CBG
Max Planck Institute
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and Genetics

A. Desalting Antigen into Coupling Buffer

1. Thaw antigen and save sample for Bradford/gel
2. Equilibrate BioRad desalting column with Coupling buffer
3. Desalt antigen into Coupling buffer; save sample for Bradford/gel.

   Use this for coupling to NHS-HiTrap

B.1. Coupling fusion proteins to Hitrap-NHS

Pump settings

- Blue:blue tubing
- Gilson setting 7 gives a flow rate of ~1.5 ml/min
- All flow rates are 1.5 ml/min

1. Wash with 1 mM HCl (ice cold) for 2 min.
2. Set-up antigen recirculation:
   - columns suspended over 15 ml conicals with Qiagen column holders (mini kit)
   - ~4 ml of antigen (anywhere from 1 to >10 mg). Dilute peptides from 100 mg/ml stocks in DMSO.
   - Recirculate at 1.5 ml/min for >30 min @ RT (can also try 4°C for 1-4 hours or O/N). Save a post coupling sample
   - Check coupling by Bradford/gel (assume 5/4 fold dilution of sample when put over HiTrap column); I find the gel to be most useful for checking whether coupling worked well.
3. Block unreacted NHS group with Blocking Buffer. Flow for 5 min, stop pump and let sit @ RT for 30 min.

Pre-elution (steps 4-8)

4. Wash with 10 mM Tris pH 8 for 5 min
5. Wash with 0.1 M glycine pH 2.0 (NOT pH 2.6!) for 5 min
6. Wash with 10 mM Tris pH 8 for 5 min
7. Wash with 0.1 M triethylamine pH 11.5 for 5 min
8. Repeat steps 5-7 with prayer and fasting
9. Wash PBS for 15 min
10. If column is to be stored at 4°C, wash into PBS + 0.1% azide.

B.2. (Variant) Coupling of peptides to Sulfolink resin

1. Pack homemade columns containing 2 ml each of Sulfolink resin.
2. Dissolve 5 mg of each peptide in 100 ul of DMSO. Just before coupling, add this to 4 ml of 50 mM Tris pH 8.5, 10 mM EDTA.
3. Run about 6 column volumes of 50 mM Tris pH 8.5, 10 mM EDTA over the column.
4. Recirculate the peptide over a column containing 2 ml of Sulfolink resin for 45 minutes.
5. Recirculate 4 ml of 50 mM Cysteine in 50 mM Tris pH 8.5, 10 mM EDTA over the columns for 30 minutes.
6. Wash with 10 mM Tris pH 8 for 5 min
7. Wash with 0.1 M glycine pH 2.0 (as opposed to pH 2.6!) for 5 min
8. Wash with 10 mM Tris pH 8 for 5 min
9. Wash with 0.1 M triethylamine pH 11.5 for 5 min
10. Repeat steps 5-8 with prayer and fasting
11. Wash PBS for 15 min

C. Serum re-circulation

1. Thaw serum at 37 °C until a small lump of ice remains.

   Add an equal volume of sterile 2X PBS and NaN3 to final concentration of 0.1 % and filter using a SteriCup (0.2 μm).
2. Replace lid on Stericup container with a special two-holed lid and attach the HiTrap column to the lid and insert the capillary tube through the other hole.
3. Recirculate overnight at room temperature.

D. Wash and elution

Wash

1. Remove HiTrap column from antiserum bottle
2. Wash with Wash Buffer for 1.5-2 hr (aim for at least 100 column volumes)
3. Wash with PBS for 30 min.

Elution (on Benchtop)

1. Wash into 10 mM Tris pH 8 for 15min
2. Set up 15 fractionation tubes containing 200 µl 2M Tris, pH 8.5 and 15 fractionation tubes containing 200 µl 2M Tris, pH 7.5
3. Start flowing in 0.1M glycine, pH 2.6 (NOT 2.0) and collect fractions in the Tris pH 8.5 tubes (total fraction volume including 2M Tris of ~1-1.2 ml)
4. As soon as fraction is collected, cap tube, mix by inversion and put on ice; the glycine should be neutralized (can check with pH paper)
5. Elute with at least 10 column volumes of glycine (15-20 fxns)
6. Wash w/ 10 mM Tris pH 8 until pH of column effluent is ~8
7. Elute with triethylamine, pH 11.5 as above, but collect in the Tris pH 7.5 tubes.

Checking Elutions

To check elutions, clean a small cuvette thoroughly and read OD280 of each fxn; put the assayed part back into the fraction tube after reading; pool all fxns with OD >0.1 and dialyze (keep glycine and triethylamine elutions separate)

Approximative calculation: 1mg/ml rabbit IgG = 1.4OD, cf Johnstone and Thorpe, Immunochemistry in Practice, Blackwell, 1982, p. 2, cited by Eric Martz.

Dialysis

At least 3X vs large volume of PBS. To concentrate, dialyze vs PBS + 50-60% glycerol (we make with the glycerol % such that total volume including dialysis sample will result in a final glycerol concentration of 50-55%)

NOTE: If elution is good, you will get some precipitation in the fraction on ice and also during dialysis; don't worry about this!

E. Collecting antibodies

Collect dialysate and centrifuge in TLA100.3 (or equivalent) rotor at 50K rpm (100,000xg) for 15min. Collect supe, mix to ensure it is homogenous and freeze aliquots in liquid N2. Keep a working stock at -20.

F. Storing Columns

1. Wash thoroughly with PBS until pH of column effluent is same as PBS.
2. Wash with PBS + 50% glycerol (reduce flow rate to 0.5 ml/min) for 30'.
3. Cap column well and store at -20.

Solutions

Prepare 1 L of each and filter through 0.2 µm Steritops

1 mM HCl (200ml)

- 1000x dilution of 1M HCl or
- 80.7µl of HCl fumans in 1l water

Coupling buffer

- 50 mM HEPES (K+) (made from 1 M pH 8 stock)
- <150 mM NaCl/li>
- 80 mM CaCl2
- 10% glycerol (optional)

Blocking Buffer

- 0.5 M ethanolamine
- 0.5 M NaCl, pH 8.3

10 mM Tris pH 8.0
0.1 M glycine pH 2.0 (pre-elution buffer)
0.1 M triethylamine pH 11.5 (Test other non-amine buffers to see if they might be more stable).
2X PBS
Wash Buffer

- PBS
- 0.5 M NaCl
- 0.1 % TritonX100

2M TrisHCl pH 8.5 (500 ml)
2M TrisHCl pH 7.5 (500 ml)
0.1 M glycine pH 2.6 (Elution buffer)
10X PBS
20 % NaN3 (sodium azide)
Storage Buffer

- 1X PBS (dilute from 10X stock of PBS)
- 55 % glycerol (v:v)

Other materials needed

- HiTrap NHS, 1ml, from Pharmacia, Cat# 17-0716-01
- Gilson pump (or other peristaltic) w/ blue-blue Tygon tubing
- SteriCups
- Glass capillary (e.g. a calibrated capillary)

On Sprint system

Connect filtered buffers to the indicated lines (A-E) and fill F with filtered H2O

Run "start-up" method to wash and fill the inlet lines.

Attach columns to first four positions on Scout.

Flow rate of 2 ml/min

Add 100 µl of 2M TrisHCl pH 8.5 to each well of 96-deep-well plate prior to collection. We do this with a short program.

Buffers

- A: 10 mM Tris pH 8
- B: 0.1 M glycine pH 2.6
- C: 0.1 M triethylamine pH 11.5
- D: 10XPBS
- E: Storage Buffer
- F: 2M Tris pH 8.5