AFFINITY HIS-TAG PURIFICATION

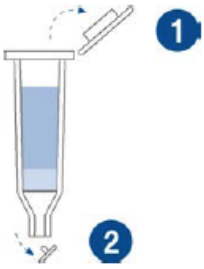
PROCEDURE FOR USE
Ni-IDA & Co-IDA Gravity Columns

DESCRIPTION

Pre-Packed Columns are in ready-to-use format for purification of histidine-tagged proteins by gravity flow. Rapid purifications and good yield of target proteins are obtainable by this method. The resin supplied in the Pre-Packed Columns is suitable for use in either native or denaturing conditions. This product is supplied as a suspension in 20% ethanol.

INSTRUCTIONS

The following procedure is for the purification of histidine-tagged protein under native conditions. To work under denaturing conditions, first check the stability table below.

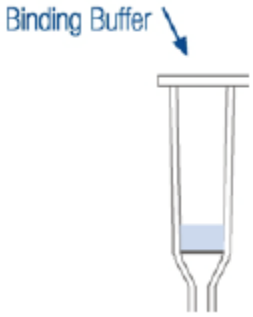


1. Elimination of the Preservative

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 - 10 column bed volume 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.



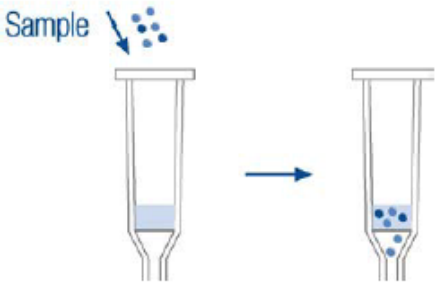
The typical binding buffer is 20 mM disodium phosphate, 500 mM NaCl, 10 mM imidazole at pH 7.5.

Note: Selection of the binding buffer depends on the characteristics of the protein to be purified. The most commonly used buffers are acetate (50 mM) or sodium phosphate (10 - 150 mM). Binding pH is usually close to neutrality (normally pH 7.0 - 8.0), however the larger range 5.5- 8.5 can be used. To avoid ionic interchange effects, 0.1 - 0.5 M NaCl may be added to the binding buffer.

Note: It is also normal to add a small amount of imidazole (10 - 40 mM) to improve the selectivity of the binding of the histidine-tagged protein. It is important to use imidazole of high purity to avoid affecting O.D. 280 nm. It is also important to avoid inclusion of reagents such as EDTA or citrate.

3. Application of the Sample

Add the sample containing the histidine-tagged protein to be purified through the top of the column, keeping sample and resin in contact at least 15 minutes before removing the bottom cap.

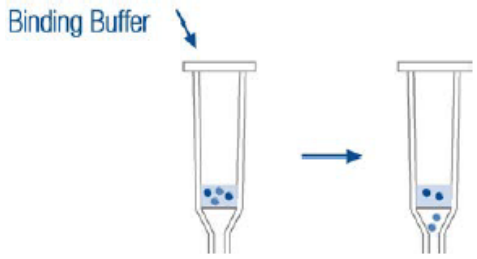


Note: In some cases a slight increase of contact time may facilitate binding.

Note: Pouring the sample down a glass rod held against the wall of the column will minimize the introduction of air bubbles.

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

4. Washing of the Pre-Packed Column



Add the binding buffer through the top to eliminate all the proteins that have not been retained in the column. Mix manually inverting the Pre-Packed column. Wash the column with binding buffer until the O.D. 280 nm of the eluent reaches the baseline level.

5. Elution of the Pure protein

Add the elution buffer to the column.

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 is 20 mM disodium phosphate. 500 mM NaCl, 500 mM imidazole at pH 7.5. This imidazole concentration is generally sufficient for elution of the target protein; if the desired result is not achieved then the concentration may be increased up to 2.0 M.

Note: Other reagents that may be used to elute the protein are histidines and ammonium chloride. Elution may also be performed by decreasing the pH to 4.0 or 3.0, or with chelating agents such as EDTA or EGTA (0.05 M). However these will also cause desorption of the metal from the resin.

6. Storage

Keep at +2°C - +8°C. Do not freeze.

The recombinant proteins often form inclusion bodies. In these cases the use of denaturing conditions is required:

STUDIES	REAGENTS	
CHEMICAL STABILITY	HCl 0.01 M	SDS 2%
	NaOH 0.1 M	2-propanol 30%
	Ethanol 20%	NaOH 1 M
	Sodium acetate 0, 1M pH 4.0	HAc 70%
DENATURING AGENTS	Urea 8 M	Guanidine-HCl 6 M
DETERGENTS	Triton X-100 2%	Chaps 1%
	Tween 20 2%	
ADDITIVES	Imidazole 2.0 M	EDTA 1 mM
	Ethanol 20% + glycerol 50%	EDTA 1 mM + $MgCl_2$ 10 mM
	Na_2SO_4 100 Mm	Citrate 60 mM
	NaCl 1.5 M	Citrate 60 mM + $MgCl_2$ 80 mM
REDUCING AGENTS (*)	Reduced glutathione 10 mM	DTE 5 mM
	B-mercaptoethanol 20 MM	DTT 5 mM
BUFFERS	Na_2HPO_4 50 mM, pH 7.5	Tris-acetate 100 mM, pH 7.5
	Tris-HCl 100 mM, pH 7.5	HEPES 100 mM, pH 7.5
	MOPS 100 mM, pH 7.5	

(*) Note: Under extended treatments with reducing agents, or in processes where high concentrations of these reagents are used, reduction of the metal ion may result - this will affect the binding capacity of the resin, so these agents should be avoided. The reagents described in the table are compatible with Nickel Activated Agarose Beads (Nickel is most commonly used) under the conditions and concentrations indicated in the table. The stability of the Nickel resin has been tested in each of the reagents separately. This resin is manufactured with imminodiacetic acid and in case of buffers that contains reducing agents we recommend to use NTA resins.
There is a very small loss of Nickel with this resin and it only happens in very drastic work circumstances. Given these conditions a slight brown discoloration may appear but does not usually affect performance. The discoloration is due to small particles of Nickel breaking away from the main body of resin and coming into contact with the reducing agents causing the Nickel to reduce and became brown in color. If there is a risk of producing this effect it can be avoided by pre-treatment of the resin before it has ever been used. This treatment eliminates the cations that are weakly attached to the resin before beginning the process, so the reducing agent doesn't affect the lost Nickel ions.

Pre-treatment:

1. Wash the resin with five column volumes of distilled water.
2. Wash the resin with five column volumes of binding buffer (without reducing agents in the buffer).
3. Wash with five column volumes of elution buffer (without reducing agents in the buffer).
4. Equilibrate with 10 column volumes of binding buffer (without reducing agents).

Once this treatment has been carried out, the resin is ready for the simple purification in such circumstances as described above.

TROUBLESHOOTING GUIDE

Problems and Solutions

Possible causes of problems that could appear during the purification protocol of biomolecules are listed below.
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.	• Increase sonication time until viscosity is reduced
	• Steric hindrance of the substrate	• Dilute the sample before its application in the column. In this case sometimes, it is preferable to carry out the purification in batch format instead of the column format. • Consult “tailor made resins” for high viscosity samples.
HIGHLY DILUTED OR CONCENTRATED SAMPLE	• Highly diluted sample.	• It is preferable to concentrate the sample before its purification in the column. • Another solution is to carry out an adsorption in batch format and pack the column with the resultant resin of the adsorption step.
	• Highly concentrated sample.	• It is preferable to make a previous dilution of the sample before its purification in the column.

2. ADSORPTION

OBSERVATION	POSSIBLE CAUSES	RECOMMENDATION
TARGET PROTEIN NOT BOUND TO THE COLUMN	• 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 inhibitors. (See chemical compatibility table).
	• It is not exposed (inaccessible).	• 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 interferes with the binding reaction. • E.g: A Zinc can lose its metal due to the presence of chelating agents in the sample and therefore, the protein will not bind. Since the presence/absence of Zinc cannot be visualized by a change of color, it would be difficult to determine this phenomenon. In case of doubt, it is advisable to regenerate the column and observe if the target protein is bound to the regenerated resin.
TARGET PROTEIN BINDS ONLY PARTALLY TO THE COLUMN	• Column capacity is exceeded.	• Apply less fused protein to the column.
	• 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 increases with the number of purification cycles of 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 slow 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.	• Optimize bacterial expression conditions.
	• The fused protein forms inclusion bodies.	• Modify bacterial growth conditions. • Work in denaturing conditions.
	• Channels have formed in the column so the sample runs	• Re-pack column.

	mainly through these undesirable channels	
	• The resin shows low binding capacity.	• Try a less selective cation.

3. ELUTION

OBSERVATION	POSSIBLE CAUSES	RECOMMENDATION
HIGH AMOUNT OF CO-ELUTED PROTEINS (CONTAMINANTS)	• Insufficient washing stage	• Increase volume of washing buffer. • Add a low concentration of imidazole (5-10 mM) in the buffer during washing and equilibrating steps.
	• Inadequate adsorption conditions.	• Check pH • Add or increase saline concentration in the binding buffer to avoid non-specific ionic interactions. • Low concentrations of non-ionic detergents can also be added. • Add small quantities of ethyleneglycol or glycerol in the binding buffer to avoid non-specific hydrophobic interactions. • Increase imidazole concentration in the binding buffer. Note: IN general, higher imidazole concentrations than 20 mM are not recommended because it can compete with the binding of the target protein. This concentration can be modified with the type of protein to be purified.
HIGH AMOUNT OF CO-ELUTED PROTEINS (CONTAMINANTS)	• Column too large.	• Reduce the resin quantity so the fused protein and contaminants will compete for les 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.	• Try a more selective cation (e.g: Cobalt). • 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.	• Increase imidazole concentration or reduce pH in the elution step. • Try, if possible, an elution at a higher temperature.
	• Sometimes protein binding with chelating metals is too strong. Note: Also the position of the	• Make an elution with a chelating agent such as EDTA. • Make an elution reducing pH (pH 4.0) in the presence of imidazole. • Purify with other chelating resins as the requirements with

	histidine tail can influence the strength of the bind binding of the target protein. • Fused protein can be precipitated.	each cation are different and the binding strength is different with each one. Also, in many cases, using a Low Density resin gives better desorption of the fused protein. • Increase imidazole concentration up to 1M in the elution buffer. • Reduce the flow in the elution step or make this step in batch format to increase contact time. • Elute in denaturing conditions. • Add solubilizing agents (see compatibilities). • Incubate the column with the elution buffer for 8-10 h 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 NOT 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. • Loss of binding capacity is observed.	• It is necessary to prepare a fresh sample. Run the protocol at 2-8°C. Add protease inhibitors (see chemical compatibilities table). • Regenerate the resin. • Prepare new buffers • Keep all the parameters and same conditions. • It is recommended to regenerate the column.

4. CHANGES IN THE RESIN

OBSERVATION	POSSIBLE CAUSES	RECOMMENDATION
LOSS OF COLOR OF THE RESIN	• Presence in the sample of chelating agents that could have caused the diminution of the content of the metal.	• Eliminate the chelating agents in the sample (e.g. by gel filtration) and after regenerate the column. Note: This is easy to see in colored resins (Cobalt, Nickel or Copper). In other cases such as Zinc the loss of the cation is not so evident by color changed and could be the cause of the non-binding of the protein.
CHANGE OF COLOR (BROWN) OF THE RESIN	• Presence in the sample of reducing agents.	• Eliminate these reducing agents and regenerate the resin.