Crosslinking Primary Antibody to Protein A/G resin using dimethylpimelimidate (DMP), for MS-Compatible Immunoprecipitation

(As an alternative to disuccinimidyl suberate (DSS) crosslinking)

Reagents:

1. PBS
2. Binding buffer: 0.2M sodium borate (adjust 0.2M Boric Acid to **pH 9** with NaOH)
3. Crosslinking reagent: 20mM DMP (dimethylpimelimidate, Pierce # 21666) dissolved in 0.2M sodium borate – *make immediately prior to use*
4. "Quenching" reagent: 0.2M ethanolamine (**pH 8.0** in 50 mM Ammonium Bicarbonate)
5. Acid Wash buffer: 0.58% v/v acetic acid with 150mM NaCl
6. Lower stringency lysis buffer for wash: 150mM NaCl, 50mM Tris, 10mM EGTA, **0.2% NP40**
7. MS Wash buffer: 50mM ammonium bicarbonate
8. Elution buffer: 0.25% Rapigest SF Surfactant in 50mM ammonium bicarbonate

Procedure:

Coupling antibody with agarose beads:

1. Couple antibody/control IgG andProtein A/G beads (Pierce # 20421) in PBS:
    a. Add 60ul (20 ug) **X** antibody to 30 ul Prot A/G beads in 1ml PBS
    b. Add 50ul (20 ug) IgG rabbit to 30 ul Prot A/G beads in 1ml PBS (control)
    c. Rock 2-3 hours or overnight at 4C
2. Wash beads 3X with 1ml of 0.2M sodium borate pH 9 – after each wash, spin at 300-500g to pellet beads, gently remove supernatant
3. Save small sample to run on gel to check efficiency (see step 15)

Crosslinking*:*

4. Make fresh DMP:
    a. Let solid warm up to room temperature ~20min
    b. Weigh out 0.0259 g DMP
    c. Immediately add DMP to 5ml of 0.2M sodium borate (pH 9) to make 20mM DMP solution
5. Immediately add 1ml 0.2M sodium borate + 20mM DMP to (1) beads + X Antibody; and (2) beads + Control IgG
6. Rock at RT for 40min to perform antibody crosslinking
7. Spin down and remove supernatant
8. Wash beads once in 0.2M ethanolamine (pH 8.0) – this removes/quenches any residual DMP
9. Resuspend in 1ml of 0.2M ethanolamine (pH 8.0)
10. Rock at RT for 1-2 hours
11. Save small sample to run on gel to check efficiency (see step 15)
12. To remove uncoupled IgGs, wash with 3X 1mL 0.58% v/v acetic acid + 150mM NaCl
13. Wash 3X with 1ml cold PBS - after each wash, spin at 300-500g to pellet beads, gently remove supernatant
14. Save small sample to run on gel to check efficiency (see step 15)

Check the efficiency of immobilization*:*

15. Sample beads (1) before and (2) after crosslinking, and (3) after the acid wash – elute by boiling resin in 2x LDS loading buffer, then run SDS-PAGE/Coomassie stain . **Ensure equal volumes of beads are analyzed for each of these samples. For example, if you are going to take out 5 uL of beads for the check and then elute in 20 uL 2X LDS and load 10 uL for SDS-PAGE, be sure to do it the same for all resin binding steps.
16. Evaluate the results of the immobilization process prior to proceeding. (please share images with us!!)

***Proceed with IP**:*

17. Quantity of input material will vary depending on the expression of the target protein/complex. Typically, between 0.5 and 5 mg input protein is utilized. Preclearing is recommended, by adding your lysate to ~25 uL of uncoupled Protein A/G resin, rock for 30 minutes, and reserve supernatant for IP.
18. Add 1ml (~1-5 mg/mL protein) of precleared lysate to approximately 25 uL coupled resin. Use same resin quantity for all samples.
19. Rock overnight at 4C (with end-over-end mixing, if possible)
20. Save supernatant for WB analysis.
21. Wash resin 3X with lysis buffer containing 150mM NaCl, 50mM Tris, 10mM EGTA, 0.2% NP40 (you may want to save these washes for troubleshooting purposes… a signal in WB at this step (but not step 20) would suggest target bound to antibody but was then washed away)
22. Elute with 50-100ul of 2X LDS loading buffer, boil 5 min at 95C, pellet beads by centrifugation, take supernatant for SDS-PAGE analysis (below). ******see step 23 for MS compatible elution protocol***.

Recommended Setup for Coomassie gel (or WB analysis):

1) Ladder
2) 50ug lysate
3) **X** Beads pre-elution
4) **X** Beads post-elution
5) **X** Elution supernatant
6) IgG Rab Beads pre-elution
7) IgG Rab Beads post-elution
8) IgG Rab Elution supernatant

*Stain with Invitrogen Colloidal Blue Staining kit (LC6025)

****Alternative elution steps once IP has been optimized:***

23. After step 21 wash, wash resin 3X with 1 mL 50mM ammonium bicarbonate
24. Add 50-100ul of 0.2% Rapigest SF Surfactant (Waters Corporation) in 50mM Ammonium Bicarbonate.
25. Boil @ 95C for 5min, pellet beads by centrifugation, take supernatant for delivery to Duke Proteomics Core Facility, or for SDS-PAGE analysis as above.

The Duke Proteomics Core Facility gratefully acknowledges the input of the following individuals in compiling this protocol:

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