AFFINITY GST PURIFICATION

Procedure for Use
Glutathione Agarose 4 Resin

DESCRIPTION

Glutathione Agarose Resin is used to purify recombinant derivatives of glutathione S-transferases or glutathione binding proteins. Resins are products that allow batch or column purifications. Purification of GST fusion proteins using Glutathione Resin provides an easy one step purification. This product is supplied as 75% (v/v) aqueous suspension (in 20% ethanol).

INSTRUCTIONS

I. Batch Purification of GST-tagged proteins

The following summarized procedure is adapted for the purification of GST-tagged protein in batch.

1. Elimination of the Preservative

Determine the quantity of Glutathione Agarose needed for your purification following the recommendations below. Gently shake the bottle of Glutathione Agarose to achieve a homogeneous suspension. Immediately pipette the suspension (1.33 ml of the original Glutathione Agarose suspension per ml of gel volume required) to an appropriate tube. Sediment the gel by centrifugation at 500 x g for 5 minutes and carefully decant the supernatant and discard it.

Recommendations

Quantity of Glutathione Agarose required. Binding capacity will vary for each GST-tagged protein. The yield of GST-tagged protein depends on various parameters, such as nature of the fusion protein, expression host, culture conditions, etc.
Glutathione Agarose has an orientative static binding capacity of 8 mg of GST tagged protein /ml gel[(1)].
[(1)] 1 ml gel corresponds to 1.333 ml of 75% (v/v) Glutathione Agarose suspension.

2. Equilibration of the Resin

Add 10 bed volumes of binding buffer to equilibrate the gel by mixing thoroughly to achieve a homogeneous suspension. Sediment the gel by centrifugation at 500 x g for 5 minutes and carefully decant the supernatant and discard it.

Note: Binding buffer: 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 140 mM NaCl, pH 7.3 (PBS buffer).

Note: A 50% suspension of pre-equilibrated Glutathione Agarose may be used directly or may be stored at 4°C for up to 1 month and used if required.

3. Application of the Sample

Once the resin is equilibrated, the sample containing the fused protein for purification is applied following the recommendations below. Add the clarified *E. coli* lysate to the equilibrated resin and mix the suspension gently for 30 min at room temperature. In some cases a slight increase of contact time may facilitate binding.

Centrifuge the suspension at 500 x g for 5 minutes to sediment the resin. Carefully decant the supernatant and discard it.

Recommendations

Protein load and culture size

The expression level of GST-tagged proteins is high ranging from 10[(a)] to 50[(b)] mg/liter of *E. coli* culture.

Table 1: Required culture volumes for 1 ml of settled Glutathione Agarose [(1)].

Expression level	*E. coli* culture	Resuspend in	Protein lysate
10 mg/l[(a)].	800 ml culture (~ 3.2 g cell pellet[(2)])	~ 16 ml PBS [(3)]	~ 20 ml
50 mg/l[(b)]	160 ml culture (~ 0.64 g cell pellet[(2)])	~ 3.2 ml PBS [(3)]	~ 4 ml

[(1)]1 ml of settled agarose corresponds to 1.333 ml of 75 % (v/v) Glutathione Agarose suspension.
[(2)]On average, 250 ml of culture will produce approximately 1g of pelleted, wet cells.
[(3)]1g cells may be lysed in 2-5 ml PBS.

4. Washing of the Resin

Wash the gel by adding 10 ml bed volumes of binding buffer. Invert to mix and centrifuge the suspension at 500 x g for 5 minutes to sediment the resin. Carefully decant the supernatant and discard it.

Repeat the washing step twice (total wash 3 x 10 bed volumes of PBS).

Note: It will be washed with the binding buffer until the 0.D 280 nm is the same as the binding buffer.

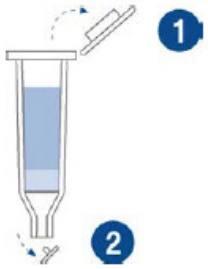
5. Elution of the Pure protein

Add 1 bed volume of elution buffer to the gel. Mix thoroughly for 10 min at room temperature. Sediment the gel by centrifugation at 500 x g for 5 minutes and carefully decant or pipette the supernatant in a new tube and store on ice. Repeat the elution step twice or more and pool the fractions containing the purified protein.

Note: Elution buffer: 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0. It is possible that a significant amount of GST fusion protein may remain 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).

6. Regeneration & Storage

See the Procedure at the end of this publication.



II. Gravity Purification of GST-tagged proteins

The following summarized procedure is adapted for the purification of GST-tagged protein in gravity columns.

1. Elimination of the Preservative

Determine the quantity of Glutathione Agarose needed for your purification *(see section I. 1- Recommendations)*.

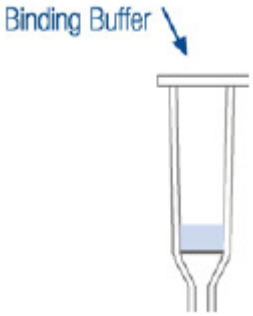
Gently shake the bottle of Glutathione Agarose to achieve a homogeneous suspension. Immediately pipette sufficient suspension to an appropriate empty column [(1)].

(1) Empty column information

Column	Cat. N°	Total Capacity
Plastic Columns	C-50	12 ml
Plastic Columns XL	CXL-50	35ml

Remove first the upper and then the lower cap of the column, to allow elimination of the preservative by gravity flow.

2. Equilibration of the Pre-Packed Column

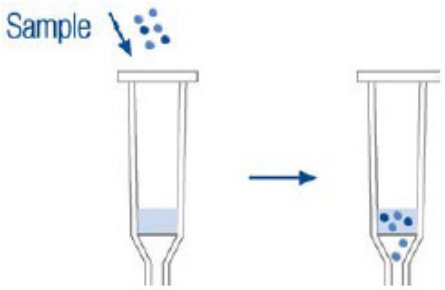


Equilibrate the column with 5ml bed volumes of binding buffer. Add the binding buffer on the upper part of the column and make sure no air has been trapped. Mix manually inverting the Pre-packed column and discard the supernatant. Repeat the equilibration step twice.

Note: Binding buffer: 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 140 mM NaCl, pH 7.3 (PBS buffer).

Note: A 50% suspension of pre-equilibrated Glutathione Agarose may be used directly or may be stored at 4°C for up to 1 month and used if required.

3. Application of the Sample



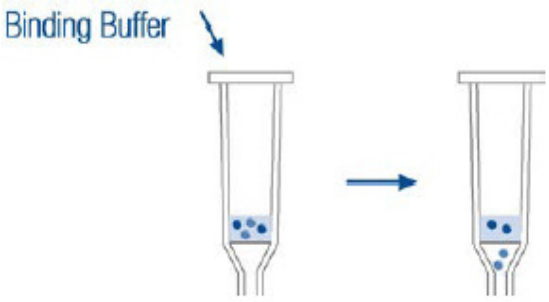
Add the sample containing the GST-tagged protein to be purified (*see section 1.3-Recommendations*) through the top of the column, keeping sample and resin in contact at least 30 minutes before removing the bottom cap. Mix manually inverting the Pre-Packed column. Remove the lower cap of the column and discard the supernatant.

Note: In some cases a slight increase of contact time may facilitate binding.

Note: Pouring the sample down a glass rod held against the wall of the column will minimize the introduction of air bubbles.

Note: Binding capacity can be affected by several factors such as sample concentration.

4. Washing of the Pre-Parked Column



Close column outlet with the cap. Add the binding buffer (10 bed volumes) through the top to eliminate all the proteins that have not been retained in the column. Close column inlet with the cap, mix manually inverting the Pre-Packed column. Remove the lower cap of the column and discard the supernatant.

Repeat the washing step twice (total wash 3 x 10 bed volumes of PBS).

Note: It will be washed with the bind1ng buffer until the O.D. 280 nm was the same as the binding buffer.

5. Elution of the pure protein

Close column outlet with the cap. Add 1 bed volume of elution buffer to the column. Close column inlet with the cap and mix thoroughly for 10 min at room temperature. Sediment the gel, remove the end cap and collect the eluate in a new tube and store on ice.

Repeat the elution step twice and pool the collected eluates.

Note: Mix manually inverting the Pre-Packed column. It is advisable to keep elution buffer and resin in contact at least 10 minutes before removing the bottom cap.

Note: Elution buffer is 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0. It is possible that a significant amount of GST fusion protein may remain 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).

6. Regeneration & Storage

See Procedure at the end of this Publication.

III. Spin Purification of GST-tagged proteins

The following summarized procedure is adapted for the purification of GST-tagged protein in spin columns [1].

Note: 50 µl Glutathione Agarose volume are used to purify up to 400 µg of GST-fused protein.

(1) In this protocol are required Empty mini spin columns with inserted frits of 10 - 20m µm pore size.

1. Elimination of the Preservative

Gently shake the bottle of Glutathione Agarose to achieve a homogeneous suspension. Remove first the upper inlet cap and immediately pipette 67 µl of the original suspension to the empty spin column. Remove the lower outlet cap and put the spin column in a collecting tube. Centrifuge at 500 x g for 30 seconds.

Note: 67 µl of the original 75% suspension corresponds to 50 µl of gel.

2. Equilibrium of the Spin Column

Equilibrate the spin column with 500 µl of binding buffer. Mix manually, centrifuge at 500 x g for 30 seconds and discard flow through.

Note: Binding buffer: 10 mM Na_2HP0_4, 1.8 mM KH_2P0_4, 2.7 mM KCl, 140 mM NaCl, pH 7.3 (PBS buffer).

Note: A 50% suspension of pre-equilibrated Glutathione Agarose may be used directly or may be stored at 4°C for up to 1 month and used if required.

3. Application of the Sample

Close spin-column outlet with cap. Add the sample containing the GST-tagged protein to be purified (700 µl of clarified *E. coli* lysate) through the top of the spin column, keeping sample and resin in contact at least 30 minutes before removing the bottom cap. Mix manually inverting the spin column. Remove bottom cap and place spin column in a collecting tube.

Centrifuge at 500 x g for 30 seconds and discard the flow through.

Note: In some cases a slight increase of contact time may facilitate binding. Binding capacity can be affected by several factors such as sample concentration.

4. Washing of the Spin Column

Add 500 µl binding buffer through the top to eliminate all the proteins that have not been retained in the column. Mix manually inverting the spin column.

Centrifuge at 500 x g for 30 seconds. Discard flow through. Repeat the washing step twice (total wash 3 x 500 ml of PBS). Discard flow through between washing steps.

Note: Wash the column with binding buffer until the O.D 280 nm of the eluent reaches the baseline level.

5. Elution of the Pure protein

Close spin column outlet with cap. Add 50 µl of elution buffer and close the lid. Mix thoroughly for 10 min at room temperature. Centrifuge the gel, remove the end cap and collect the eluate in a new tube and store on ice. Repeat the elution step twice. Pool the collected eluates.

Note: Elution buffer is 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0. It is possible that a significant amount of GST fusion protein may remain 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).

6. Regeneration & Storage

See the Procedure at the end of this publication.

IV. FLPC Purification of GST-tagged proteins

Glutathione Agarose Resin is 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 (see Table 1).

1. Colum Packaging, Elimination of the preservation and Equilibration of the Resin

a. Manually shake the bottle to obtain a homogeneous suspension of Glutathione Agarose/preservative. Place a funnel in the head of column and slowly run the suspension down the walls of the column.

   Note: It is advisable to make the addition slowly to avoid the formation of bubbles. The product may also be degassed before adding to the column.

   Decant the product 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.

b. Repeat previous steps until the desired column height is obtained.

c. Insert the adapter gently in the column head until it begins to displace the liquid.

   Note: Make sure no air is trapped under the net.

d. Add distilled water to the purification stream until a constant height (corresponding to the height of the column) is achieved.

   Note: If the desired height is not achieved, repeat steps 1 through 4.

e. When a constant height has been obtained, maintain the flow with the addition of 5 volumes of distilled water to completely eliminate the preservative.

f. Equilibrate the column with at least 5 column volumes of binding buffer until the baseline at 280 nm is stable.

   Note: It is advisable to previously de-gas all the solutions before adding to the column to avoid the formation of bubbles.

Table 1: WORKING CONDITIONS

		Volumetric flow rate (ml/min)		
Column diameter (mm)	Bed Volume (ml)	Packing	Equilibrium/Washing/Elution	Binding
6.6	1	1.4	1	0.3 - 1
16	10	7	5	0.5 - 5
			Linear flow rate (cm/h)	
		≤250	≤180	≤180

(1) Converting from linear [cm/h] to volumetric flow rates [ml/min].

Converting from linear flow rate [cm/h] to volumetric flow rate [ml/min].

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

(2) Converting from volumetric flow rate [ml/min] to linear flow rate [cm/h].

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

3. Application of the Sample

Once the resin is equilibrated, the centrifuged or filtered sample is applied (*see section 1.3- Recommendations*). In some cases a slight increase of contact time may facilitate binding. Therefore use low rates for the loading step to allow maximal binding of the GST-tagged protein.

Note: Binding capacity can be affected by several factors, such as sample concentration or the flow rate during sample application. Collect flow through and verify that GST has bound.

4. Washing of the Resin

It will be washed with the binding buffer (5-10 bed volumes of PBS) until the 0.D. 280 nm reaches the baseline level.

5. Elution of the Pure protein

Elute the GST-tagged protein with 5-10 bed volumes of Elution buffer and collect the fractions on ice.

Note: Is important to identify (SDS-PAGE & Bradford protein assay) the fractions that contain the majority of pure protein.

6. Regeneration & Storage

During the life of the resin, it may lose binding points because some protein is retained. Hence a loss of the binding capacity may be observed in successive cycles. To return to the starting state, regeneration may be necessary. In general, column regeneration is always necessary when changing proteins. When continuing with the same protein it is recommended to do a regeneration when an appreciable diminution in the yield is observed. The frequency of these stages varies with the protein and the conditions used.

After use, regenerate the resin by sequentially applying 10 bed volumes of 100 mM Tris-HCl, 0.5 M NaCl, pH 8.5 followed by a second step with 10 bed volumes of 100 mM sodium acetate, 0.5 M NaCl pH 4.5. Repeat the above wash cycles 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°C.

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
LOW PROTEIN YIELD	• Problems with vector construction.	• Ensure that protein and tag are in frame.
	• Poor protein expression.	• Optimize bacterial expression conditions.
	• Fusion protein forms inclusion bodies.	• Lower the growth temperature from 37°C to 30 -15°C.
	• Extraction may be 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.
HIGHLY DILUTED OR CONCENTRATED SAMPLE	• Highly diluted sample.	• It is preferable to concentrate the sample before its purification in the column. • Another solution is to carry out an adsorption step in batch format and pack the column with the resultant resin of the adsorption step.
	• Highly concentrated sample.	• It is preferable to make a previous dilution of the sample before its purification in the column.

2. ADSORPTION

OBSERVATION	POSSIBLE CAUSES	RECOMMENDATION
TARGET PROTEIN DOES NOT BIND EFFICIENTLY	• Sonication may have been too severe.	• Choose milder sonication conditions. Over sonication can alter the conformation of the GST moiety and prevents the fusion protein from binding to Glutathione Agarose
	• Reducing agent missing.	• By adding DTT to the lysis buffer (final concentration 5 mM) prior to cell lysis can significantly increase binding of some fusion proteins.

	• Concentration of fusion protein is too dilute.	• Concentrate the sample. If the sample is too dilute, target protein may not bind efficiently. Yield depends on the concentration of the fusion protein in the lysate.
	• Flow rate too high.	• Try to use slower flow rates or make the adsorption in batch to allow a better contact between resin and fused protein.
TARGET PROTEIN DOES NOT BIND EFFICIENTLY	• Channels have formed in the column so the sample runs mainly through these undesirable channels.	• Re-pack column.
	• Inadequate binding conditions	• Check the conditions.
	• Column capacity exceeded.	• Apply less fused protein to the column
	• The resin has been previously used during several purification cycles. 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.	• Clean resin according to the Procedure of use or use fresh resin. Note: Immobilized glutathione can be degraded by Y-glutamil transpeptidase activity in E. coli cell lysates.

3. ELUTION

OBSERVATION	POSSIBLE CAUSES	RECOMMENDATION
POOR PROTEIN PURITY	• Sonication may have been too severe.	• Choose milder sonication conditions. Over-sonication can lead to the co-purification of host proteins with the GST-fused protein.
	• Degradation of GST fusion protein	• 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. Keep the samples and buffers on ice to reduce the activity of proteases. • Add protein inhibitors • Use a protease-deficient host. Multiple bands may be the result of partial degradation by host proteases during cell growth.
	• Insufficient washing stage.	• Increase the number of washed with PBS.
	• Co-purification of chaperonins	• Several chaperonins that are involved in protein folding may co-purify with GST fusion proteins, e.g. DnaK (~70 kDa), DnaJ (~37kDa), GrpE (~40kDa),

		GroEL (~57 kDa), GrpE (~40kDa), GroEL (~57kDa), GroES (~10kDa). Several additional purification steps have been described. E.g. co-purification of DnaK can be avoided by treating the cell lysate with 5 mM $MgCl_2$ and 5mM ATP prior to purification. DnaK can be dissociated from other components in the presence of ATP and Mg^{2+}.
TARGET PROTEIN ELUTES POORLY	• Too smooth elution (low elution volume).	• Increase the volume of elution Buffer. Depending on the nature of the fusion protein and the amount of protein loaded, additional elution steps or buffer volume is required.
	• Flow rate too high	• Decrease flow rate during elution
	• Inadequate elution conditions	• Check the buffer and pH of the elution buffer. Note: In some cases increase to 50 mM reduced glutathione improves the elution.