

Affinity purification of chicken or rabbit anti-peptide

antibodies (using Sulfolink Coupling Gel from Pierce (product no. 20401 and 20402)
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Solutions and hardware needed:

Sulfolink coupling gel

Lyophilized peptide

10 mls total IgY fraction from appropriate chicken per column

Plastic 10 ml capacity columns

Column rack with plastic trays

L-Cysteine-HCl (light sensitive)—used for blocking the column

1 M Tris pH 8.0

glycerol

Bradford reagent (diluted 1:5 in ddH₂O, 190 ul per fraction collected)

Filter sterilize all solutions:

1x PBS pH 7.2

1x PBS with 0.05% Na azide pH 7.2

1x PBS with 250 mM NaCl pH 7.2

Elution buffer 100 mM glycine pH 2.5 (for 500 mls 3.75 g in 500 mls ddH₂O, pH to 2.5 with HCl)

Coupling buffer 50 mM Tris 5 mM EDTA pH 8.5 (for 500 mls: 6 g Tris, 5 ml 0.5 M EDTA pH 8.0, up to 500 mls with ddH₂O and pH to 8.5 with several drops of concentrated NaOH)

Wash buffer 1 M NaCl (for 500 mls 29.22 g in 500 mls ddH₂O)

Notes:

The principle behind the column is covalent linkage of your peptide to an agarose supported gel via the cysteine residue present at either the C-terminus or N-terminus of your peptide. In almost all cases the cysteine is at the N-terminus of the C-terminus-directed peptides and the C-terminus of the N-terminus-directed peptides. For practical purposes, we use 1 mg of peptide with 1 ml bed volume of coupling gel (2 ml slurry). The binding capacity of the column is about 1 mg peptide per ml of coupling gel.

One very important consideration prior to linking the peptide to the column is to make sure the peptide is reduced. This is because the covalent linkage occurs on the sulfur residues of the cysteine. If the cysteines in the peptides have formed disulfide bridges with one another in solution, the coupling efficiency will be bad, since these are needed to provide the linkage to the agarose matrix. The Pierce protocol states that if a peptide is lyophilized it is safe to assume it is reduced. Once it is resuspended, you

should proceed immediately to the linking step. If this is not the case, a reduction step followed by removal of the reductant must be performed prior to linkage. See the Pierce protocol associated with product no. 20401 for details.

This is a two day protocol, but most of the time is spent watching the column drip or incubate at RT. Therefore, you can multi-task if you want to save time or alleviate the boredom. In this case, its highly recommended that you have a step by step protocol handy for each column you plan to make so you can keep track of what solutions have been added to each column. This is particularly important if some columns have slower flow rates due to small bubbles introduced into the column matrix or insoluble materials that find their way into the matrix.

On day one, the peptide will be linked to the column and the unbound sites on the column will be blocked. After washing and equilibration, you add your total IgY and let it incubate overnight at 4C.

On day two, the unbound IgY is flowed thru the column and several washes are performed prior to eluting the specifically bound antibodies. The column is then immediately regenerated and can be stored or used again immediately.

Protocol:

B. Coupling Protein/Peptide to Gel

1. Equilibrate SulfoLink ® Coupling Gel to room temperature (RT) and add 2 ml of gel slurry to a disposable plastic column on a rack (Biorad #731-1550 poly-prep chromatography columns work well). This will give a gel bed of 1 ml.
2. Equilibrate column with 4 column volumes of Coupling Buffer. Let drip into a plastic tray waste container.

Note: Throughout the entire procedure, do not allow the gel bed to run dry; instead add additional solution or replace the bottom cap on the column whenever the buffer drains down to the top of the gel bed.

3. Replace the bottom cap. Dissolve 1 mg of the sulfhydryl-containing peptide in 1 ml of Coupling Buffer**. Add the remaining dissolved peptide to the column. (Use 1 ml of protein solution per ml of SulfoLink ® Coupling Gel). Optional: Retain about 50 ul of sample of the peptide or protein solution for later determination of coupling efficiency.

**Note: Make sure the peptide is compatible with the coupling buffer prior to dissolving. If not, use appropriate solvent (DMSO, 10% acetic acid) to bring into solution and then add at least 1 volume of coupling buffer to bring the pH close to the coupling buffer before adding to the column.

4. Replace the top cap and mix column (by rocking or end-over-end mixing) at RT for 15 minutes.
5. Incubate the column at RT for an additional 30 minutes without mixing.

6. Sequentially remove top and bottom column caps and allow the solution to drain from the column into a **clean** tube. Optional: Save the flow thru in this tube.
7. Place the column over a new collection tube and wash column with 3 column volumes of Coupling Buffer.
8. Optional: Determine the coupling efficiency by comparing the protein/peptide concentrations (e.g, by absorbance at 280 nm) of the unbound fraction (Step 6) to the starting sample (Step 3).

C. Blocking Nonspecific Binding Sites on Gel

1. Replace the bottom cap on column.
2. Prepare a solution of 50 mM L-Cysteine•HCl in Coupling Buffer--light sensitive.
3. Apply 1 ml of 50 mM cysteine solution to the column for each ml of gel.
4. Replace the top cap and mix for 15 minutes at RT, then incubate the reaction without mixing for an additional 30 minutes at RT.

D. Washing the Column

1. Sequentially remove the top and bottom caps and allow the buffer to drain from the column.
2. Wash the column with at least 6 column volumes of Wash Solution (1 M NaCl).

Note: If intending on proceeding with the incubation of the IgY fraction, proceed to beginning of **Protocol for Affinity Purification** below. If intending on storing the column, proceed with steps 3-5 below:

3. Wash the column with 2 column volumes of 1x PBS pH7.2.
4. Replace the bottom cap and add an additional 2 ml of 1x PBS pH 7.2 + 0.05% Na azide. Cap the top and bottom and wrap the top with parafilm. Store upright at 4C.
5. The column can be stored for months at 4C and used repeatedly for affinity purification.

Protocol for Affinity Purification

Adapted from M. Preuss

TR 3/27/06

1. Wash the column with:

2x 10 ml of 1x PBS + 250 mM NaCl pH 7.2

1x 10 ml of Elution buffer

2x 10 ml 1x PBS pH 7.2

2. Add total IgY protein to column. Total IgY/IgG should be in 1x PBS with or without Na azide. If not, then column should be buffered with what the Ig-- is stored in. Adding 100 ul 10x PBS to 10 mls of anti-sera is sufficient for rabbit bleeds.

3. Cap the column, wrap with parafilm, and rotate the column O/N at 4C to mix the gel and total IgY.

Next day:

4. Take off parafilm, top cap and then bottom cap. Allow Ig-- to drip into a clean 15 ml corning tube.

5. Pass flow thru over the column once at RT. Optional: For good antisera, this step may be skipped. However, for first time trials, it may be better to perform this step. If necessary, one can run the flow thru two times over the packed gel bed. However, this lengthens the protocol by 30 min-1 hr.

6. Wash column with:

20 ml 1x PBS pH 7.2

10 ml 1x PBS + 250 mM NaCl pH 7.2

7. Stop liquid just as it reaches the top of the packed column by capping the bottom.

8. Prepare eppendorf tubes labeled with fraction numbers (1-20 is sufficient). Put 50 ul 1M Tris pH 8.0 in each tube.

9. Elute with 5 mls of Elution buffer. Capture 250-500 ul elutions in labeled tubes. Mix immediately to neutralize the pH and prevent the antibodies from denaturing.

10. Perform a Bradford assay on the fractions to identify the peak fractions containing antibody. Use 190 ul of 1:5 diluted Bradford reagent stock in each well and add 25 ul of each fraction to a corresponding well. Pipet up and down to mix.

11. Pool peak fractions into a labeled tube.

12. Add the peak fractions to a 2 ml eppendorf tube labeled with animal ID # and antibody name containing glycerol at a final concentration of 50%. Store at -80C.

Optional: Snap freeze in liquid nitrogen and store at -80C.

14. Regenerate the column for future use by going through step 1 from the affinity purification protocol above. However, in the last two washes, include 0.05% Na azide in the 1x PBS.