Protocol

TD-P Revision 3.0

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Affinity GST Purification Protocol

utilizing Glutathione Agarose Bulk Resin

Introduction

Glutathione Agarose Resin is used to purify recombinant derivatives of glutathione S-transferases (GST) or glutathione-binding proteins. In this assay, GST-tagged proteins bind glutathione (GSH) immobilized to agarose. These proteins can then be eluted and characterized. Our product allows for batch or column purification and provides an easy one-step purification method for the isolation of GST fusion proteins. Glutathione Agarose Bulk Resin is supplied as a 75% (v/v) aqueous suspension in 20% EtOH. Here, we describe a protocol for batch, gravity, spin and fast protein liquid chromatography (FPLC) purification of GST-tagged proteins. We also describe the procedure needed for storage and regeneration of Glutathione Agarose Resin.

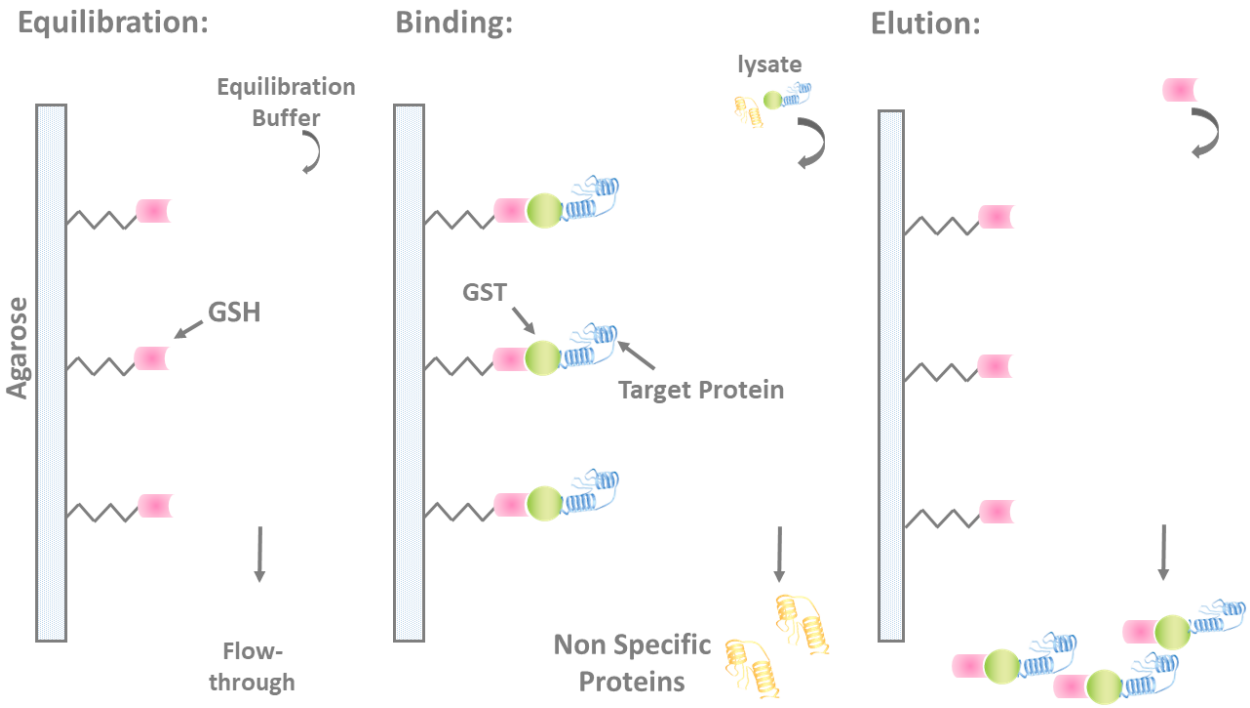

Figure 1. Purification of proteins fused to glutathione S-transferase (GST).

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Materials

- Glutathione Agarose Resin (GoldBio Catalog # G-250)
- Plastic Columns (GoldBio Catalog # P-301) or Plastic Spin Columns (GoldBio Catalog # P-300)
- Phosphate Buffered Saline (PBS) (GoldBio Catalog # P-271) pH 7.3
- Distilled Water

Buffers

Binding Buffer (PBS):

- 10mM Na_2HPO_4
- 1.8mM KH_2PO_4
- 2.7mM KCl
- 140mM NaCl

Elution Buffer

- 10mM L-Glutathione, Reduced (GoldBio Catalog # G-155)
- 50mM Tris-HCl (GoldBio Catalog # T-095), pH 8.0

Method

Procedure for Batch Purification of GST-Tagged Proteins

1. Determine the quantity of Glutathione Agarose Resin needed for your purification.

   Note: Binding capacity varies for each GST-tagged protein and depends on parameters such as the nature of the fusion protein, expression host, etc. Glutathione agarose has an orientative static binding capacity of 8 mg of GST-tagged protein per milliliter of resin (8 mg GST-tagged protein/1 ml resin). Typically, 1 ml of resin corresponds to approximately 1.33 ml agarose suspension.

2. Gently swirl the bottle of Glutathione Agarose to generate a homogeneous suspension before pipetting a sufficient amount to an appropriate tube.

3. Centrifuge the resin at 500 g for 5 minutes. Carefully decant and discard the supernatant.

4. Equilibrate the resin by adding 10 bed volumes of binding buffer. Mix thoroughly to achieve a homogeneous suspension.

5. Centrifuge the resin at 500 g for 5 minutes to form a sediment bed. Carefully decant and discard the supernatant.

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.

6. Prepare your sample according to Table 1.

Table 1. Required *E. coli* culture volumes for 1 ml of settled Glutathione Agarose resin[a]

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

[a]1 ml of settled agarose corresponds to 1.33 ml of glutathione agarose suspension.
[b]On average, 250 ml of culture will produce approximately 1 g of pelleted cells.
[c]1 g of cells may be lysed in 2-5 ml PBS.

7. Add the clarified *E. coli* lysate to the equilibrated resin, and mix the suspension gently for 30 minutes at room temperature on a shaker.

Note: In some cases, a slight increase in incubation time may facilitate binding.

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

9. Repeat step 8 with fresh binding buffer 3 times (for a total of 3 x 10 bed volumes of binding buffer).

Note: To ensure beads are thoroughly washed, compare final supernatant against fresh buffer at OD 280.

10. To elute the pure protein, add 1 bed volume of elution buffer to the resin. Mix thoroughly for 10 minutes at room temperature on a shaker. Then, centrifuge the resin at 500 x g for 5 minutes. Carefully decant or pipette the supernatant into a new tube and place on ice.

11. Repeat step 10 twice, and pool the fractions containing the purified protein.

Note: The elution step may need to be repeated because 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. Thus, eluates should be monitored (absorbance, SDS-PAGE, etc.) to determine protein yield.

12. For Regeneration, please see the Regeneration and Storage section.

Gravity Purification of GST-tagged proteins

The following summarized procedure is adapted for the purification of GST-tagged protein using gravity columns (GoldBio Catalog # P-301 or P-302).

1. Determine the quantity of glutathione agarose needed for your purification.

Note: Binding capacity varies for each GST-tagged protein and depends on parameters such as the nature of the fusion protein, expression host, etc. Glutathione agarose has an orientative static binding capacity of 8 mg of GST tagged protein per milliliter of resin (8 mg GST-tagged protein / 1 ml resin). One ml of resin corresponds to approximately 1.33 ml agarose suspension.

2. Gently swirl the bottle of glutathione agarose to ensure a homogeneous suspension and pipette a sufficient amount to an appropriate column.

Note: Remove both the upper and lower caps of the column to allow the elimination of preservative by gravity flow.

3. Replace the bottom cap and equilibrate the column by adding 5 bed volumes of binding buffer. Remove all air bubbles. Replace the top of the column and mix by inverting. Discard the supernatant by removing the top and bottom caps.

4. Repeat step 3 as described twice with fresh binding buffer.

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

5. Prepare your sample according to Table 1.

6. Replace the bottom cap and add the clarified *E. coli* lysate. Replace the top cap and mix by gently inverting the column for at least 30 minutes on a shaker.

Note: In some cases, a slight increase in incubation 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, including sample concentration.

7. Remove the top and lower caps to allow flow through the column. Discard the flow-through. Wash the column to remove any protein that has not been retained by replacing the bottom cap and adding 10 bed volumes of binding buffer. Replace the top cap and mix by inversion. Remove lower and top caps and allow the binding buffer to flow through the column. Discard the flow-through.

8. Repeat step 7 twice as described with fresh binding buffer (for a total of 3 washes).

Note: To ensure beads are thoroughly washed, compare final supernatant against fresh buffer at OD 280.

9. Replace the bottom cap, add 1 bed volume of elution buffer and replace the top cap. Mix thoroughly by gently inverting the column for 10 minutes at room temperature in a carousel shaker. Centrifuge the resin, remove the bottom cap and collect the eluate in a new tube. Store the eluate on ice.

Note: When mixing the column, it is advisable to keep elution buffer and resin in contact for at least 10 minutes before removing the bottom cap.

Note: Conditions (volumes, times, temperatures) used for elution may vary among GST fusion proteins. It is possible that a significant amount of GST fusion protein may remain bound to the resin.

Note: Eluates should be monitored (absorbance, SDS-PAGE, etc.) to determine yield of the protein.

10. Repeat step 10 twice with fresh elution buffer and pool the collected eluates.

11. For Regeneration, please see the Regeneration and Storage section.

Spin Purification of GST-Tagged Proteins

The following summarized procedure is adapted for the purification of GST-tagged protein using spin columns (GoldBio Catalog # P-300).

1. Gently swirl the bottle of Glutathione Agarose to ensure use of a homogeneous suspension in the following steps.

2. Add 67 µl Glutathione Agarose to a new spin column and replace the top cap.

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

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

3. Remove the lower cap and place the spin column in a collecting tube. Centrifuge at 500 g for 30 seconds.

4. Equilibrate the spin column by adding 500 µl of binding buffer. Replace the top cap and mix manually. Centrifuge at 500 g for 30 seconds and discard flow-through.

   Note: A 50% suspension of pre-equilibrated glutathione agarose may be used immediately or stored at 4°C for up to 1 month.

5. Replace the bottom cap of the spin-column, add the sample (700 µl of clarified *E. coli* lysate) and replace the top cap. Incubate for at least 30 minutes.

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

   Note: In some cases, a slight increase of incubation time may increase binding.

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

6. Mix briefly by manually inverting the spin column. Remove the bottom cap, and place the spin column in a collecting tube. Centrifuge at 500 x g for 30 seconds and discard the flow-through.

7. Add 500 µl binding buffer to eliminate all the proteins that have not been retained in the column. Mix by manually inverting the spin column. Centrifuge at 500 x g for 30 seconds and discard the flow-through.

Note: To ensure beads are thoroughly washed, compare final supernatant against fresh buffer at OD 280.

8. Repeat step 7 with 500 ml of fresh PBS 3 times (total of 4 washes).

9. Replace the bottom cap and add 50 µl elution buffer and replace the top cap. Gently mix for 10 minutes at room temperature on a shaker. Remove the bottom cap, and place the column in a new collection tube. Centrifuge at 500 x g for 30 seconds, and place the resulting eluate on ice.

10. Repeat step 9 twice and pool the collected eluates.

Note: 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 (absorbance, SDS-PAGE, etc.) to determine yield of the protein.

11. For Regeneration, please see the Regeneration and Storage section.

FPLC 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 the loading step to allow maximal binding of the GST-tagged protein. The flow rate for equilibration, washing and elution can be increased to reduce purification time (see Table 2).

Table 2. Working conditions.

Column diameter (mm)	Bed Volume (ml)	Volumetric flow rate (ml/min)		
		Packing	Equilibration/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

For conversion between flow rates, see Calculations section.

1. Gently shake the bottle of Glutathione Agarose Resin to ensure use of a homogeneous suspension in the following steps.

2. Place a funnel in the head of the column and run the Glutathione Agarose Resin down the walls of the column slowly, to avoid bubbles.

   Note: The product may be degassed before adding to the column if desired.

3. Decant the product leaving 1 cm above the column head by passing it through the column or pipetting it from the top of the column to prevent the column from drying out.

4. Repeat step 3 until desired column height is achieved.

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

   Note: Do not allow air to become trapped under the net.

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

   Note: If desired height is not achieved, repeat steps 1-6.

7. When a constant height has been obtained, maintain the flow with the addition of 5 volumes of distilled water.

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

   Note: Degas all solutions before adding to the column to avoid bubbles.

9. Refer to Table 1 to prepare your sample.

10. Once the resin is equilibrated, apply the filtered or centrifuged sample. Use a low rate for the loading step to allow maximal binding of the GST-tagged protein. A slight increase of contact time may facilitate binding.

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

11. Wash the resin with binding buffer (5-10 bed volumes of PBS) until O.D. reaches baseline level.

12. Elute the GST-tagged protein with 5-10 bed volumes of elution buffer, collect the fractions and place on ice.

Note: It is important to identify (using SDS-PAGE and Bradford protein assay) the fraction that contains the majority of pure protein.

Regeneration and Storage

Long term use of resin can lead to decreased binding capacity and diminished protein yield. In addition, the use of the same resin to purify different types of protein can cause contamination of elution. To avoid these effects, the column should be regenerated when a change in protein yield is observed. The column should also be regenerated before using it in the isolation of a different type of protein to obtain stable and pure yields.

1. Apply 10 bed volumes of 100mM Tris-HCl, 0.5M NaCl at pH 8.5 followed by 10 bed volumes of 100mM sodium acetate, 0.5M NaCl at pH 4.5.

2. Repeat step 1 twice.

3. Wash the bed 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.

Calculations

Converting from linear flow rate (in cm/h) to volumetric flow rate (in ml/min)

$$VF\left(\frac{ml}{\min}\right) = \frac{Linear\ Flow\ (\frac{cm}{h})}{60} \times \frac{\pi\ \times d(cm^2)}{4}$$

Converting to volumetric flow rate (in ml/min) to linear flow rate (in cm/h)

$$LF\left(\frac{cm}{h}\right) = \frac{VF(\frac{ml}{min}) \times 60 \times 4}{\pi \times\ d(cm^2)}$$

Where:

d = diameter

VF = Volumetric Flow
LF = Linear Flow

Troubleshooting

If low protein yield is observed:

- Ensure protein and tag are in frame.
- Optimize bacterial expression conditions.
- Lower the growth temperature from 37°C to 30-15°C.
- 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.

If sample is highly diluted or concentrated:

- Concentrate the sample before purification in the column.
- Perform the adsorption step as instructed for batch purification, and pack the column with resulting resin from the adsorption step.
- Dilute the sample prior to purification in the column.

If target protein does not bind efficiently:

- Change sonication conditions. Over-sonication may alter GST moiety and prevent binding of the fusion protein to Glutathione Agarose.
- Add DTT to lysis buffer for a final concentration of 5mM prior to cell lysis.
- Concentrate the sample. Sample might be too dilute, preventing efficient binding of target protein.
- Increase contact between resin and fusion protein by decreasing flow rates or performing the adsorption in batch.
- Repack the column.
- Check that appropriate binding conditions are used.
- Add less fusion protein to the column.
- Regenerate resin according to Regeneration procedure or use fresh resin. Immobilized glutathione may be degraded by ϒ-glutamil transpeptidase activity in *E. coli* lysates.

If low protein purity is observed:

- Change sonication conditions. Over-sonication may lead to purification of other proteins in addition to the fusion protein.
- Perform the purification at lower temperatures (4°C) to reduce possible degradation of the fusion protein.
- Reduce time of purification steps to reduce possible degradation of the fusion protein.
- Keep the samples on ice to reduce protease activity and prevent degradation of the fusion protein.

- Add protease inhibitors to prevent degradation of the fusion protein.
- Use a protease-deficient host. Multiple bands may result from partial degradation by host proteases during culture.
- Increase number of washes with PBS.
- To prevent co-purification of chaperones (DnaK [~70 kDa], DnaJ [~37 kDa], GrpE [~40 kDa], GroEL [~57 kDa] or GroES [~10 kDa]) with the GST-fusion proteins, additional purification steps may be performed. Prior to purification, treat cell lysate with $MgCl_2$ 5mM and ATP 5mM to prevent co-purification with DnaK.

Associated Products

- Plastic Columns (GoldBio Catalog # P-301 or P-302)
- Plastic Spin Columns (GoldBio Catalog # P-300)
- PBS Tablets (GoldBio Catalog # P-271)
- Tris HCl (GoldBio Catalog # T-095)
- L-Glutathione, Reduced (GoldBio Catalog # G-155-25)

References

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Marston, F. A. O. (1986). The purification of eukaryotic polypeptides synthesized in Escherichia coli. *Biochem. J*. 240(1):1-12. Celltech Ltd.

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