Instructions for use

ROTI®Garose His/Ni NTA-Cartridges

Cartridges with matrix of Nickel charged agarose beads for high pressure affinity chromatography.
Suitable for denaturing or reducing as well as native conditions. IMAC matrix, nickel charged. NTA ligand.

I. Characteristics

The matrix of ROTI®Garose His/Ni NTA-Cartridges are prepacked and may be used directly. The matrix consists of cross-linked and beaded 6 % agarose, NTA-conjugated and charged with divalent Nickel ions.
The ROTI®Garose His/Ni NTA-matrix results in eluates with considerably low metal contamination. The tetradentate NTA cross-linker binds his-tagged proteins very efficiently, leading to high recovery rates with minimized nickel bleeding into the eluate.
Optimal for automated liquid chromatography, or if proteins shall be isolated under pressure. Suitable for ÄKTA™FPLC™ via standard 10-32 fittings without additional connectors, and for sample application via peristaltic pump or syringe.
The Matrix is stable in all commonly used reagents including denaturing reagents (like 8 M urea, 6 M guanidinium hydrochloride) and (dependent on the respective buffer) reducing substances (for instance ≤30 mM glutathion, ≤10 mM DTT, ≤10 mM DTE, ≤20 mM β-mercaptoethanol and ≤0,3 % SDS).
Matrix: Slurry in ethanol (15 %).
May repeatedly be regenerated.

Cartridge material made from polypropylene and polyethylene (frit).
Inner diameter 1,6 cm, height 2,5 cm, inlet port 10-32 (1/16") female, outlet port 10-32 (1/16") male.

II. Run of the affinity chromatography under native conditions

The following procedure is for the purification of histidine-tagged protein under native conditions. To work under denaturing conditions, first check *III. Elution from inclusion bodies.*
The strength of binding of the protein to the resin will depend on the accessibility of the His-tag, the pH and the buffer composition.
Binding capacity: Ø ≥50 mg/ml matrix, max. total binding capacity 250 mg.

1. Connecting the cartridge to the chromatography system and elimination of the preservative
Purge the pump with binding buffer. Assure that all air is displaced.
Remove the snap-off end at the cartridge outlet and save it for further use.
Remove the upper plug from the cartridge. Fill the inlet port of the cartridge with several drops of buffer to remove air to form a positive meniscus.
Start the pump and insert the fitting “drop-to-drop” into the cartridge port to avoid introducing air bubbles.
Wash the beads with at least 25 ml of distilled water to eliminate the preservative.

2. Equilibration of the cartridge
Equilibrate the cartridge with at least 25 ml of the chosen binding buffer (see VI. Buffers and general comments).
Check $OD_{280\ nm}$, and equilibrate until this OD is stable.

3. Application of sample
In order to remove residual particles from the sample, filter the sample through a 0.45 µm filter and/or centrifuge it immediately pror to application to the cartridge.
Add the filtered or centrifuged sample containing the histidine-tagged protein to be purified through the top of the cartridge. Collect the flow-through and monitore by SDS-PAGE in order to make sue that the protein has bound to the matrix.
Note: In some cases, a slight increase of contact time may facilitate binding.

4. Washing of matrix
Wash the cartirdge with 20-30 volumes of washing buffer. Monnitor washing efficiency by measuring the $OD_{280\ nm}$. Washing can be stopped as soon as this OD is stable.

5. Elution of target protein
Add the elution buffer to the cartridge using a one-step buffer (e.g. 25 ml) or linear gradient (e.g. 100 ml).
Note: Conditions (volumes, times, temperatures) used for elution may vary and habe to be optimised for each specific protein.
Eluates should be monitored (Bradford protein assay, SDS-PAGE or measuring of the absorbance at 280 nm) in order to determine the yield of the eluted protein.

The recommended flow rate is 5 ml/min.
The recommended pressure is 3 bar [0.3 MPa] (40 psi)
Maximum pressure is 5 bar [0,5 MPa] (70 psi)

III. Purification of His-tagged proteins under <u>denaturing conditions</u> / Elution from <u>inclusion bodies</u>

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. Following centrifugation, the fused protein is extracted and solubilized from the pellet by using a denaturant agent (urea or guanidine).

Isolation of inclusion bodies

1. Thaw the cell pellet from an *E. coli* expression culture on ice (if frozen).
2. Resuspend 1 g of pelleted, wet cells in 5 ml binding buffer on ice (see VI. Buffers and general

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comments). Pipette up and down, or use stirring until complete resuspension without visible cell aggregates.

3. Add lysozyme to a final concentration of 1 mg/ml. Stir the solution on ice for 30 min.
4. Sonicate the suspension on ice (e.g. 10x 15 sec. bursts with 15 sec. cooling period between). Check samples appearance after sonication. If the lysate is still viscous add 5 µg/ml DNase I and stir on ice for 15 min.
5. Centrifuge the crude lysate at 10,000 x g for 30 mins at +4 ºC to collect the inclusion bodies. Discard supernatant and keep the pellet on ice.

Solubilization of inclusion bodies

6. Resuspend the pellet in 10 ml binding buffer (per g wet cells).
7. Centrifuge at 10,000 x g for 30 mins at +4 ºC and discard supernatant.
8. Add 2 ml (per g wet cells) lysis buffer (see VI. Buffers and general comments. Resuspend the cells by vortexing or sonication.
9. Lyze the inclusion bodies by stirring on ice for 60 mins.
10. Centrifuge at 10,000 x g for 30 min at moderate room temperature to eliminate insoluble material, and transfer supernatant to a clean tube.
11. Centrifuge until the supernatant is clear, then freeze. Optionally, filter the supernatant through a 0,45 µm membrane.

Isolation of Proteins

Use the instructions given above under II / native conditions. Make sure that all buffers used contain the same denaturing agent as indicated in **VI.B. Buffers for denaturing protein isolation / isolation from inclusion bodies**

IV. Cleaning and Regeneration:

In order to preserve the optimum binding capacity during repeated binding and elution cycles, we recommend cleaning and/or regenerating the resin once in a while. During **cleaning**, contaminants originating from loaded samples are removed like, for instance, unpsecifically bound proteins, precipitated proteins and lipoproteins. This procedure is recommended only if the same recombinant protein is to be isolated.

During **regeneration**, the metal charge and, therefore, the retained protein are completely eliminated. Regeneration frequency cannot be recommended in general, but should be performed if either the binding rate decreases. Regeneration is obligatory, when another protein is to be eluted.

A. Cleaning of the cartridge resin

Flood the cartridge with cleaning buffer and incubate for 30 mins. Wash with 10 volumes distilled water. Wash with 10 volumes binding buffer, then use or, if the cartridge shall be stored, wash with 2 volumes of 30 % ethanol and store.

B. Regeneration of the cartridge resin:

Wash the cartridge with 10 volumes of distilled water. Wash the cartridge with 10 volumes of regeneration buffer (see VI. Buffers and general comments). Again, wash with 10 volumes of distilled water, in order to remove residual EDTA.
Add 2 column volumes fo metal solution (0.1 M of metal chloride or -sulphate solution, for instance nickel chloride of cobalt sulphide). Then wash with at least 10 volumes of distilled water.

D. Preparation of the column:

D.1) In case the column shall be used immediately: Equilibrate with at least 25 ml of the binding buffer to be used.
D.2) In case the column shall be stored for some time: Rinse the cartridge matrix with ethanol (conc. 30 %), close the cartridge thoroughly and store at 4-8 °C. Wash the cartridge prior to use with 10 volumes binding buffer.

VI. Buffers and general comments

Determination of the quantity required depends on the amount of His-tagged protein which is to be isolated. The strength of binding of the protein to the resin as well as the yield of His-tag protein will depend on the amino acid composition, the accessibility of the His-tag, 3D structure, molecular weight etc.

The binding capacity was tested using purified Green fluorescent protein GFPuv-$(His)_6$, expressed in *E. coli*. This is, however, only an indicative value because binding capacity can be affected by several factors such as sample concentration, binding buffer and the flow rate during sample application. Binding capacities and recovery rate is always optimal and for best results in your assays, all parameters will have to be optimised. The choice of buffer mainly depends on the particular properties of the protein and has to be optimised for each protein. In general, buffer substances used most frequently are phosphates (10-150 mM). The pH of binding buffers generally leads to neutrality (pH 7.0-8.0), but can vary from 5.5 to 8.5. In order to avoid ionic interchange, add 0.15-0.5 M of NaCl.
Addition of imidazole increases the selectivity of the binding of target protein (10-40 mM). It is important to use high purity imidazole to avoid affecting photometric measurement at $OD_{280\ nm}$.
Important: Avoid presence of agents like EDTA or citrate!

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, make sure your His-tag is spliced to the protein at a protease cleavage site.

VI.A. Buffers for native protein isolation

Binding buffer: A typical binding buffer includes 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0
In general, buffer substances used most frequently are acetate (50 mM) or phosphate (10-150 mM). The pH of binding buffers generally leads to neutrality (pH 7.0-8.0), but can vary from 5.5 to 8.5. In order to avoid ionic interchange, add 0.1-0.5 M of NaCl.
Addition of imidazole increases the selectivity of the binding of target protein (10-40 mM). It is important to use high purity imidazole to avoid affecting photometric measurement at $OD_{280\ nm}$.
Important: Avoid presence of agents like EDTA or citrate!

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

Elution buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0.
In general, addition of a competitive ligand (generally imidazole), allows the elution of the retained protein. In most cases, 0.25 M of imidazole is enough to

efficiently elute the target protein. However, in case the elution is not as efficient as required, the imidazole concentration in the elution buffer may be increased to 0.5 or even 1.0 M.

VI.B. Buffers for denaturing protein isolation / isolation from inclusion bodies

Use the same denaturing agent for all buffers involved in one isolation procedure.

Lysis buffer (for inclusion bodies): 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, denaturing agent (e.g. 8 M urea), pH 8.0

Binding buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH of 8.0. (w/o denaturing agent)

Washing buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, denaturing agent (e.g. 8 M urea), pH 8.0.

Elution buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, denaturing agent (e.g. 8 M urea), pH 8.0.

VI.C. Buffers for cleaning and regeneration

Cleaning buffer: 0.5 M NaOH

Regeneration buffer: EDTA (100 mM), pH 8.0.

VII. Compatibility of reagents:

Reagents	Comments
Buffers	
Sodium phosphate	Sodium phosphate buffer 50 mM 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 2 M can be used
Denaturing Reagents	
Urea	Solubilizes protein. Use 8 M for purification under denaturing conditions.
Guanidine-HCl	Solubilizes protein. Up to 6 M may be used.
Additives	
Imidazole	Competes with the His-tag protein. Reduces non specific binding (20 mM). Elute the His-tag protein (up to 100 mM).
Glycerol	Avoids hydrophobic interactions between proteins. Up to 50% can be used.
EDTA	Coordinates with cations, causing a decreasse in capacity. Not recomended, 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 clumn clogging and low flow rates. Up to 20% can be used.
Reducing Agents	
Reduced glutathione	Can reduce Ni^{2+} ions at higher concentrations. Up to 30 mM in samples has been used successfully in some cases.
ß-Mercapto-ethanol	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	
Non ionic detergents (Tween, Triton, etc.)	Remove background proteins. Up to 2% can be used

VIII. Recommended columns

Empty cartridges for protein isolation

In case longer column beds are needed, 2 or 3 cartridges may be interconnected using the Luer adapter (0198.1, 10-32, male/female luer connector), resulting in a matrix of up to 10 cm. Compatible with common chromatography instruments like ÄKTA™FPLC™.

Art. No.	Volume*	Inner Ø	Frit pore size	Method
1345	1 ml	6,2 mm	12 µm	FPLC

* The actual filling volume depends on the respective resin used, varying between approx. 1 and 1.3 ml.

Empty columns for protein isolation

Art. No.	Type	Fig.	Matrix vol.	Total vol.	Frit pore size	Method
1541	Spin	1	100-250 µl	1,5 ml	20 µm	Spin
1515	Grav S	2	100-200 µl	1 ml	20 µm	Gravity
1516	Grav M	3	0,5-2 ml	12 ml	20 µm	Gravity
1518	Grav L	3	2-6 ml	35 ml	20 µm	Gravity
1527	Luer	4	50-100 µl	0,8 ml	35 µm	Pressure
1532	Inject	5	0,8 ml	0,8 ml	10 µm	Pressure

Empty cartridges FPLC

Art. No.	Type	Vol. (ml)*	Cartridges (no.)	Frits (no.)	End caps (no.)	Ø (mm)	Height (mm)
0860	FPLC-8	8	3	9	6	12	70
0862	FPLC-30	30	2	6	4	21	87
0866	FPLC-45	45	2	6	4	21	137
0868	FPLC-80	80	1	3	2	26	144

*packed column matrix

IX. Trouble Shooting

IX.A. Sample Application

Putative cause	Recommendation
Sample of high viscosity	
Presence of DNA in the sample	Sonify sample until viscosity is reduced or degrade DNA via DNAse
Steric hindrance of the substrate	Dilute the sample prior to application to the column. Purification in batch format may be method of choice.
Highly diluted or concentrated sample	
Highly diluted sample	Concentrate sample prior to application to the column. Carry out an adsorption step in batch format and pack the column with the pre-adsorbed resin
Concentr. sample	Dilute sample prior to loading onto the column

IX.B. Adsorption

Putative cause	Recommendation
No binding of target protein to the column	
His-tag is not present or has been degraded	Purify protein at lower temperature (4°C). Reduce time needed for purification. Add Protease inhibitors. Double-check recombinant vector for multi-His site.
His-tag is not exposed (inaccessible)	Purify in denaturing conditions. Redesign vector in order to change site of the His-tag {N-terminus, C-terminus, or in both positions).
Inadequate binding conditions	Check buffers and binding pH. Check reagents used (best before dates). Use fresh chemicals. Reduce imidazole concentration or omit completely in binding buffer. Recheck reagents used in binding buffer for interference with binding reaction. Check whether protein may be found in inclusion bodies (see IV.) Regenerate column/resin
Inefficient binding of target protein to the column	
Column capacity is exceeded	Apply less protein/sample. Increase matrix volume.
Matrix has been used too often	Apply a regeneration step
Loss of chelating metal in the resin	Apply a regeneration step. Avoid use and chelating agents.
Histidine tail is not well exposed	Apply slower flow rates. Apply or increase incubation time of sample in the column. Invert column after application of sample to disperse the matrix.
	Choose batch format for binding in order to allow increased contact between resin and rec. protein. Chose buffer with denaturing conditions.
Poor protein expression	Optimize bacterial expression conditions.
Rec. protein expressed in inclusion bodies.	Modify bacterial growth conditions. Purify under denaturing conditions (see IV.)
Matrix bed disturbed (channel-formation)	Re-pack column.

IX.C. Elution

Putative cause	Recommendation
High amount of co-eluted proteins (contaminants)	
Insufficient washing	Increase volume of washing buffer. Increase number of washing steps. Increase concentration of imidazole in washing buffer. Invert column during washing in order to disperse matrix beads.
Inadequate adsorption conditions	Check pH. Add or increase saline concentration in binding buffer in order to avoid non-specific ionic interactions. Add low concentration of non-ionic detergent. Add small quantities of ethylene glycol or glycerol to the binding buffer in order to avoid non-specific hydrophobic interactions. Increase imidazole conc. in binding buffer or apply imidazole gradient (*note:* keep imidazole ≤20 mM).
Column / matrix volume too large	Reduce resin quantity. Proteins will compete for less binding sites, increasing binding selectivity. Employ an imidazole concentration gradient to separate the target protein from the rest of retained proteins.
Target protein poorly eluted	
Too smooth elution conditions	Increase imidazole concentration up to 2.0 M in elution buffer. Increase temperature during elution. Apply slower flow rates.

	Invert column during elution in order to disperse matrix HPBeads more thoroughly. Apply or increase incubation time after application of sample to the column. Choose batch format for binding in order to allow increased contact between resin and rec. protein.
	Change elution conditions / buffer: - Replace imidazole by other chelating reagents (see VI.). - Elute under pH-reduction (pH 4.0) in the presence of imidazole. - Elute under denaturing conditions. - Use chelating agent such as EDTA.
Recombinant protein precipitates / can be precipitated	Add solubilizing agents. Incubate column with elution buffer for 8-10 h and then elute from the column. Choose batch format in order to reduce local concentration of protein.
Elution profile is not reproducible	
Sample has been modified (e.g. lost His-tag)	Recheck conditions of bacterial growth / protein expression and sample preparation. Prepare fresh sample, standardise sample preparation. Run the protocol at 2-8 °C. Add protease Inhibitors.
Precipitation of proteins or lipids	Regenerate resin/column.
PH or ionic forces could have been modified	Prepare new buffers.
Loss of binding capacity	Regenerate resin/column.

IX.D. Changes of the Resin

Putative cause	Recommendation
Loss of colour	
Chelating reagents present in sample may have caused diminution of metal content	Eliminate chelating reagents in the sample (e.g. by gel filtiation) and regenerate resin/column.
Colour shift to brown	
Presence of reducing agents in sample	Eliminate reducing agents and regenerate resin/column.
Loss of binding capacity	Regenerate resin/column.

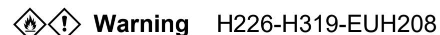
X. Storage

We recommended to wash the cartridge with at least 25 ml of 15 % ethanol prior to storage.
Store at +2 to +8 °C. Do not freeze.

For research use only. Not approved for human or veterinary use, for application to humans or animals, or for use in clinical or in vitro *diagnostics.*

Hazard and Precautionary Statements

Please note safety data given on label and MSDS.



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ROTI®Garose His/Ni NTA-/Ni NTA-Cartridges

1x5 ml 0804.1
5x5 ml 0804.2