AFFINITY HIS-TAG PURIFICATION

PROCEDURE FOR USE
Nickel NTA Agarose Beads

DESCRIPTION

Resins are products that allow batch or column purifications.
This product is supplied as a suspension in 50% aqueous suspension containing 30 vol % ethanol.

INSTRUCTIONS

The resins are adapted to work mainly in native conditions like denaturing.

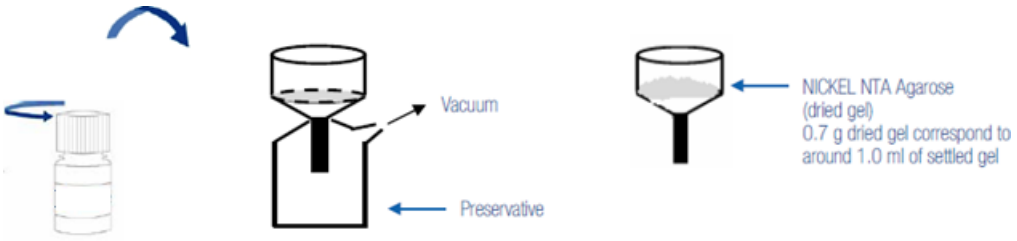
I. Batch Purification of His-tagged proteins under native conditions

The following summarized procedure is adapted for the purification of His-tagged protein in batch and under native conditions.

1. Elimination of the Preservative

Determine the quantity of NICKEL NTA Agarose Resin needed for your purification following the Recommendations below.

Option A: Gently shake the bottle of NICKEL NTA Agarose Resin to achieve a homogeneous suspension. Immediately pipette the suspension (2 ml of the original NICKEL NTA Agarose suspension per ml of gel volume required) to an appropriate tube. Sediment the gel by centrifugation at 500 x g for 5 minutes and carefully decant the supernatant and discard it.
Option B: Manually shake the bottle of the resin to obtain a homogeneous suspension of beads and preservative. Invert the bottle of resin several times and then filter the resin and put it in a container.



Recommendations

Quantity of NICKEL NTA Agarose Resin required: Binding capacity will vary for each His- tagged protein. The yield of His-tagged protein depends on various parameters, such as amino acid composition, 3-D structure, molecular weight. etc. NICKEL NTA Agarose Resin has an orientative binding capacity of up to 50 mg/ml gel (6 x His -GFPuv. ~32 kDa)[1]
[1] 1ml gel corresponds to 2 ml of 50% (v/v) NICKEL NTA Agarose suspension.

2. Equilibration of the Resin

Add 10 bed volumes of binding buffer to equilibrate the gel by mixing thoroughly to achieve a homogeneous suspension. Sediment the gel by centrifugation at 500 x g for 5 minutes and carefully decant the supernatant and discard it.

Note: Binding buffer: 50 mM Na_2PO_4 , 300 mM NaCl, 10 mM imidazole, pH 8.0.

Binding buffer: The choice of buffer depends on the particular properties of the protein. The buffer used most frequently is phosphate (50 mM is recommended). The pH of binding buffers generally leads to neutrality (7.0 - 8.0).

Note: In some cases to increase the selectivity of the binding of target protein it is necessary to add to the binding buffer a small concentration of imidazole (10-40mM). It is important to use high purity imidazole to avoid affecting the O.D. 280 nm. It is important to avoid the presence of agents like EDTA or citrate at all times.

3. Application of the Sample

Once the resin is equilibrated, the sample containing the fused protein for purification is applied. In some cases a slight increase of contact time may facilitate binding.

Note: Binding capacity can be affected by several factors, such as sample concentration, binding buffer or the flow rate during sample application.

Once the resin is equilibrated, add the clarified *E coli* lysate or protein extract. Mix the suspension gently for 30 -60 min at room temperature. In some cases a slight increase of contact time may facilitate binding.

Centrifuge the suspension at 500 x g for 5 minutes to sediment the resin. Carefully decant the supernatant and discard it.

4. Washing the Resin

Wash the gel by adding 10 ml bed volumes of wash buffer. Invert to mix and centrifuge the suspension at 500 x g for 5 minutes to sediment the resin. Carefully decant the supernatant and discard it.

Repeat the washing step twice (total wash 3 x 10 bed volumes of binding buffer).

Note: Washing buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0.

5. Elution of the Pure Protein

Add 1 bed volume of elution buffer to the gel. Mix thoroughly for 10 min at room temperature. Sediment the gel by centrifugation at 500 x g for 5 minutes and carefully decant or pipette the supernatant in a new tube and store on ice. Repeat the elution step twice or more and pool the fractions containing the purified protein.

Note: Elution buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole pH 8.0. It is possible that a significant amount of His tagged protein may remain bound to the resin. Conditions (volumes, times, temperatures) used for elution may vary among His-tag proteins. Eluates should be monitored (Bradford protein assay, SDS-PAGE or measure the absorbance at 280 nm to determine the yield of the eluted His-fused protein).

For proper storage it is recommended to remove the imidazole by ultrafiltration or dialysis.

Note: For most of the applications it is not necessary to eliminate the His-tag. However, this elimination is necessary for certain applications such as X-ray crystallography or RMN, where the protein structure is to be determined later.

For these purposes, a His-tag is usually spliced to the protein, at a protease cleavage site.

6. Regeneration & Storage

See the Procedure at the end of this publication.

II. Gravity Purification of His-tagged proteins under native conditions

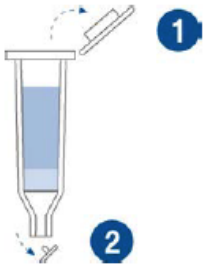
The following summarized procedure is adapted for the purification of His-tagged protein under native conditions.

1. Elimination or the Preservative

Determine the quantity of NICKEL NTA Agarose Resin needed for your purification (see section *I.1-Recommendations*).

Gently shake the bottle of NICKEL NTA Agarose Resin to achieve a homogeneous suspension. Immediately pipette sufficient suspension to an appropriate empty column.

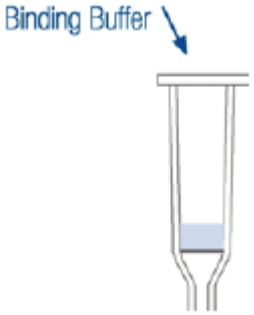
Column	Cat. N°	Total Capacity
Plastic Columns	C-50	12 ml
Plastic Columns XL	CXL-50	35ml



Remove first the upper and then the lower cap of the column, to allow elimination of the preservative by gravity flow.

2. Equilibration of the Pre-Packed Column

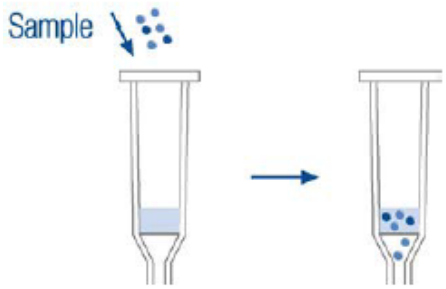
Equilibrate the column with 5 ml bed volumes of binding buffer. Add the binding buffer on the upper part of the column and make sure no air has been trapped. Mix manually inverting the Pre-packed column and discard the supernatant. Repeat the equilibration step twice.



Note: Binding buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0.

3. Application of the Sample

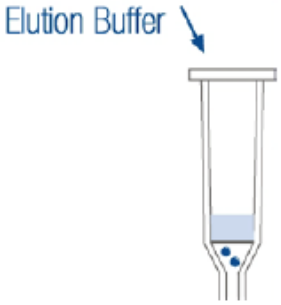
Add the sample containing the His-tagged protein to be purified (see section *I.1-Recommendations*) through the top of the column, keeping sample and resin in contact 30- 60 minutes before removing the bottom cap. Mix manually inverting the Pre-Packed column. Remove the lower cap of the column and discard the supernatant.
Note: In some cases a slight increase of contact time may facilitate binding.
Note: Binding capacity can be affected by several factors such as sample concentration.



4. Washing of the Pre-Packed Column

Close column outlet with the cap. Add the washing buffer (10 bed volumes) through the top to eliminate all the proteins that have not been retained in the column. Close column inlet with the cap, mix manually inverting the Pre-Packed column. Remove the lower cap of the column and discard the supernatant.
Repeat the washing step twice (total wash 3 x 10 bed volumes of washing buffer).
Note: Washing buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0.
Note: It will be washed with the wash1ng buffer until the O.D. 280 nm was the same as the washing buffer.

5. Elution of the Pure Protein



Close column outlet with the cap. Add 1 bed volume of elution buffer to the column. Close column inlet with the cap and mix thoroughly for 10 min at room temperature. Sediment the gel, remove the end cap and collect the eluate in a new tube and store on ice.
Repeat the elution step twice and pool the collected eluates
Note: Mix manually inverting the Pre-Packed column. It is advisable to keep elution buffer and resin in contact at least 10 minutes before removing the bottom cap.
Note: Elution buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0. It is possible that a significant amount of His tagged protein may remain bound to the resin. Conditions (volumes, times, temperatures) used for elution may vary among His-tag proteins. Eluates should be monitored (Bradford protein assay, SDS-PAGE or measure the absorbance at 280 nm to determine the yield of the eluted His-fused protein).
For proper storage it is recommended to remove the imidazole by ultrafiltration or dialysis.
Note: For most of the applications it is not necessary to eliminate the His-tag. However, this elimination is necessary for certain applications such as X-ray crystallography or RMN, where the protein structure is to be determined later.
For these purposes, a His-tag is usually spliced to the protein, at a protease cleavage site.

6. Regeneration & Storage

See the Procedure at the end of this publication.

III. Spin Purification of His-tagged proteins under native conditions

The following summarized procedure is adapted for the purification of His-tagged protein in spin columns [(1)].

(1) In this protocol are required Empty mini spin columns with inserted frits of 10-20 µm pore size.

1. Elimination of the Preservative

Gently shake the bottle of NICKEL NTA Agarose to achieve a homogeneous suspension. Remove first the upper inlet cap and immediately pipette 100 µl of the original suspension to the empty spin column. Remove the lower outlet cap and put the spin column in a collecting tube. Centrifuge at 500 x g for 30 seconds.

Note: 100 µl of the original 50% suspension corresponds to 50 µl of gel.

2. Equilibration of the Spin Column

Equilibrate the spin column with 500 µl of binding buffer. Mix manually, centrifuge at 500 x g for 30 seconds and discard flow through.

Note: Binding buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0

3. Application of the Sample

Close spin-column outlet with cap. Add the sample containing the His-tagged protein to be purified through the top of the spin column, keeping sample and resin in contact 30 - 60 minutes before removing the bottom cap. Mix manually inverting the spin column. Remove bottom cap and place spin column in a collecting tube.

Centrifuge at 500 x g for 30 seconds and discard the flow through.

Note: In some cases a slight increase of contact time may facilitate binding. Binding capacity can be affected by several factors such as sample concentration.

4. Washing of the Spin Column

Add 500 µl washing buffer through the top to eliminate all the proteins that have not been retained in the column. Mix manually inverting the Spin column.

Centrifuge at 500 x g for 30 seconds. Discard flow through. Repeat the washing step twice. Discard flow through between washing steps.

Note: Washing buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0

Note: Wash the column with washing buffer until the O.D. 280 nm of the eluent reaches the baseline level.

5. Elution or the Pure protein

Close spin column outlet with cap. Add 500 µl of elution buffer and close the lid. Mix thoroughly for 10 min at room temperature. Centrifuge the gel, remove the end cap and collect the eluate in a new tube and store on ice. Repeat the elution step twice. Pool the collected eluates.

Note: Elution buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0. It is possible that a significant amount of His tagged protein may remain bound to the resin. Conditions (volumes, times, temperatures) used for elution may vary among His-tag proteins. Eluates should be monitored (Bradford protein assay, SDS-PAGE or measure the absorbance at 280 nm to determine the yield of the eluted His-fused protein).

For proper storage it is recommended to remove the imidazole by ultrafiltration or dialysis

Note: For most of the applications it is not necessary to eliminate the His-tag. However, this elimination is necessary for certain applications such as X-ray crystallography or RMN, where the protein structure is to be determined later.

For these purposes, a His-tag is usually spliced to the protein, at a protease cleavage site.

6. Regeneration & Storage

See the Procedure at the end of this publication.

IV. FLPC Purification of His-tagged proteins under native conditions

NICKEL NTA Agarose Resin is compatible with common low-pressure chromatography columns and FPLC™ applications. We recommend columns equipped with an adjustable plunger/flow adapter. Use low rates for loading step to allow maximal binding of the His-tagged protein. The flow rate for equilibration, washing and elution can be increased to reduce the purification time.

1. Column Packaging, Elimination of the preservative and Elimination of the Resin

a. Manually shake the bottle to obtain a homogeneous suspension of NICKEL NTA Agarose Beads/preservative. Place a funnel in the head of column and slowly run the suspension down the walls of the column.

   Note: It is advisable to make the addition slowly to avoid the formation of bubbles. The product may also be degassed before adding to the column.

   Decant the product and discard most of the leftover liquid, leaving 1 cm above the column head to prevent drying out. This is done either by passing it through the column, or pipetting it from the top of the column.

b. Repeat previous steps until the desired column height is obtained.

c. Insert the adapter gently in the column head until it begins to displace the liquid.

   Note: Make sure no air is trapped under the net.

d. Add distilled water to the purification stream until a constant height (corresponding to the height of the column) is achieved.

   Note: If the desired height is not achieved, repeat steps 1 through 4.

e. When a constant height has been obtained, maintain the flow with the addition of 5 volumes of distilled water to completely eliminate the preservative.

f. Equilibrate the column with at least 5 column volumes of binding buffer until the baseline at 280 nm is stable.

   Note: It is advisable to previously de-gas all the solutions before adding to the column to avoid the formation of bubbles.
   Note: Binding buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0.

2. Application of the Sample

Once the resin is equilibrated, the centrifuged or filtered sample is applied (*see section I- Recommendations*). In some cases a slight increase of contact time may facilitate binding. Therefore use low rates for the loading step to allow maximal binding of the His- tagged protein.
Note: Binding capacity can be affected by several factors, such as sample concentration or the flow rate during sample application. Collect flow through and verify that fused protein has bound.

3. Washing of the Resin

It will be washed with the washing buffer (10- 20 bed volumes) or until the O.D. 280 nm reaches the baseline level.
Note: Washing buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0.

4. Elution of the Pure Protein

Elute the His-tagged protein with 5-10 bed volumes of Elution buffer and collect the fractions on ice.
Note: It is important to identify (SDS-PAGE & Bradford protein assay) the fraction that contains the majority of pure protein.
Note: Elution buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0.
For proper storage it is recommended to remove the imidazole by ultrafiltration or dialysis.

Note: For most of the applications it is not necessary to eliminate the His-tag. However, this elimination is necessary for certain applications such as X-ray crystallography or RMN, where the protein structure is to be determined later.

For these purposes, a His-tag is usually spliced to the protein, at a protease cleavage site.

5. Regeneration & Storage

See the Procedure below at the end of this publication.

V. Purification of His-tagged proteins under denaturing conditions

The recombinant proteins often form insoluble inclusion bodies. If so these need to be rendered soluble by purification under denaturing conditions, using for example urea or guanidine chloride at relevant stages (see table).
Cells are disrupted under native conditions using lysozyme together with sonication. After centrifugation the fused protein is extracted and solubilized from the pellet by using a denaturant agent (urea or guanidine).

Isolation of inclusion bodies

Thaw the cell pellet from an *E.coli* expression culture on ice (if frozen). Resuspend 1 g of pelleted, wet cells in 5 ml buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0.) on ice. Pipette up and down, or use stirring until complete resuspension without visible cell aggregates.
Add lysozyme to a final concentration of 1mg/ml. Stir the solution on ice for 30 min.
Sonicate the suspension on ice and check samples appearance after sonication. If the lysate is still viscous add 5 µg/ml DNase I and stir on ice for 15 min.
Centrifuge the crude lysate at 10,000 x g for 30 min at 4°C to collect the inclusion bodies. Discard supernatant and keep pellet on ice.

Solubilization of inclusion bodies

- Resuspend the pellet in 10 ml 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0. Centrifuge at 10,000 x g (30 min at 4°C) and discard supernatant.
- Add 2.0 ml (per g wet cells) 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 8 M urea pH 8.0. Homogenization or sonication may be necessary to resuspend the pellet. Dissolve the inclusion bodies by stirring on ice for 60 min.
- Centrifuge at 10,000 x g for 30 min (at 20°C) to eliminate insoluble material and transfer the supernatant to a clean tube. Centrifuge until the supernatant is clear and save it.

Procedure

The procedure is similar to purification under native conditions except that the sample and the buffers loaded on the column contain 8M urea.

- Binding buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 8 M urea pH 8.0.
- Washing buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 8 M urea pH 8.0.
- Elution buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 8 M urea pH 8.0.

COMPATIBILITY OF REAGENTS

STUDIES	REAGENTS	COMMENTS
BUFFERS	• Sodium phosphate	• Sodium phosphate buffer 50mM pH 8.0 is recommended.
	• Tris, HEPES, MOPS	• Coordinate with metal ions, causing a decrease in binding capacity. Up to 100mM may be used.
	• Sodium Chloride	• Avoids unspecific binding (ionic interactions). • At least 0.3M should be used. Up to 2M can

		be used.
DENATURING AGENTS	• Urea	• Solubilizes protein. Use 8M for purification under denaturing conditions.
	• Guanidine-HCl	• Solubilizes protein. Up to 6 M can be used
ADDITIVES	• Imidazole	• Competes with the His-tag protein. • Reduce nonspecific binding (20 mM)
	• Glycerol	• Elute the His-tag protein (up to 100 mM) • Avoids hydrophobic interactions between proteins. • Up to 50% can be used.
	• EDTA	• Coordinates with cations, causing a decrease in capacity. • Not recommended, but up to 1 mM in samples has been used successfully in some cases.
	• Ethanol	• Avoids hydrophobic interactions between proteins but may precipitate proteins causing column clogging and low flow rates. • Up to 20% can be used.
REDUCING AGENTS	• Reduced glutathione	• Can reduce Ni2+ ions at higher concentrations. • Up to 30 mM in samples has been used successfully in some cases.
	• B-mercaptoethanol	• Avoids formation of disulfide bonds. Can reduce Ni^{2+} ions at higher concentrations. • Up to 20 mM in samples has been used successfully in some cases.
	• DTT, DTE	• Can reduce Ni^{2+} ions at higher concentrations. • Up to 10 mM in samples has been used successfully in some cases.
	• SDS	• Avoids hydrophobic interactions between proteins. • Coordinates with cations, causing a decrease in capacity. Not recommended, but up to 0.3% in samples has been used successfully in some cases.
DETERGENTS	• Nonionic detergents (Tween, Triton, etc.)	• Removes background proteins. • Up to 2% can be used.

NTA RESIN RCGENERATION PROCEDURE

During the life of the resin, it may lose binding points because some protein is retained. Hence a loss of the binding capacity may be observed in successive cycles. To return to the starting state, regeneration may be necessary. Regeneration consists of the complete elimination of the retained protein.

In general, column regeneration is always necessary when changing proteins. When continuing with the same protein it is recommended to do regeneration when an appreciable diminution in the yield is observed. The frequency of these stages varies with the protein and the conditions used.

A. Solubilize and desorb contaminants: It is necessary to wash the resin with 0.5 M NaOH (30 min).
B. Remove the NaOH solution by washing with 10 bed volumes of distilled water.
C. If you are using the resin directly, wash with 10 bed volumes of 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0.)
D. For storage wash with 2 volumes of 30% ethanol, resuspend the resin in 30% ethanol and store at 4 - 8°C.

In some cases (depending on the nature of the sample) the above procedure may not be enough. For example, when the color of the resin changes (due to loss or reduction of nickel ions). Regeneration consists of the complete elimination of the metal.

- It is necessary to wash the resin with 10 column volumes of distilled water.
- Elimination of the metal from the resin by washing with 10 column volumes of 100 mM EDTA, pH 8.0.
- Elimination of the excess EDTA: In order to eliminate the residual EDTA before reloading the resin with the metal, the column should be washed with 10 column volumes of distilled water.
- Load the column with the corresponding metal: once the excess EDTA has been eliminated, add 2 column volumes of 100 mM metal ion aqueous solution (normally chlorides or sulphates are used).
- Elimination of the excess of metal: wash with 10 column volumes of distilled water.
- Preparation of the column: add 10 column volumes of the binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0).

Note: If the resin is not going to be used for a while it is recommended to replace the last step by the addition of the preservative.

TROUBLESHOOTING GUIDE
Problems and Solutions

Possible causes of problems that could appear during the purification protocol of biomolecules are listed below. The causes described are theoretical and it is always advisable to contact our team with your specific problem.
The table delineates the potential problems at each step in the protocol that might explain poor performance.

1. SAMPLE APPLICATION

OBSERVATION	POSSIBLE CAUSES	RECOMMENDATION
HIGH VISCOSITY SAMPLE	• Presence of DNA in the sample/lysate.	• Lysate may remain viscous. Add 5 µg/ml DNase I and incubate on ice for 10 min.
	• Presence of insoluble material in the sample/lysate.	• Use centrifugation or filtration (0.45 µm membrane) to avoid clogging of the column.
HIGHLY DILUTED OR CONCENTRATED SAMPLE	• Highly diluted sample.	• It is preferable to concentrate the sample before its purification in the column.
	• Highly concentrated sample.	• It is preferable to make a previous dilution of the sample before its purification in the column.
TARGET PROTEIN NOT BOUND TO THE	• His-tag is not present or has been degraded.	• Check it. If it has been degraded, make the purification at lower temperatures (4°C) reducing the degradation. Try to reduce the purification step times. Add protease

COLUMN	• It is not exposed (inaccessible).	inhibitors. (See chemical compatibility table). • Purify in denaturing conditions or add the tag in other site (*N-terminus, C-terminus*, or in both positions).
	• Inadequate binding conditions.	• Check the buffer and binding pH. • If the binding has been done in presence of imidazole, reduce its concentration or eliminate it in this step. • Verify if some of the reagents used in the adsorption step interfered with the binding reaction.
TARGET PROTEIN BINDS ONLY PARTIALLY TO THE COLUMN	• Column capacity is exceeded. • The resin has been previously used during several purification cycles without regeneration. This causes a diminution of the binding capacity This diminution varies in each case and increase with the number of purification cycles of the resin.	• Apply less fused protein to the resin. • Apply a regeneration step in the column when a decrease of the binding capacities is observed.
	• Loss of chelating metal in the resin.	• Apply a regeneration step in the column. Avoid use of reducing and chelating agents
	• Histidine tail is not very exposed.	• Try to use slower flow rates or make the adsorption in batch to allow a better contact between resin and fused protein. Note: a greater exhibition would be obtained working in denaturing conditions.
	• Poor protein expression • The fused protein forms inclusion bodies.	• Optimize bacterial expression conditions. • Modify bacterial growth conditions • Work in denaturing conditions.
	• Channels have formed in the column so the sample runs mainly through these undesirable channels.	• Repack column
HIGH AMOUNT OF CO-ELUTED PROTEINS (CONTAMINANTS)	• Column too large.	• Reduce the resin quantity so the fused protein and contaminants will compete for less binding sites, increasing the binding selectivity of the tagged protein.
	• The resin used in the purification shows low selectivity to bind the fused protein. In some cases Nickel resin is not as selective as ones loaded with other metals. It may also bind proteins with histidine, cysteine and tryptophan residues.	• Employ an imidazole concentration gradient to separate the target protein from the rest of retained proteins. Also "Single Step Elution" procedures can be used.
TARGET PROTEIN ELUTES POORLY	• Too smooth elution conditions. Sometimes protein binding with chelating metals is too strong. Note: Also the position of the histidine tails can influence the strength of the binding of the target protein.	• Increase imidazole concentration or reduce pH in the elution step. • Try, if possible, an elution at a higher temperature. • Make a elution with a chelating agent such as EDTA • Increase imidazole concentration up to 1M in the elution buffer. • Elute in denaturing conditions. • Add solubilizing agents (see compatibilities).

	• Fused protein can be precipitated	• Incubate the column with the elution buffer for 8-10 hr and elute with the elution buffer. • Run binding and elution steps in batch format to avoid local concentration of protein and therefore its potential precipitation.
ELUTION PROFILE IS NOTE REPRODUCIBLE IN DIFFERENT CYCLES OF PURIFICATION	Samples nature could have been modified. The histidine tail could have been lost due to protease action. • Proteins or lipids could have precipitated. • pH or ionic forces could have been modified. • The sample to apply could be different than the first one.	It is necessary to prepare a fresh sample. Run the protocol at 2-8 degrees C. Add protease inhibitors (see chemical capabilities table) • Regenerate the resin • Prepare new buffers • Keep all the parameters and same condi

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