cells subsequently expand and rupture, thereby releasing the cellular components through the fractionally open valve.

freeze-thawing – a method that is sometimes used to break open cells by successive periods of slow freezing and thawing. Ice crystals are generated during the freezing stage, which disrupt the cells when they melt during thawing. The method, however, is slow and releases a limited amount of subcellular components.

his - a 3 letter symbol for L-histidine

his-tag – a permanent affinity tag engineered into the expression vector upstream or downstream of the gene of interest to facilitate the purification of the recombinant protein. The His-tag doesn't normally have any effect upon the protein structure or function, it comprises 6-8x Histidine residues (polyhistidine) and has a molecular weight of 0.7-0.9 kDa

iminodiacetic acid (IDA) – chelating group used for immobilizing metal ions.

immobilized metal ion affinity chromatography (IMAC) – method of protein affinity purification using immobilized metal ions.

immobilized - bound to a surface, usually through covalent linkages.

inclusion bodies – quite a lot of proteins form insoluble crystalline aggregates known as inclusion bodies when they are expressed at high levels inside bacteria. The proteins can be solubilized using denaturants such as 8 M urea or 6 M guanidine hydrochloride.

ion exchange chromatography - chromatographic separation based on different charge properties of macromolecules.

isoelectric point - the pH at which the protein has no net charge.

lysozyme – an enzyme than hydrolyzes β-1,4-linkages between N-acetylmuramic acid and 2-acetamido-2-deoxy-D-glucone in peptidoglycan heteroploymers of prokaryotic cell walls. An example is egg white lysozyme and this enzyme is used to disrupt cells in order to liberate expressed proteins. 1 mg/ml lysozyme is normally added to *E.coli* cells in lysis buffer and incubated for 30 min to aid cell disruption. The pH optimum for lysozyme is pH 9.2 (Davies *et al* 1969).

nitrilotriacetic acid (NTA) – chelating group used for immobilizing metal ions.

recombinant protein – a protein coded for by a cloned gene which has often been modified *to increase the expression of that protein or to alter the properties of the protein.*

sonication – this technique uses ultrasonic energy to generate high transient pressures that are believed to disrupt the cells.

tris(2-chloroethyl) phosphate (TCEP) – strong irreversible reducing agent used to break disulphide bonds.

truncate - terminate prematurely or to shorten by cutting.

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