Protein purification with PureCube Ni-NTA FPLC columns

Automated purification of His-tag fusion proteins

1. Description

The PureCube Ni-NTA FPLC column is an IMAC column prepacked with PureCube Ni-NTA agarose resin for the purification of His-tagged recombinant proteins. It is available in 1 mL and 5 mL bed volumes, making it suitable for both analytical and preparative applications. The column is compatible with most standard FPLC systems, including ÄKTA chromatography systems.

The Ni-NTA resin selectively binds His-tagged proteins through coordination between immobilized nickel ions and the imidazole side chains of histidine residues. It exhibits a binding capacity of approximately 80 mg/mL, determined using a 27 kDa 6×His-tagged GFP protein. The Ni-NTA resin is compatible in presence of 1 mM DTT, 1 mM EDTA, 100% methanol, 100% ethanol, 8 M urea, 6 M guanidinium hydrochloride, 30% (v/v) acetonitrile. Furthermore, the Ni-NTA resin can be regenerated using an established regeneration protocol, allowing repeated use while maintaining consistent binding capacity.

Overall, the PureCube Ni-NTA FPLC provides a straightforward, reliable, and high-performance purification strategy for His-tagged proteins within automated FPLC workflows.

2. General information & required material

All necessary buffers for protein purification and subsequent regeneration of the resin are listed in the following table and should be prepared freshly.

Buffers/solutions	
Binding buffer	50 mM phosphate buffer, 300 mM NaCl, 10 mM imidazole
Wash buffer	50 mM phosphate buffer, 300 mM NaCl, 20 mM imidazole
Elution buffer	50 mM phosphate buffer, 300 mM NaCl, 250 mM imidazole
Regeneration wash buffer	0.5 M NaOH, 2 M NaCl, 2% (v/v) LDAO Note: LDAO is only required if a membrane protein was purified on the resin being washed or regenerated. An alternate detergent may be used but generally we recommend LDAO because it is non-ionic, harsh to proteins, and easily washed off the resin.
100 mM EDTA	-
10 mM $NiSO_4$	-
Regeneration buffer	100 mM Acetic acid (97%), 150 mM NaCl
20 mM Tris buffer, pH 8.0	-
20% (v/v) ethanol	-

The composition of all purification buffers can be modified to suit the properties of the target protein. A list with compatible reagents is available at https://cube-biotech.com/products/protein-purification-products/his-tag-affinity-resins-magbeads/ni-nta/purecube-ni-nta-fplc-column/31302. Please note that the resin is very stable and can resist

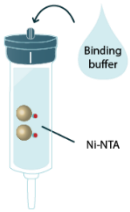
the following conditions in most situations: pH 2-14, 100% methanol, 100% ethanol, 8 M urea, 6 M guanidinium hydrochloride, 30% (v/v) acetonitrile.

3. Protocol

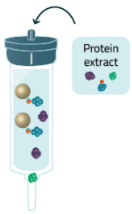
3.1. FPLC column-based protein purification

PureCube Ni-NTA columns are compatible with all common liquid chromatography instruments, such as Äkta® systems, and can be directly connected to the chromatography workstation. If fittings other than 10-32 are required, connect adapters to the FPLC column beforehand.

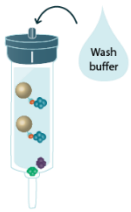
- In all steps, use recommended volumes or wash until the baseline is stable
- Depending on the FPLC column used, a flow rate of 0.5–3 ml/min is recommended:
  0.5-1 ml/min for 1 ml cartridges (operational pressure of up to 5 bar)
  1-3 ml/min for 5 ml cartridges (operational pressure of up to 3 bar)
- Use **freshly prepared buffers** for all steps.
- All solutions have a temperature of 4 °C or ambient temperature! Hot sodium hydroxide can harm resin and chromatography material!



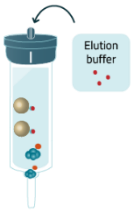
3.1.1. Equilibrate the FPLC column with 5 CV (column bed volumes) of Binding Buffer.
3.1.2. Monitor the flow-through at 280 nm; the baseline should be stable after washing with 5 CV.



3.1.3. Apply sample to FPLC column. Begin with a flow rate of 1 ml/min. Monitor pressure at this step. If the sample is very viscous and pressure is increased significantly, reduce viscosity of the sample by dilution with Binding buffer or reduce flow rate.
Collect the flow-through for SDS-PAGE analysis.



3.1.4. **Wash** with **Wash Buffer until A_{280} is stable**. Usually, 10-20 CVs are sufficient to reach the baseline.
Collect fractions for SDS-PAGE analysis.



3.1.5. **Elute** the protein with **Elution buffer until baseline at A_{280} is reached**.
Collect fractions for SDS-PAGE analysis.
Note: Due to an increased imidazole concentration in the elution buffer, the baseline is expected to be higher than at the end of the column wash.

3.2. Washing of the FPLC column

Ni-NTA agarose resins should be washed after each run and regenerated at the latest after 5 runs (though we recommend regenerating the resin after each run, if possible).

3.2.1	Wash the FPLC column with 10 CV ddH_2O or until baseline is stable.
3.2.2	Wash the FPLC column with 10 CV regeneration wash buffer.
3.2.3	Rinse the FPLC column with 10 CV ddH_2O.
3.2.4	Wash the FPLC column with 10 CV 20% (v/v) ethanol. The matrix is now ready to be re-used.

3.3. Washing and Regeneration and storage of the FPLC column

Ni-NTA agarose resins should be washed after each run, and regenerated latest after 5 runs (though we recommend regenerating the resin after each run, if possible).

3.3.1	Wash the FPLC column with 10 CV ddH_2O or until baseline is stable.
3.3.2	This step is only required after using DTT: Wash the FPLC column with 10 CV 1-3% (v/v) HCl. Minimize the exposure time of the resin to HCl. Afterwards, wash the columns with 10 CV ddH_2O. Note: The concentration of HCl depends on the extent to which the resin is reduced. For example, 1% HCl was sufficient to strip Ni-NTA and Ni-IDA resin exposed to 1 mM DTT, 2% HCl for 5 mM DTT, and 3% for 10 mM DTT.
3.3.3	Wash the FPLC column with 10 CV 100 mM EDTA
3.3.4	Rinse the FPLC column with 10CV ddH_2O.
3.3.5	Wash the FPLC column with 10 CV regeneration wash buffer.
3.3.6	Rinse the FPLC column with 10 CV ddH_2O.
3.3.7	Wash the FLPC column with 10 CV 10 mM $NiSO_4$ to recharge the matrix.
3.3.8	Rinse the FPLC column with 5 CV ddH_2O.
3.3.9	Regenerate the FPLC column by washing with 5 CV regeneration buffer.
3.3.10	Wash the column twice with 5 CV ddH_2O.
3.3.11	Wash with 5 CV 20 mM Tris pH 8.0.
3.3.12	Rinse the column twice with 5 CV ddH_2O.
3.3.13	For Storage: Equilibrate the FPLC column in 20% (v/v) ethanol and store at 2–8 °C.

4. Troubleshooting

No or weak binding to Ni-INDIGO column	
pH is not correct	The resin is stable at pH 2-14.
His-tag is not present.	Add protease inhibitors during cell lysis and work quickly at 2-8 °C. If E. coli is used as expression host, use a protease deficient expression strain.

His-tag is not accessible.	Fuse the tag with the other protein terminus, use a different linker, or increase the number of histidine (His_6 to His_{10}).
His-tag has been degraded.	Check if the tag is associated with a portion of the protein that is processed. If it is the case, change the position of the tag. Avoid purification in discontinuous batch mode. Prolonged batch incubations increase the risk of proteolytic degradation of the target protein including cleavage of the tag.
His-tag is partially accessible.	Reduce the washing volume.
Protein Degradation and Aggregation	Protein of interest is degraded by proteases that can be found inside the cell lysate. These need to be inhibited. As some proteases usually work with cations as co-factors, EDTA will chelate these cations and thus inactivate the proteases. If this is the case, we recommend adding 1 mM EDTA to the Binding Buffer. It is recommended to combine 5 kinds of different protease inhibitors or utilize a ready-to-use commercial protease inhibitor cocktail. When the protein of interest needs divalent cations to function or fold properly, do not use EDTA.
Protein Aggregation	Sometimes proteins can aggregate in a lysate, which prevents proper binding to the Ni-NTA resin. You should screen the buffer conditions with regard to co-factors, detergents and stabilizers such as glycerol. To counter protein aggregation, you can also add around 1 mM DTT to all buffers, if the target protein contains cysteines.
Ni-NTA column is inactive.	Regenerate and recharge the resin.
The column is not properly regenerated.	For removal of contaminations with very hydrophobic proteins or lipids, or precipitated proteins, incubate the matrix with one of the following chemicals for 1-2 h: 100% methanol, 100% ethanol, 8 M urea, 6 M guanidinium hydrochloride, 30% acetonitrile, or 1 M NaOH. Thoroughly wash with distilled water.
Flow rate is too fast.	Reduced flow rates may increase yields depending on the given recombinant protein.

Contaminating proteins

Contaminants derive from remaining lysate.	Check the column side and remove any remaining sample before proceeding with the next step.
Contaminants are short forms of the tagged protein	When working with *E. coli*, use protease deficient expression strains. Add protease inhibitors before/after cell lysis. Fuse His-tag with the other protein terminus. Check if internal translation initiation starts (only in case of C- terminal tag) or premature termination sites (only in case of N- terminal tag) are present. Add another tag to the other terminus and use both tags for purification.
Contaminants are covalently linked to the recombinant protein via disulfide bonds.	Add reducing reagents to all buffers necessary for cell lysis and protein purification.
Contaminants are non-covalently linked to the recombinant protein.	Increase the ionic strength of all buffers (up to 5 M NaCl) or add mild detergents (up to 2% Triton X-100, 2% Tween 20, 0.1% CHAPS, etc.).

Contaminants are histidine-rich proteins.	Increase the number of histidine (His_6 to His_{10}) and repeat the wash steps multiple times

Air bubbles in the column

When the column is taken from the cold storage room to the bench, the different temperatures can cause small air bubbles in the column. The reason is that the cold buffer can take up more gas than buffers at ambient temperature. Generally, it is recommended performing chromatography at 2-8 °C. Dependent on the individual equipment this is not always possible, and protein purification has to be performed at room temperature. If the protein purification occurs at room temperature, use degassed buffers, and wash the column immediately with buffers at ambient temperature once the column is removed from the cold.