Instructions for use

ROTI®Garose His HPBeads

Agarose-Beads for one-step isolation of His-tagged proteins by affinity chromatography under low or middle pressure, or also in big volumes.
IMAC matrix, nickel- or cobalt charged. IDA ligand.

I.a. Characteristics

Nickel or cobalt charged IDA-agarose HPBeads for affinity chromatography under medium pressure, with high flow rate or with big sample-/matrix volume. In ROTI®Garose-His HPBeads, the advantages of the highly efficient His binding have been combined with a bead technology allowing a very rapid flow rate.
Those beads are perfectly suitable for MPLC and FPLC, or if His-tag proteins shall be isolated either in particularly short time or in big amounts.
The matrix of ROTI®Garose-His HPBeads consists of crosslinked and beaded 6 % agarose, IDA-conjugated and charged with divalent nickel or cobalt ions.
The tridentate IDA cross-linker provides easy elution with low amounts of imidazole. Additionally, ROTI®Garose-His HPBeads may repeatedly be regenerated, making them very cost-effective.
The Matrix is stable in all commonly used reagents including denaturing reagents (like 8 M urea, 6 M guanidinium hydrochloride, 5 mM DTT).
50 % bead slurry in 20 % ethanol.

Carl Roth GmbH + Co. KG
Schoemperlenstraße 3-5 • 76185 Karlsruhe
P.O. Box 100121 • 76231 Karlsruhe
Phone: +49 (0) 721/ 5606-0
Fax: +49 (0) 721/ 5606-149
info@carlroth.com • www.carlroth.com
ip 07/2021

I.b. Selection of adequate ion

Nickel charged HPBeads:

Suitable for **all general applications**, **or if proteins of very small amount** shall be isolated without loss.

Nickel chelates recognize two exposed histidine tags with good specificity and very high affinity, making the Ni^{2+} charged matrix the first choice for all standard applications. ROTI®Garose His HPBeads give very high yields of pure His-tagged protein with considerably low metal contamination.

Cobalt charged HPBeads:

Perfect choice **if extremely pure proteins are required**, **or if hard-to-separate proteins** shall be isolated.

Cobalt chelates recognize two exposed, vicinal histidine tags with superior specificity and good affinity, resulting in acceptable to good recovery rates only, but yielding proteins of superior purity.

Important – please note

In the following protocols, *'1 volume'* always refers to *'volume of matrix'*, which is the *amount of bead suspension* in batch mode, or the *bed volume* for packed columns.
For 1 ml gel volume (column bed), 2 ml resuspended agarose beads is necessary.

II. Column packaging

Determine the amount of resin needed for isolation of the required His-tagged protein (see VII. Buffers and general comments)

1. Manually shake the bottle to obtain a homogenous suspension of ROTI®Garose His HPBeads resin. Place a funnel in the head of the column and *slowly* run the suspension down the walls of the column. Avoid formation of bubbles.
2. Let the matrix settle. Decant the resin 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.
3. Repeat previous steps until the desired column height is obtained, considering the required binding amount and the sample volume.
4. In case the upper end of the column is to be capped (e.g. for storage of the prepacked column), insert the adapter or cap gently in the column head until it begins to displace the liquid. Make sure no air is trapped.
5. Apply distilled water to the column until the column matrix has completely settled and height is constant. In case the desired height is not achieved, add some more material by repeating steps 1 through 4.
6. When the required height of the column matrix is obtained, wash the column with 5 volumes of distilled water in order to completely eliminate the preservative.
7. Equilibrate the column with 5 to 10 volumes of binding buffer (see VII. Buffers and general comments). Cap the column.

Note: in gravity flow columns, matrix height should not exeed ¼ of the column height. We recommend to de-gas all solutions prior to adding to the column in order to avoid formation of bubbles.

III. Run of the affinity chromatography

Please note:

a) The following protocol has been optimised for the purification of His-tagged proteins under native conditions. In case of denaturing conditions, minor adaptations may be necessary.
b) The strength of binding of the protein to the resin will depend on: The resin used (both the number of chelate groups and the chelant metal), the accessibility of the His-tag, pH and buffer composition.
c) Application of the buffers and sample has to be performed by pipetting or injection, depending on the column chosen (gravity flow or pressure).
d) In case a gravity flow column is used, cap the column between steps as soon as the last buffer has just run into the surface of the matrix. During application of buffers or sample, make sure to *not disturb* the matrix surface. After application, remove cap in order to run chromatography by gravity. Pouring sample and buffers down a glass rod held against the wall of the column will minimise the introduction of air bubbles. Efficiency of washing may be enhanced by closing the bottom

and top of the column and inverting the column in order to disperse the resin.

e) Binding capacity of the resin is due to several factors, such as sample concentration, buffer composition, flow rate during sample application etc.

f) Steps 1 and 2 may be omitted if column has been self-packed directly prior to use according to II. Column packaging.

1. Elimination of preservative
Apply 5-10 bed volumes of distilled water and let them flow through by gravity or pressure.
2. Equilibration of resin/column
Equilibrate the column with 5-10 volumes of binding buffer (see VII. Buffers and general comments).
3. Application of sample
Apply sample onto the top of the matrix or by injection without stirring the surface of the matrix.
Note: An increase in contact time may facilitate binding. In order to do so, let the sample introduce into the matrix and then cut the flow by capping the lower column end for at least 15 mins.
4. Washing of matrix
Wash with binding buffer until the $OD_{280\ nm}$ reaches the baseline level of the binding buffer. Usually, this takes washing with 5-10 volumes.
5. Elution of target protein
Apply elution buffer to the column (see VII. Buffers and general comments).
Note: In order to enhance elution efficiency, keep elution buffer and resin in contact at least 10 minutes.
The amount of elution buffer used mainly depends on the protein amount to be eluted and the matrix volume. As a rule of thumb, 1 matrix volume may be applied.

Column flow recommendations:
Recommended maximum flow rate
(100 kPa, 15 cm bed height): 600 cm/h.
Recommended maximum pressure
(15 cm bed height): 300 kPa (3 bar, 40 psi)
The resin may be used with batch methods, gravity flow, and FPLC.

IV. 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 metal charged ROTI®Garose HPBeads under the conditions and concentrations indicated. The stability of the metal 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 metal ion may result. This will then affect the binding capacity of the resin, so these agents should be avoided.
In these cases we recommend to use
ROTI®Garose His/Ni NTA Beads,
perfectly suited for use under **reducing conditions**.

V. 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 5 column volumes of regeneration buffer (see VII. 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 5 column volumes 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 matrix / column has already been used.
B.1 Elimination of ionic interactions:
Remove the resin from the column and wash in batch for approximately 20 minutes in a 1.5 M NaCl. Wash three times with distilled water, repack and again wash with 10 column volumes of distilled water.
B.2 Elimination of precipitated proteins
(Putative cause for column pressure changes).
Remove the resin from the column and wash in batch for at least 2 hours in 1.0 M NaOH. Wash three times with distilled water, repack and again wash with 10 column volumes of distilled water.
B.3 Elimination of strong hydrophobic interactions:
Remove the resin from the column and wash in batch for approximately 20 minutes in isopropanol 30 %. Wash ten times with distilled water. Then wash for 2 hours in 0.5 % of non-ionic detergent, acetic acid 0.1 M. Wash twice with approx. 10 column volumes of ethanol 70 %, then wash three times with distilled water. Repack and again wash with 10 column volumes of distilled water.

C. Recharge the column with the corresponding metal:
Add 5 volumes of metal solution (0.1 M of metal chloride or -sulphate solution). Then wash with 5 column volumes of distilled water.

D. Preparation of the column:
D.1) In case the column shall be used immediately:
Equilibrate in 5 column volumes of the binding buffer used.
D.2) In case the column shall be stored for some time:
Add 1 column vol. ethanol (conc. 20 %), close column thoroughly and store at 4-8 °C.

VI. Pretreatment of Ni^{2+}-charged resin/columns in particular (optional)
Due to some minor metal bleeding and metal reduction in the 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* metal 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 10 volumes of binding buffer (without reducing agents).

VII. 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, pH, buffers used etc.
As a start one may use a general binding capacity of ca. 80-100 mg/ml gel volume (40-50 mg/ml suspension volume).

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 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 0.15-0.5 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!

Binding buffer: A typical binding buffer includes 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0

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, there are several ways of protein elution from the columns.

a) Addition of a competitive ligand (generally imidazole), for instance in the following composition: disodium phosphate (20 mM), NaCl (500 mM), imidazole (500 mM), pH 7.5.
   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.
   Note: 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.
b) Reduction of pH to 3.0-4.0 (with or without gradient)
c) Elution with EDTA or EGTA (0.05 M). Please note that the latter causes the elution of both the protein and chelating metal.

Regeneration buffer: Sodium phosphate (20 mM), NaCl (0.5 M), EDTA (50 mM), pH 7.0.

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.

VIII. Bibliography

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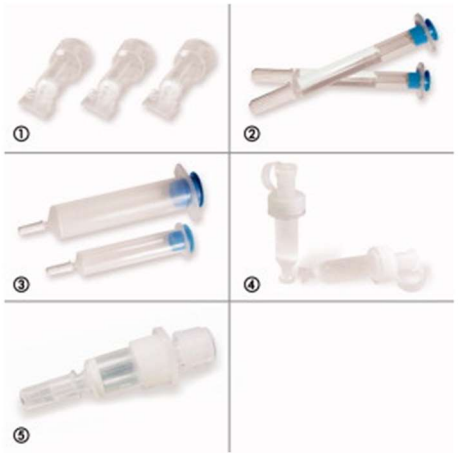
IX. Recommended columns



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)*	Cartri-dges (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

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.

X. Trouble Shooting

X.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 applica-tion 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

X.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.)
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. 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.

X.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 HPBeads.
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 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 VII.). - 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.

X.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.

XI. Storage

Store at +2 to +8 °C. Do not freeze.
HPBeads may be autoclaved at 121 °C for 30 mins.

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.

- **ROTI®Garose His/Co HPBeads** (Art. No. 0838) **Danger** H226-H319-H350-H411-EUH208
- **ROTI®Garose His/Ni HPBeads** (Art. No. 0835) **Warning** H226-H319- EUH208

SDB-Versionen: 08/2021

ROTI®Garose His/Co HPBeads	**25 ml**	**0838.1**
	100 ml	**0838.2**
ROTI®Garose His/Ni HPBeads	**25 ml**	**0835.1**
	100 ml	**0835.2**