AFFINITY HIS-TAG PURIFICATION

PROCEDURE FOR USE
NICKEL NTA Magnetic Agarose Beads (5%)

DESCRIPTION

Nickel NTA Magnetic Agarose Beads are products that allow rapid and easy small-scale purification of histidine-tagged proteins. This product is supplied as a suspension in 20% Ethanol and 5% magnetic resin.

The product is available in the following pack sizes:

- Nickel NTA Magnetic Agarose (5%) 2ml	- Nickel NTA Magnetic Agarose (5%) 5ml	- Nickel NTA Magnetic Agarose (5%) 10ml
The bottle slurry contains 100 µl of magnetic beads in a 2ml total volume. Preservative: 20% Ethanol.	The bottle slurry contains 250 µl of magnetic beads in a 5ml total volume. Preservative: 20% Ethanol.	The bottle slurry contains 500 µl of magnetic beads in a 10ml total volume. Preservative: 20% Ethanol.

INSTRUCTIONS

The resins are adapted to work in native and denaturing conditions. The imidazole concentration used in the binding buffer will be optimized in each case. A low imidazole concentration during equilibration and washing steps will provide a higher binding capacity and a higher concentration will improve the purity.
The following summarized procedure is adapted for a compromise between both objectives, but it is not a fixed protocol and can be optimized by user.
Each user has to optimize the procedure for each specific protein; however some general recommendations can help to optimize the purification.

General Recommendations

Native Conditions

The presence of a low concentration of imidazole in the lysis and binding buffers: usually up to 20mM concentration will not affect the target-fused protein. In case the tagged protein doesn't bind to the magnetic beads the imidazole concentration should be reduced to 5-10mM.
In order to prevent non-specific interactions during binding and washing, buffers should contain 300mM NaCl as minimum amount avoid increasing this concentration up to 2M.

Denaturing Conditions

The recombinant proteins often form insoluble inclusion bodies. If so, these need to be rendered soluble by purification under denaturing conditions, using 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 denaturing agent such as urea.

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 stir until resuspension is completed 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 sample 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.

The procedure is similar to purification under native conditions except that the sample and the buffers loaded on the column contain 8M urea. In some cases 8M urea is not sufficient to completely solubilize inclusion bodies. In these cases the urea in the Denaturing Lysis Buffer can be replaced with 6 M Guanidine Hydrochloride (Glu-HCl). In this case, samples containing Glu-HCl cannot be applied to SDS-PAGE directly. Dilute the sample or subject it to a precipitation step with trichloracetate to remove the Glu-HCl.

1. Magnetic beads preparation

Determine the quantity of NICKEL NTA Magnetic Agarose Beads (5%) needed for your purification. This procedure is described to work with 10µl of magnetic beads.
Gently shake the bottle of the product to achieve a homogeneous suspension. Immediately pipette the suspension (200 µl of the original NICKEL NTA Magnetic Agarose (5%) suspension) to an appropriate tube.
Place the tube in a Magnetic Separator (see accessories) or use a magnet to remove the preservative from the beads.

Recommendations

Quantity of magnetic beads 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 70 mg/ml gel (6 x His -GFP).
Sample preparation: adjust the sample to the composition and pH of the Equilibration Buffer. Clarification of sample may be needed before applying it to the magnetic beads.
Magnetic bead suspension volumes suitable for given protein expression levels: the following table should be used as a guide. Volumes can be linearly scaled up or down for smaller or larger culture volumes.

Protein expression level	µg of His-tagged protein / 10 ml culture	Volume NICKEL NTA Magnetic Agarose (5%) suspension /10 ml culture	Minimum Elution Volume / 10 ml culture
< 0.5 mg/L	< 5 µg	10 µL	25 µL
1 mg/L	10 µg	20 µL	25 µL
5 mg/L	50 µg	100 µL	50 µL
10 mg/L	100 µg	200 µL	100 µL
50 mg/L	500 µg	1 ml	500 µL

2. Equilibration of the Resin

Remove the tube from the magnetic separator and add 500µl equilibration buffer. Mix the bead slurry thoroughly to achieve a homogeneous suspension.

Place the tube in the magnetic separator and discard the supernatant.

Note: Binding buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 20 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: to increase the selectivity of the binding of target protein it is necessary to add to the binding buffer a small concentration of imidazole (10-40 mM). We recommend 20mM as a guideline, but it will depend on each case. If the tagged protein doesn't bind under these conditions, the amount of imidazole should be reduced to 5-1OmM.

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 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 contact time.

Once the resin is equilibrated, add the clarified E coli lysate or protein extract. Mix the suspension gently for 30 min at room temperature or 1h at 4°C. In some cases a slight increase of contact time may facilitate binding.

Place the tube in a Magnetic Separator (see accessories) or use a magnet to remove the supernatant and discard it.

4. Washing of the Resin

Wash the magnetic beads by adding 500 µl of washing buffer and mix by vortexing. Place the tube again in the magnetic separator and discard the supernatant.

Repeat the washing step twice.

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

5. Elution of the Pure Protein

Add 100 µl elution buffer to the magnetic beads. Mix thoroughly for 10 min and place tube in the magnetic separator and collect the elution fraction and store on ice.

Repeat the elution step 2x or more and collect each fraction in a separate tube and determine the protein concentration of each fraction.

Note: Elution buffer: 50 mM NaH_2PO_4. 300 mM NaCl, 500 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 ultrafilltration 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.

Denaturing conditions: The procedure is similar to purification under native conditions except the buffers.

- Binding buffer: 100 mM NaH_2PO_4, 10 mM Tris base, 8 M urea pH 6.3.
- Washing buffer: 100 mM NaH_2PO_4, 10 mM Tris base, 8 M urea pH 6.3.
- Elution buffer: 100 mM NaH_2PO_4, 10 mM Tris base, 8 M urea pH 4.5.

Note: Due to urea dissociation, adjust the pH immediately before use.

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 Ni2+ ions at higher concentrations. • Up to 20 mM in samples has been used successfully in some cases.
	• DTT, DTE	• Can reduce Ni2+ 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.

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 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 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 interfered with the binding reaction.
TARGET PROTEIN BINDS ONLY PARTIALLY TO THE COLUMN	• Resin 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 increases with the number of purification cycles of the resin.	• Apply less fused protein to the resin. • Use fresh resin.
	• Loss of chelating metal in the resin.	• Apply a regeneration step in the resin. Avoid use of reducing and chelating agents.
	• Histidine tail is not very exposed.	• Increase the contact time 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.
HIGH AMOUNT OF CO-ELUTED PROTEINS (CONTAMINANTS)	The resin used in the purification shows low selectivity to bind the fused protein.	• Employ Single Step Elution procedures to separate the target protein from the rest of retained proteins.
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). • Incubate the column with the elution buffer for 8-10 hr and elute with the elution buffer.

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) • Use fresh resin. • Prepare new buffers • Keep all the parameters and same conditions

For laboratory use only. Not for use in diagnostic or therapeutic procedures.