Instructions for use

ROTI®Garose His Cartridges

Cartridges with matrix of Nickel charged agarose beads for high pressure affinity chromatography.
IMAC matrix, nickel-charged.

I. Characteristics

The matrix of ROTI®Garose His cartridges consists of crosslinked and beaded 6 % agarose, IDA-conjugated and charged with divalent nickel ions. The ROTI®Garose His matrix results in eluates with considerably low metal contamination. The tridentate IDA cross-linker provides easy elution with low amounts of imidazole.
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 has been optimised for native and denaturing conditions, being stable in all commonly used reagents including denaturing and reducing reagents like 8 M urea, 6 M guanidinium hydrochloride, 5 mM DTT etc.
Cartridge material made from polypropylene and polyethylene (frit). Matrix: Slurry in ethanol (20 %).
May repeatedly be regenerated.

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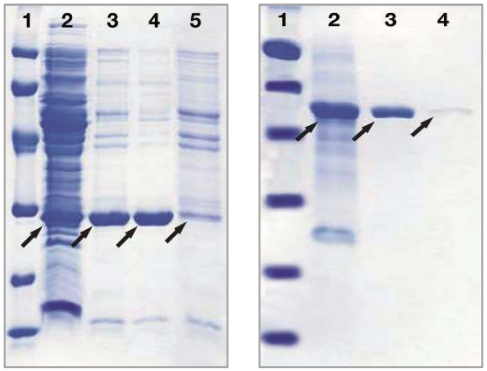

Figures
Left: SDS-PAGE of 6xHis-fuculose aldolase, 28 kDa (arrows). Very high yield of well purified proteins.
1: Marker, 2: Protein raw extract, 3,4: 6xHis target protein purified with ROTI®Garose His/Ni Beads or Column, respectively, 5: 6xHis target protein purified by competitor product.
Right: SDS-PAGE of highly expressed 6xHis-glutaryl acylase, 58 kDa (arrows). Good yield of extremely pure proteins.
1: Marker, 2: Protein raw extract, 3: 6xHis target protein purified with ROTI®Garose His/Co Beads, 4: 6xHis target protein purified by competitor product.

II. Run of the affinity chromatography

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.

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).
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.
Note: In some cases, a slight increase of contact time may facilitate binding.
4. Washing of matrix
Wash the cartirdge with big amounts of binding buffer. A good marker for efficient washing is measurement of 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 5 bar [0.5 MPa] (70 psi)
Maximum pressure is 10 bar [1 MPa] (140 psi)

Note on binding capacity: The binding capacity was tested using purified dehydroxyacetone kinase (6xHis), measuring 110 mg DHAK-(6xHis) purified per ml medium. 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.

III. Elution from inclusion bodies

Recombinant proteins often form insoluble inclusion bodies. In these cases, purification under denaturing conditions has to be applied using one of the following reagents:

Reducing chemical stability: HCl 0.01 M, SDS 2 %, NaOH 0.1 M, 2-propanol 30 %, ethanol 20 % NaOH 1 M, sodium acetate 0.1 M (pH 4.0), acetic acid 70 %.
Denaturing agents: Urea 8 M, guanidine-HCl 6 M.
Detergens: Triton X100 2 %, Tween 20 2 %, Chaps 1 %.
Additives: Imidazole 2.0 M, ethanol 20 % + glycerol 50 %, Na_2SO_4 100 mM, NaCl 1.5 M, EDTA 1 mM, EDTA 1 mM + $MgCl_2$ 10 mM, Citrate 60 mM, Citrate 60 mM + $MgCl_2$ 80 mM.
Buffer substances: Na_2HP0_4 50 mM (pH 7.5), Tris-HCl 100 mM (pH 7.5), MOPS 100 mM (pH 7.5), Tris-acetate 100 mM (pH 7.5), HEPES 100 mM (pH 7.5).
Reducing agents:* Reduced glutathione 10 mM, β-mercaptoethanol 20 mM, DTE 5 mM, DTT 5 mM.
*Note: The reagents described here are compatible with nickel charged ROTI®Garose beads under the conditions and concentrations indicated. The stability of the nickel charged resin has been tested with each reagent separately.
However, in general under extended treatments with reducing agents, or when high concentrations of these reagents are used, reduction of the nickel ion may result. This will then affect the binding capacity of the resin, so these agents should be avoided.

IV. Regeneration:
In order to preserve the optimum binding capacity during repeated binding and elution cycles, we recommend regenerating the resin once in a while. During this process, 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, or when another protein is to be eluted.

A. Elimination of the metal from the resin:
Wash the resin with 25 ml of regeneration buffer (see VI. Buffers and general comments).

B. Elimination of the excess EDTA:
In order to eliminate the residual EDTA before recharging the resin with the corresponding metal ions, the column should be washed with at least 25 ml binding buffer, followed by at least 25 ml of distilled water.

In few cases it may be necessary to additionally apply a more drastic regeneration procedure, in order to eliminate denatured proteins or lipids. This process is only necessary if the column has already been used.
B.1 Elimination of ionic interactions:
Wash the cartridge matrix in at least 50 ml of 1.5 M NaCl followed by at least 50 ml of distilled water.
B.2 Elimination of precipitated proteins
(Putative cause for cartridge pressure changes).
Wash the cartridge matrix for at least 2 hours with 1.0 M NaOH. Remove the NaOH by washing with in at least 50 ml of 1.5 M NaCl followed by at least 50 ml of distilled water.
B.3 Elimination of strong hydrophobic interactions:
Wash the cartridge matrix for approximately 30 minutes with isopropanol 30 %. Wash with at least 50 ml distilled water in order to eliminate the isopropanol. Then wash withat least 50 ml of a solution of 0.5 % of non-ionic detergent, acetic acid 0.1 M. Wash twice with 50 ml of ethanol 70 %, then finally wash with at least 50 ml of distilled water.

C. Recharge the column with the corresponding metal:
Add at least 25 ml of a nickel solution (0.1 M of metal chloride or -sulphate solution). Then wash with at least 25 ml of distilled water. Prepare the cartridge for use by washing with at least 25 ml of binding buffer.

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. 20 %), close the cartridge thoroughly and store at 4-8 °C. Wash the cartridge prior to use according to II.1

V. Pretreatment of Ni^{2+} charged cartridge matrix (optional)
Due to some minor metal bleeding and metal reduction in the nickel charged resin – in most cases after use of reducing reagents in the buffers – a slight brown discolouration may appear. Under most circumstances, this does *not* affect performance.
However, in order to avoid this effect the resin may be pretreated prior to first use, eliminating the *free* nickel cations and hence preventing their reduction, subsequent colouring of the resin, and unwanted effects during protein elution.
1. Wash the resin with five volumes of distilled water.
2. Wash the resin with five volumes of binding buffer (without reducing agents in the buffer),
3. Wash the resin with five volumes of elution buffer (without reducing agents in the buffer).
4. Equilibrate with 50 volumes of binding buffer (without reducing agents).

VI. Buffers and general comments

Binding buffer: The choice of buffer depends on the particular properties of the protein as well as of the type of chelate used. The typical binding buffer includes disodium phosphate (20 mM), NaCl (500 mM) and imidazole (10 mM), at a pH of 7.5.
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!

Elution buffer: Typical elution buffer is disodium phosphate (20 mM), NaCl (500 mM), imidazole (500 mM), pH 7.5.
In general, addition of a competitive ligand (generally imidazole), allows the elution of the retained protein. In most cases, 0.5 M of imidazole is enough to efficiently elute the target protein, since most proteins are efficiently eluted at an imidazole concentration of approx. 250 mM. However, in case the elution is not as efficient as required, a concentration gradient of 0-0.5 M, or elution buffer with imidazole of 2.0 M may be applied. Other reagents that can be used as competitive ligands are histidine and ammonium chloride.
Generally, the subsequent elimination of imidazole is not necessary. In case it may hinder downstream processes, it may be eliminated by dialysis, by precipitation with ammonium sulphate or by ultrafiltration using ROTI®Spin centrifugation devices.
Other elution methods included reduction of pH to 3.0-4.0 (with or without gradient), and elution with EDTA or

EGTA (0.05 M). Please note that the latter causes the elution of both the protein and chelating metal.
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.

Regeneration buffer:
Sodium phosphate (20 mM), NaCl (0.5 M), EDTA (50 mM), pH 7.4.

VII. Bibliography

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VIII. Trouble Shooting

VIII.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	Apply diluted sample onto column

VIII.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 III.)
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 of reducing 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.
Poor protein expression	Optimize bacterial expression conditions.
Rec. protein expressed in inclusion bodies.	Modify bacterial growth conditions. Purify under denaturing conditions (see III.)
Matrix bed disturbed (channel-formation)	Re-pack column.

VIII.C. Elution

Putative cause	Recommendation
High amount of co-eluted proteins (contaminants)	
Insufficient washing	Increase volume of washing buffer. Increase number of washing steps. Add a bit imidazole (5-10 mM) to buffer used for washing. 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.
Column / matrix volume too large	Reduce resin quantity. Proteins will compete for less binding sites, increasing binding selectivity.
Metal ion chosen is not adaquate	Use Cobalt charged resin rather than Nickel charged resin.
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 beads 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.
Buffers have changed	Prepare new buffers
Loss of binding capacity	Regenerate resin/column.

VIII.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.

IX. Storage

We recommended to wash the cartridge with at least 25 ml of 20 % 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.

 **Warning** H226-H319-EUH208

SDB-Version: 08/2021

ROTI®Garose His Cartridges	**1x5 ml**	**1318.1**
	5x5 ml	**1318.2**