Instructions for use

ROTI®Garose Glu/GST Beads

Agarose-Beads for one-step isolation of glutathione S-transferase(GST)-tagged molecules or other glutathione binding molecules by affinity chromatography under low or moderate pressure.

I. Characteristics

The matrix of ROTI®Garose-Glu/GST Beads consists of beaded 4 % agarose without crosslinking, coupled with glutathione. The matrix is easy to pack and results in very evenly packed columns. Elution is carried out via a mild Tris/glutathione buffer, enabling even recovery of native protein complexes.
Efficient and highly specific isolation of GST-tagged proteins via affinity chromatography is based on the affinity of the glutathion-S-transferase to it's substrate glutathione. Merely no unspecific binding of proteins without GST-tag takes place, even during purification from raw extracts, while the biological activity of the purified proteins is preserved by chromatography conditions.
Recommended for isolation of glutathione-S-transferase (GST) or GST fusion proteins in batch mode or via columns. Suitable for low to medium pressure, FPLC, or if GST-tag proteins shall be isolated either in particularly short time or in big amounts.

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II. Suitablilty

	Glu/GST Beads
Batch mode	suitable
Gravity flow	suitable
Spin isolation	suitable
Max. pressure	1 bar to 500 g
Max. flow rate	250 cm/h
Recommended flow rate	30-180 cm/h
Reducing conditions	not applicable
Bed volume	as required (also large scale)
Concentration	75 % beads
Preservative	20 % ethanol
Cross-linking	no
Agarose conc.	4 %
Ligand	glutathione
Binding capacity (approx..)	8 mg/ml settled matrix

Please note: The binding between GST-tag and glutathione is highly selective but slow. Thus, low flow rates and an increase in contact time during binding is strongly recommended in order to facilitate binding efficiency.

III. General notes

a) The following protocol has been optimised for the purification of GST-tagged molecules under native (mild, unreducing, undenaturing) conditions. However, these are general guidelines. Please optimise for each specific application.
b) The resin may be used with batch methods, gravity flow, and moderate FPLC.
c) Batch purification or flow through columns are the most common way to perform purifications with glutathione resins. However, in case cartridges shall be used, please refer to ROTI®Garose Glu/GST cartridges (Art. No. 0843.1)
d) Determine the amount of resin needed for isolation of the required tagged protein (see VI. Buffers and general comments).
e) Application of the buffers and sample has to be performed by pipetting.
f) 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.
g) 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.
h) Additional equilibration may be omitted if column has been self-packed directly prior to use.
i) Binding capacity of the resin is due to several factors, such as sample concentration, buffer composition etc. The binding between GST-tag and glutathione is highly selective but slow. Due to the optimised bead size, however, the flow through the matrix in general is fast.
Thus, low flow rates and an increase in contact time during binding is strongly recommended in order to facilitate binding efficiency.
j) 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.
k) In order to optimise the amount of protein isolated, we recommend to elute 3times and pool the eluates. However, since the yield of eluted proteins decreases with each elution, fractions may be analysed separately regarding the yield, and pooled only if they contain significant amount of isolated protein.
l) In some cases, a significant amount of GST fusion protein remains bound to the resin. Conditions (volumes, times, temperatures) used for elution may vary among GST fusion proteins. Eluates should be monitored (Bradford protein assay, SDS-PAGE or measure the absorbance at 280 nm to determine the yield of the eluted GST-fused protein.
m) 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.
n) For 1 ml gel volume (column bed), 1,33 ml resuspended agarose beads is necessary.

o) A 50 % suspension of pre-equilibrated ROTI®Garose-Glu/GST Beads may be stored at 4 ºC for up to 1 month and used if required.

IV.A. Purification of GST-tagged proteins by batch method

Particular parameters are dependent on the molecules that are to be isolated, may vary from the protocol given here, and should be determined for each assay.

Elimination of preservative

1. Determine the quantity of ROTI®Garose-Glu/GST Beads needed for your isolation according to VI. Buffers and general comments.
2. Gently shake the bottle of ROTI®Garose-Glu/GST Beads to achieve a homogeneous suspension. Immediately pipette the suspension to an appropriate tube.
3. Sediment the gel by centrifugation at 500 x g for 5 mins. and carefully decant and discart the supernatant.
4. Add 10 bed volumes of binding buffer to equilibrate the gel by mixing thoroughly to achieve a homogeneous suspension.
5. Sediment the gel by centrifugation at 500 x g for 5 mins. and carefully decant and discart the supernatant.

Binding of GST-tagged proteins

6. Add the clarified *E. coli* lysate or other sample to the equilibrated resin and mix the suspension gently for 30 mins. in minimum at room temperature.
   Note: An increase in contact time may facilitate binding.
7. Sediment the gel by centrifugation at 500 x g for 5 mins. and carefully decant the supernatant. Keep the supernatant until the isolation has been finished with good recovery rate.
8. Wash the gel by adding 10 ml bed volumes of binding buffer. Invert to mix.
9. Sediment the gel by centrifugation at 500 x g for 5 mins. and carefully decant and discart the supernatant.
10. Repeat the washing step at least twice (overall 3x washing). Wash the column with binding buffer until the $OD_{280\ nm}$ (nearly) reaches the baseline level of the binding buffer (<0.01).

Elution of target proteins

11. For elution add 1 bed volume of elution buffer to the gel. Mix thoroughly for 10 min at room temperature.
12. Sediment the gel by centrifugation at 500 x g for 5 mins. and carefully pipette the supernatant into a new tube. Store the supernatant on ice.
13. Repeat the elution step at least twice and pool the fractions containing the purified protein.

IV.B. Purification of GST-tagged proteins by gravity column chromatography

Particular parameters are dependent on the molecules that are to be isolated, may vary from the protocol given here, and should be determined for each assay.

Gravity column packaging

1. Determine the amount of resin needed for isolation of the required protein (see VI. Buffers and general comments)
2. Manually shake the bottle to obtain a homogenous suspension of ROTI®Garose Glu/GST-Beads resin. Close the lower column cap. Immediately pipet the required amount of bead solution onto the bottom membrane.
3. Let the matrix settle for some seconds. Remove the bottom cap and let the preservative flow by gravity.

Elimination of preservative

4. Equilibrate the column 3x with 5 bed volumes of binding buffer (see VI. Buffers and general comments): Close the bottom cap and add the buffer. Close the upper cap and invert a few times for mixing. Let the matrix settle for some seconds. Remove the bottom cap and let the preservative flow by gravity, making sure no air has been trapped and no bubbles are formed.

Prepacked columns may be equilibrated in binding buffer with 20 % ethanol and be stored for up to 1 month at 4 °C. In case the column has been stored in ethanol containing buffer, it has to be equilibrated as described above prior to use.

Binding of GST-tagged proteins

5. Apply the sample onto the top of the matrix without stirring the surface of the matrix. Open the bottom cap and let it just enter the matrix. Stop the flow through by closing the bottom cap.
   Incubate for at least 30 mins.
   Note: An increase in contact time may facilitate binding. Binding may also be increased by mixing of the column matrix, for instance by inverting the capped column carefully several times.
6. Remove the lower cap and keep the entire flow through.
7. Wash the column with 10 beds of binding buffer by carefully adding it on top of the matrix. Keep the first flow through. For efficient washing we recommend to close the caps after addition of the binding buffer and mix by inverting. Let the matrix settle shortly, then remove the caps for gravity flow through.
   Wash until the $OD_{280\ nm}$ (nearly) of the flow through reaches the baseline level of the binding buffer (<0.01). Usually this takes 3-4 washing cycles.

Elution of target proteins

8. For elution close bottom cap and carefully add 1 bed volume of elution buffer to the column. Close upper cap too and mix thoroughly for 10 mins. at room temperature.
   Note: An increase in contact time may facilitate elution. We recommend manual mixing.
9. Let the gel settle, remove the caps and collect the flowing through eluate in a new tube. Immediately store on ice.
10. Repeat the elution twice and pool all three eluates.

IV.C. Purification of GST-tagged proteins by spin column chromatography

Particular parameters are dependent on the molecules that are to be isolated, may vary from the protocol given here, and should be determined for each assay.
67 µl ROTI®Garose-Glu/GST Beads (corresponding to 50 µl settled gel matrix) are used to purify up to 400 µg of GST-fused protein. This protocol has been optimised for mini spin columns with inserted frits of 10-20 µm pore size.

Column packaging and equilibration

1. Gently shake the bottle to obtain a homogenous suspension of ROTI®Garose Glu/GST-Beads resin. Remove first the upper inlet cap and immediately pipette 67 µl suspension (corresponding to 50 µl gel beads) to an empty spin column.
2. Remove the lower outlet cap and put the spin column into a collecting tube. Centrifuge at 500 x g for 30 seconds.
3. Close the bottom cap and apply 500 µl of binding buffer. Close the upper cap and mix manually by inverting the column.

4. Remove both caps, centrifuge at 500 x g for 30 seconds and discard the flow through.

Prepacked columns may be equilibrated in binding buffer with 20 % ethanol and be stored for up to 1 month at 4 °C. In case the column has been stored in ethanol containing buffer, it has to be equilibrated as described above prior to use.

Binding of GST-tagged proteins

5. Close the bottom cap. Apply 700 µl of the clarified *E. coli* lysate or protein extract onto the matrix and let the sample mix with the resin.
6. Close the upper cap and mix the resin by inverting the column. Incubate for 30 to 60 mins.
7. Remove both caps and centrifuge at 500 x g for 30 seconds. Keep the flow through at +4 °C until the isolation has been successfully finished.
8. Close the bottom cap, then add 500 µl washing buffer (=binding buffer, see VI. Buffers and general comments). Close the upper cap and mix by inverting the column.
9. Remove both caps, centrifuge at 500 x g for 30 seconds and discard the flow through.
10. Repeat the washing steps 8-9 twice (total wash 3 x 500 µl of wash buffer).
    Note: Check the $OD_{280\ nm}$ of the flow through. Wash with wash buffer until this OD reaches the baseline level of the wash buffer.

Elution of target proteins

11. Close the bottom cap. Apply 50 µl elution buffer to the column (see VI. Buffers and general comments). Close the upper cap and incubate at room temperature for 10 mins. under constant agitation.
    Note: Longer incubation time during elution may increase the yield during the first elution round.
12. Remove both caps and centrifuge at 500 x g for 30 seconds. Collect the flow through (eluate) in an appropriate new tube. Store at +4 °C.
13. Repeat the elution steps twice and pool the collected eluates containing the purified protein.

IV.D. Purification of GST-tagged proteins by <u>FPLC</u>

Particular parameters are dependent on the molecules that are to be isolated, may vary from the protocol given here, and should be determined for each assay.

Column packaging

1. Gently shake the bottle to obtain a homogenous suspension of ROTI®Garose Glu/GST-Beads 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. Remove most of the supernatant from the top of the column by pipetting or flow through. Leave 1 cm above the top pf the matrix in order to prevent drying out.
3. Repeat previous steps until the desired column height is obtained, considering the required binding amount and the sample volume.
4. Insert the adapter or upper 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 stream until the column matrix has completely settled and height is constant (corresponding to the height of the column).

Elimination of preservative and equilibration

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 VI. Buffers and general comments).
   Note: Check the $OD_{280\ nm}$ of the flow through. Equilibrate with binding buffer until this OD reaches the baseline level of the binding buffer.

Binding of GST-tagged proteins

8. Apply the clarified (filtred or centrifuged) *E. coli* lysate or protein extract to the column. Use *low* flow rates in order to allow maximal binding of the GST-tagged proteins.
   Note: Keep the flow through and check whether the protein has been successfully removed from the lysate. In case too much protein is still present, reduce the flow rate further and apply the flow through again to the column.
9. Apply 5 to 10 volumes of washing buffer (see VI. Buffers and general comments)
   Note: Check the $OD_{280\ nm}$ of the flow through. Wash with wash buffer until this OD reaches the baseline level of the wash buffer.

Elution of target proteins

10. Apply 5 to 10 volumes of elution buffer (see VI. Buffers and general comments) and collect the flow through in fractions. Keep on ice.
11. Check the fractions for presence of the GST-tagged proteins (SDS-PAGE, Bradford) and pool the fractions containing the majority of the pure proteins.

Column flow recommendations

ROTI®Garose Glu/GST-Beads are compatible with common low pressure chromatography columns and FPLC™ applications. We recommend columns equipped with an adjustable plunger/flow adapter.
Use low rates for loading step to allow maximal binding of the GST-tagged protein.

The flow rate for equilibration, washing and elution can be increased to reduce the purification time as follows:

Column Ø (mm)	Bed vol. (ml)	Packing	Flow rate (ml/min)	Binding
6.6	1	1.4	1 (vol.)	0.3 - 1
16	10	7	5 (vol.)	0.5 - 5
			Flow rate (cm/h)	
		≤ 250	≤ 180 (lin.)	≤ 180

Vol.: volumetric lin.: linear

Conversion of
linear flow rate [cm/h] to volumetric flow rate [ml/min]:

$$VF\ [ml/min] = \frac{LF\ [cm/h]}{60} \times A\ [cm^2] = \frac{LF\ [cm/h]}{60} \times \frac{\pi \times (d[cm])^2}{4}$$

Convertion of
volumetric flow rate [ml/min] to linear flow rate [cm/h]:

$$LF\ [cm/h] = \frac{VF\ [ml/min]}{A\ [cm^2]} \times 60 = \frac{VF\ [ml/min] \times 60 \times 4}{\pi \times (d[cm])^2}$$

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. 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.
Regeneration of the resin is performed by sequentially applying 10 bed volumes of Regeneration buffer 1, followed by a second step with 10 bed volumes of Regeneration buffer 2 ('regeneration washing cycle'). Repeat this regeneration washing cycle twice and finally wash with 5 bed volumes of binding buffer. If you will not be using the resin immediately wash with additional bed volumes of 20 % ethanol and store at 4 ºC.

VI. Buffers and general comments

Determination of the quantity required depends on the amount of tagged molecule which is to be isolated.
The strength of binding of the protein to the resin as well as the yield of protein will depend on the amino acid composition, the 3D structure, molecular weight, pH, buffers used etc.
As a start one may use a general binding capacity of ca. 8 mg/ml gel volume (approx. 11 mg/ml suspension volume). Binding selectivity is high.
The expression level of GST-tagged proteins is high ranging from 10 to 50 µg/ml of *E. coli* culture.
Recommended gel volume per 100 ml culture volume:

Level of expression	Protein per vol	Settled gel	Bead suspension
10 mg/l	1 mg	0,125 ml	1,67 ml
50 mg/l	5 mg	4 ml	5,33 ml

Average cell pellet of 100 ml culture volume is 0.4 g.
Cells of 100 ml max be resuspended in 0.5-2 ml PBS.

Binding buffer: PBS buffer
We recommend to use ROTI®Stock 10x PBS buffer (Art. No. 1058). Or prepare: 10 mM NaH_2PO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 140 mM NaCl, pH 7.3

Washing buffer: Same as binding buffer

Elution buffer: 10 mM glutathione, 50 mM Tris-HCl, pH 8.0

Regeneration buffer 1: 100 mM Tris-HCl, 0.5 M NaCl, pH 8.5

Regeneration buffer 2: 100 mM sodium acetate, 0.5 M NaCl pH 4.5.

VII. 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

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 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
Highly concentrated sample	Dilute sample prior to loading onto the column

VIII.B. Adsorption

Putative cause	Recommendation
No binding of target protein to the column	
Inadequate binding conditions	Check buffers and binding pH. Check reagents used (best before dates). Use fresh chemicals. Recheck reagents used in binding buffer for interference with binding reaction. Check whether protein may be found in inclusion bodies.
GST-tag (or whole fusion protein) 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.
Extraction not optimal	Choose milder sonication conditions. Over-sonication can alter the conformation of the GST moiety. Add 5 mM DTT to the cell lysis buffer. Reducing agents may significantly increase the binding efficiency of some fusion proteins.
Resin used too often	Regenerate or use freh resin. Immobilized glutathione can be

	degraded by γ-glutamil-transpeptidase activity present in *E. coli* cell lysates.
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
GST-tag is not binding well	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.
Protein concen-tration too low	Concentrate the sample prior to application
Flow rate too high	Use lower flow rate or skip to batch isolation in order to allow better contact between resin and fused protein
Matrix bed disturbed (channel-formation)	Re-pack column.

VIII.C. Elution

Putative cause	Recommendation
High amount of co-eluted proteins (contaminants) / poor protein purity	
Insufficient washing	Increase volume of washing buffer. Increase number of washing steps. Invert column during washing .
Inadequate adsorption conditions	Check above.
Co-purification of chaperonins	Several chaperonins involved in protein folding may co-purify with GST-fusion proteins, e.g. DnaK (~70 kDa), DnaJ (~37kDa), GrpE (~40 kDa), GroEL (~57 kDa), GrpE (~40 kDa), GroEL (~57 kDa), GroES (~10kDa). Co-purification of DnaK, for instance, can be avoided by treating the cell lysate with 5 mM $MgCl_2$ and 5 mM ATP prior to purification. For other chaperonins please see the common literature.
Target protein poorly eluted / low protein yield	
Too smooth elution conditions	Increase temperature moderately during elution. Reduce flow rate. Apply or increase binding time. Invert column during elution incub. in order to disperse matrix beads. Choose batch format for binding in order to allow increased contact between resin and fusion protein. Add 1 or 2 elutions steps and pool the fractions.
Flow rate too high	Decrease flow rate during elution
Inadequate elution conditions	Check formualtion and pH of the elution buffer. Use fresh reagents. For some fusion proteins, increase to up to 50 mM glutathione increases elution yield.
Problems with vector construction	Ensure that protein and tag are in frame
Poor protein expression	Optimize bacterial expression conditions. Check OD_{600} – overgrowth (too long incubation times) may dramatically lower protein yield.
Fusion protein forms inclusion bodies	Lower the growth temperature from 37 ºC to 30 ºC in maximum.
Extraction insufficient	Check extraction conditions (lysozyme, sonication). Use up to 2% of a non-ionic detergent to improve cell disruption and/or solubilization of the fusion protein.
Fusion proteins degraded	Sonication during extraction too severe. Chose milder conditions. Purify at lower temperatures (4 ºC). Reduce purification step times. Keep the samples and buffers on ice in order to reduce the activity of proteases. Add protease inhibitors. Use a protease-deficient host.
Elution profile is not reproducible	
Sample has been modified (e.g. lost 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 or use freh resin. Immobilized glutathione can be degraded by γ-glutamil-transpeptidase activity present in *E. coli* cell lysates.

IX. Storage

Store at +2 to +8 °C. Do not freeze.
Beads may not be autoclaved.

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.*

Warning H226-H319
P210-P280.7-P305+P351+P338

ROTI®Garose Glu/GST Beads	**10 ml**	**0841.1**
	100 ml	**0841.2**