Buffers Needed:

Lysis Buffer (LB): TBS; 0.25 M NaCl; 1 mM EGTA; 1 mM EDTA; 0.1% Tween-20; 1 mM PMSF; 1 mM Benzamidine-HCl.

Wash Buffer 1 (WB1): TBS; 0.25 M NaCl (final); 1 mM DTT; 0.1% Tween-20.

Wash Buffer 2 (WB2): TBS; 0.25 M NaCl (final); 1 mM DTT.

Elution Buffer (EB): 50 mM Tris, pH 8.0; 1 mM DTT; 0.25 M NaCl; 5 mM reduced glutathione. You must double check the pH of this buffer after adding glutathione.

Borate Buffer: 0.1 M borate, pH 8.5; 0.5 M NaCl

Acetate buffer: 0.1 M acetate, pH 4.0; 0.5 M NaCl

Preparation of Resin: (Glutathione Agarose- G-4510 from Sigma)

You need 70 mg of lyophilized resin/ml of column desired. The binding capacity is on the order of 10 mg of GST/ml of resin so you don’t need a large column. If we purchase the 10 ml size bottle, I usually swell the entire bottle and save the rest of the resin in a 50 ml conical tube at 4 °C.

1. Swell resin in TBS with 0.5 M NaCl (final); 0.1% Tween 20; 5 mM reduced glutathione in a total volume of 50 ml for 3 hrs. To swell, put contents of bottle into a 50 ml conical and add about 25 ml of buffer. Rinse glutathione agarose bottle several times with remaining 25 mls of buffer in several small batches. Seal conical, parafilm the top, and put on a rotator at 4 °C.

2. Wash resin 2 X 50 ml with WB1 by pelleting resin for 2-3’ in a clinical centrifuge on a setting of 4 and carefully aspirating off the supernatant.

3. After second wash, resuspend the resin in 50 ml of WB1 containing 0.1% NaN3. Take out the desired amount of resin and move to a fresh 50 ml conical tube- it is ready to go.

4. Parafilm the top of the conical and store the remaining resin at 4 °C.
Growth of Cells and Preparation of Lysate:

Usually 500 ml or 1 liter of culture is sufficient to generate plenty of fusion protein.

1. Grow 25 ml ON culture of bacteria in LB-AMP at 37 °C.

2. Dilute 1/50 into fresh LB-AMP.

3. Grow at 37 °C to OD600 of 0.5 to 0.7 (usually takes 3-5 hrs but monitor carefully).

4. Add IPTG to 0.5 mM (or the optimum amount you determined) and induce expression for 4 hrs at 37 °C or the temperature that you determined to be optimal for solubility.

5. Pellet cells by centrifugation at 5000 rpm for 15’ in the superspeed centrifuge.

6. Weigh the bacterial pellet and resuspend the pellet in 10 ml of LB/g pellet weight. Add lysozyme to 0.5 mg/ml and freeze in a 50 ml conical tube by dropping it in liquid nitrogen. Alternatively the bacterial suspension can be put in a ziplock bag, and the cell suspension can be rapidly frozen by placing the ziplock bag between slabs of dry ice- this results in rapid freezing and thawing which minimizes ice crystal formation and thus minimizes protein degradation (I recommend this method).

7. Place tube or ziplock bag in –80 and store until ready to use.

8. To prepare lysate, thaw cell suspension by immersing the ziplock bag or conical tube in cool water. Mix often to insure even thawing and avoid ice chunks or warm spots.

9. Transfer cell suspension to a small plastic beaker- it will be quite goopy from the DNA at this point. Glass is supposed to be better for sonication, but I’m always afraid that it will crack. Sonicate at least 2 X 20 sec with a medium to large tip sonicator. You want to set the sonicator at a setting that gives good sonication but avoids frothing. Monitor extent of sonication by reduction in the amount of goopy DNA- the solution should become quite liquid. Save a 25 µl aliquot of this sample as lysate.

10. Add DTT to 1 mM and then centrifuge at 18K for 20’ in an SS-34 or JA-20 rotor.

11. Remove sup to a fresh tube and save a 25 µl aliquot of this sample as column load.
Running the Column:

It is simplest to actually batch bind the protein to the resin and pour it into a column and do the washes in column format, and I usually do it this way. I do then pour it into a column and do the last washes and elution in column format- I often find that you get better elution from a column than in batch. Alternatively, you can run the entire procedure like a classical column, but this takes longer. All of the column steps should be done in the cold room to keep everything cold. Also, I don’t set up any fancy parastaltic pumps- I just let the columns flow by gravity.

5. Take the desired amount of GST agarose in a 50 ml conical and pellet to remove excess buffer.

6. Add the clarified lysate to the resin and batch bind at least 1 to 2 hrs or ON at 4 °C. If you batch bind ON then I sometimes add NaN3 to 0.02% to prevent growth in the extract which is very high in protein and other goodies.

7. Pour the resin into a column and let the resin settle into the bottom of the column.

8. Wash out the 50 ml conical with 15 ml of WB1.


10. Wash resin with 50 ml WB2.

11. Stop flow of the column when the last of WB2 has just entered the bed of the resin so there is no buffer sitting on top of the resin bed.

12. Gently pipet on a small volume (1-2 ml) of EB onto the surface of the resin. Begin to flow the column and collect 1 1.5 ml fraction. What this does is basically let the elution buffer enter into the resin of the column. Let sit 30-60’ at 4 °C.

13. Begin eluting column. Collect 10-15 1.5 ml fractions depending on the size of the column. The protein will usually elute in a sharp peak in the first 1-2 column volumes.

14. To see the elution profile, you can do a nitrocellulose spot test. Take a small piece of NC paper and number for column fractions. Spot 1 µl of each fraction on the paper, and let it air dry. Then stain the NC paper with Ponceau S and destain in 5% acetic acid as if you were doing a western blot. The protein containing fractions will turn bright pink which the other fractions will be blank.
15. Run out all protein containing fractions on SDS-PAGE and coomassie stain to determine purity of protein. Generally you should get very pure protein with just some lower bands which are either proteolysis or inefficient translation.

16. Pool all fractions that contain sufficient protein and dialyze against your desired buffer. For injection into rabbits, I’ve found that 50 mM MOPS, pH 7.2; 50 mM NaCl; 0.1 mM EGTA; 0.1 mM EDTA works well. For frog extract experiments, dialyze into 10 mM Hepes, pH 7.2; 100 mM KCl; 25 mM NaCl; 50 mM sucrose; 0.1 mM EDTA; 0.1 mM EGTA. There may be some precipitation after the dialysis. For the immunogen, this is fine- a few solids are good for the immune system. For extract experiments, precipitation is bad so the final sample should be centrifuged and then filtered thru a 0.2 um filter before use. Samples can be stored at 4 °C short-term and in aliquots at –80 for longer term storage.

17. To determine concentration, use Bradford with BSA as a standard. Alternatively, you can use the Von-Hippel equation to calculate the molar extinction coefficient for a given protein. When measuring A280, a 1 mg/ml solution has an OD equivalent to the ((#trpX5690 + #tyrX1280)/ MW of the protein) X 1.05.

18. For injection into rabbits, you need at least a 1 mg/ml solution so that you can inject at least 0.5 mg/injection. For extract experiments, it is preferable to have at least a 1-2 mg/ml solution so that it is sufficiently concentrated when diluted into the extract. If necessary, concentrate by microcon or centricon- you will take a loss. For extracts, you will need to refilter after concentration which may generate more precipitates.

Regeneration of Resin:

After use, it is necessary to regenerate the resin to really clean it. I don’t find it necessary to do this every time- maybe every three runs. I also never use the same resin for purification of more than one fusion protein- this may seem like a waste of resin but I’m always afraid of just a little bit of contamination which would really screw up antibody preparation or extract experiments so I just make one column per fusion protein. If you don’t regenerate the resin, equillibrate the column back into TBS with 0.25 M NaCl final and 0.1 % NaN3. Leave a good head of buffer and seal the column with parafilm for storage. To regenerate resin:

12. Wash with 25 ml of borate buffer

13. Wash with 25 ml of water.

15. Wash with 25 ml of TBS with 0.25 M NaCl final and 0.1 % NaN3.

16. Leave a good head of buffer over the column and seal with parafilm.

17. Store at 4 °C.