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REAGENTS

cross linker: dimethyl pimelimidate (DMP), Sigma D-8388, mw = 259.2

quencher: ethanolamine, Sigma E-9508, 98% solution (~16.04 M), mw = 61.08

triethanolamine, Sigma T-1377, 98% solution (~6.57 M), mw = 149.2

Protein G immobilized on 4% agarose beads, Sigma P-7700

1 g resin expands to ~4 ml

1 ml resin binds >20 mg human IgG

Antibody of interest: binding to Protein G optimal for mouse IgGs

SOLUTIONS10x PBS: 80g NaCl, 2 g KH₂PO₄, 11.1 g Na₂HPO₄ (anhydrous), 2 g KCl, to 1 L w/ H₂O; dilute 1/10 to use

1 M glycine (pH 3) [for 1 L, will need to add ~5 Pasteur pipetfuls of conc. HCl]

D (dilution) buffer: PBS + 1 mg/ml BSA

W (wash) buffer: 0.2 M triethanolamine in PBS (3.04 ml triethanolamine per 100 ml buffer)

Q (quench) buffer: 50 mM ethanolamine in PBS (311.7 ul per 100 ml)

DAY 1 - Preparation1. Swell beads.

Weigh 25 mg aliquots of dry beads into each of 10 (or more) microfuge tubes. Add 1 ml PBS to each tube (10x volume; swelled beads ~100 µl/25 mg). Shake to mix well, then rotate O/N @ 4°C.

2. Aliquot DMP.

Want 13 mg/ml DMP, 1 ml per microfuge tube, for each of three cross-linking reactions. So, if doing 10 microfuge tubes, weigh out 143 mg DMP (13 mg x 11 ml) in each of three 15 ml Falcon tubes.

3. Make Dilution, Wash, and Quench buffers; also 1 M glycine. (see Reagents/Solutions)**DAY 2 – Cross-linking**1. Wash beads.

Spin beads (14,000 rpm, 1 min) to pellet. Aspirate out PBS with pipetmen: use 1ml tip to remove 750 µl, then 200µl *gel loading tip* to remove 150 µl. *Be very careful not to aspirate out beads*; this is especially true if immunoprecipitation will be used for quantification. Add 1 ml D-buffer, shake to mix, and rotate 10 min @ 4°C. Spin and aspirate (850+150 µl) as before.

2. Bind Ab to beads.

Make Ab solution: 6 µg/ml in D-buffer, need 1ml/tube *for only half the tubes* (other tubes will get D-buffer alone). Add 1 ml (each) to half the tubes, and 1ml D-buffer (each) to the other half (label which tubes have which!). Shake and rotate 1 hr @ 4°C.

3. Wash out unbound Ab.

Spin and aspirate (850+150 µl), then add 1 ml D-buffer to each. Rotate 5 min @ 4°C. Repeat, using PBS. Spin and aspirate this wash.

4. Cross-link.

DMP is unstable in aqueous solution, so dissolve each batch with 11 ml W-buffer just prior to addition to beads, vortex quickly, and aliquot 1 ml to each microfuge tube. Rotate 30 min @RT.

Verify pH is between 8-9 before and after addition to beads (cross-linking efficiency is greatly reduced outside this pH range).

Wash with W-buffer (rotate 5 min RT, then spin and aspirate).

Add 2nd DMP aliquots, rotate 30 min RT, wash as before.

Add 3rd DMP aliquots, rotate 30 min RT, wash as before.

5. Quench and wash.

Add 1 ml (10x volume) Q-buffer, rotate 5 min RT, spin and aspirate; repeat.

Wash with PBS.

6. Remove un-cross-linked Ab.

Wash with 1 M glycine pH 3. Rotate 10 min RT. Repeat.

7. Storage washes.

Wash with buffer to be used for immunoprecipitation (usually PBS+TWEEN). Rotate 5 min RT.

Do this 3x (total), and store in final wash (after rotation). Store beads in fridge; if it will be for more than a few days, add azide to prevent bacterial growth.

DAY 3 – Immunoprecipitation

1. Prepare beads.

Use at least 1 tube of bead blank and 1 tube of AbXlink, or more if there are a lot of samples (more than seven or eight).

Spin, aspirate, and wash with 1 ml PBS+TWEEN, just to refresh buffer. Then resuspend beads in 100 µl PBS+TWEEN. If using more than 1 tube of each type of beads, move all beads of each type to one tube. Do this by shaking and then transferring the bulk of the beads to one tube, then spin all tubes to pellet the primary tube and pull down residual beads from the other tubes. Use the supernatant from the primary tube to resuspend the residual beads in the secondary tubes, and transfer.

2. Prepare samples and incubate.

Dilute samples, as necessary, in PBS+TWEEN. For these beads, will need at least 1 µg sample per tube. A nice sample volume is 500 µl, but more or less can be used. To add beads to samples, resuspend using vortex, and pipet quickly, 40 µl bead suspension per tube. Rotate 1 hr @4°C.

3. Wash beads.

Wash 4x with PBS+TWEEN.

4. Elute bound antigens.

Add 20 µl 3x SDS sample buffer directly to aspirated beads, boil 5-10 minutes, and load 20 µl on polyacrylamide gels. *Note:* This is the harshest elution, and will also elute any non-covalently bound Abs and Ab fragments, which will appear in the gel and make western blotting a nightmare (for Coomassie staining, this is usually not a problem).

Alternatively, antigens can be gently eluted with a glycine gradient (up to 1 M).

REFERENCES

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