LP105 – Purification of Phospho-specific Polyclonal Antibodies Using Peptide-affinity Columns

The following is a general protocol for affinity purification of phosphospecific antibodies from serum. First, peptides are covalently bound to agarose beads by the free sulfhydryl group. Pierce SulfoLink Coupling Resin can bind approximately 1 mg sulfhydryl containing peptide per 1 ml of settled resin. The nonphosphopeptide column is used first to remove the nonphosphospecific antibodies, and to collect the phosphospecific antibodies in the flow-through. This fraction is then loaded on the phosphopeptide column to purify the phosphospecific antibodies from the other immunoglobulins. An affinity column can typically be re-used up to 10 times depending on the stability of the immobilized peptide.

Materials

Pierce SulfoLink Coupling Resin (20401)
Peptide or Protein
Rainin Rabbit Plus Peristaltic pump
1.6mm ID Silicone tubing (Bio-rad #7318211)
2mm ID peristaltic tubing connector (Gilson F1825113)
Kontes Flex-Column 1.5 x 5cm (VWR 420400-1505)
Luer locks (Bio-rad #7318222, 7318225, 73128102)
5ml collection tubes and rack (Sarstedt 55.526)
Eppendorf BioPhotometer and Uvettes
Dialysis tubing and closures (Spectra Por 4 - 132700)
4L Beaker
Magnetic stirrer and stir bar
Filtration units (Nalgene 166-0045)

Solutions

Coupling Buffer: 50 mM Tris, 5mM EDTA-Na, pH8.5

Quenching Reagent: 50 mM L-Cysteine HCl in Coupling Buffer, prepare fresh each time

Wash Solution: 1M Sodium Chloride

Storage Buffer: PBS + 0.05% Sodium Azide

Binding/Wash buffer: 50 mM 1 x TBS

Elution buffer: 100 mM Glycine, pH2.5

50 mM Diethanolamine pH11. Store at 4°C

1 x TBS: 50 mM Tris, 100 mM NaCl, pH7.4

1 x TBS/Azide: 1 x TBS + 0.1% Azide

1 x PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH7.4

Filter-sterilize all buffers before use.

Protocol

Coupling Peptide to the Resin

1. Equlibrate Sulfolink Resin, peptide and reagents to room temperature.
2. Set pump to 0.5-1 ml/min (Speed 4 = 1 ml/min. Will be different each time depending on size of column, tubing, etc.)
3. Swirl resin to evenly resuspend and then transfer 10 ml to a column to give a 5-ml bed volume.
4. Equilibrate column with 20 ml (4 column volumes) Coupling Buffer, do not allow resin bed to dry.
5. Resuspend 5 mg peptide in 5 ml Coupling Buffer, take OD280 reading.
6. Drain remaining buffer from column and add 5 ml of peptide into top of column.
7. Replace cap and mix by inverting until beads are completely in suspension. Continue to mix by placing on a rocker for 15 min at room temperature.
8. Stand the column upright and incubate at room temperature for 30 min to allow beads to settle.
9. Remove bottom cap and allow buffer to drain, keep flow through and take OD280 reading again to assess coupling efficiency.
10. Wash column with 15 ml Coupling Buffer and allow to drain.
11. Replace bottom cap and add 5 ml Quenching Reagent to top off column and repeat steps 7 and 8.
12. Wash the column with 50 ml Wash Solution (1M NaCl)
13. Wash with 15 ml Storage Buffer
14. Column can now be stored upright at 4°C or proceed with affinity purification.

Purification of the polyclonal antibodies

Run the diluted centrifuged serum or purified Ig through the non-phosphopeptide column first. The flow-through from the non-phosphopeptide column is applied to the phosphopeptide column. This cycle can be repeated if resulting antibodies are still recognizing the nonphospho-peptide.

1. Equilibrate column with 15 ml (3 column volumes) of Binding/Wash buffer.

2. Centrifuge serum at 4000 rpm for 10 min at 4°C to eliminate aggregates and debris.
3. Dilute sample 1:1 in Binding/Wash buffer and apply 20 ml (~100 mg of IgG) to the column. Recirculate once then collect flow-through in 1 fraction. The flow-through contains the phosphospecific antibodies, store at 4°C until ready for step 13.
4. Wash the column with 25 ml TBS. Check OD280 periodically
5. Elute the antibody with 20 ml Elution Bf.
6. Collect 1-ml fractions in collection tubes containing 0.1 ml 1M Tris, pH8.0, antibody will come off quickly in a small peak.
7. Mix each tube gently to neutralize, avoid foaming as this denatures the protein.
8. Check fractions after 20 ml. Identify the immunoglobulin containing fractions by measuring absorbance at 280 nm. Pool concentrated fractions together and pool other fractions separately.
9. Wash and equilibrate column with 40 ml Binding/Wash Buffer.
10. Between rabbit sera, wash column with 20 ml 50 mM Diethanolamine, pH11. Collect in 2-ml fractions and check by measuring absorbance at 280 nm.
11. Equilibrate phosphopeptide column with 15 ml (3 column volumes) of Binding/Wash buffer.
12. Apply flow-through fraction from step 3 to the column. Recirculate once then collect flow-through in 1 fraction. The flow-through contains the nonphosphospecific antibodies, store at 4°C.
13. Wash the column with 25 ml TBS. Check OD280 periodically
14. Elute the antibody with 20 ml Elution Bf.
15. Collect 1-ml fractions in collection tubes containing 0.1 ml 1M Tris, pH8.0, antibody will come off quickly in a small peak.
16. Mix each tube gently to neutralize, avoid foaming as this denatures the protein.
17. Check fractions after 20 ml. Identify the immunoglobulin containing fractions by measuring absorbance at 280 nm. Pool concentrated fractions together and pool other fractions separately.
18. Wash and equilibrate column with 40 ml Binding/Wash Buffer.
19. Between rabbit sera, wash column with 20 ml 50 mM Diethanolamine, pH11. Collect in 2-ml fractions and check by measuring absorbance at 280 nm.
20. Before storage, wash both columns with 4 column volumes (20 ml) of glycine buffer and then 20 ml of diethanolamine followed by 20 ml TBS and then 2 column volumes (10 ml) of TBS/Azide. Store columns in TBS/Azide buffer at 4°C immediately after use.
21. Pool appropriate fractions together
22. Dialyze overnight in 100x volume PBS, repeat dialysis in fresh PBS.
23. Measure absorbance again at 280nm and calculate concentration.
24. QC – Run 1µg on a reducing and non-reducing gel to check for degradation, concentration.
25. Perform Direct ELISA using peptide coated plate.