ADMEbio
for proteomic research

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Immunoprecipitation (IP) Protocol

I. Materials and Reagents

1. **Pierce Protein A/G Plus Agarose or equivalent:**
   0.55ml settled resin supplied as a 50% slurry (e.g., 100µL of 50% slurry is equivalent to 50µL of settled resin).

2. **Cell Lysis Buffer:**
   20mM Tris HCl pH 8; 137mM NaCl; 1% Nonidet P-40 (NP-40) and 2 mM EDTA. Note: It is necessary to adjust IP reaction volume and add the inhibitors immediately prior to use.

3. **Wash Buffer:**
   Tris-buffered saline (TBS)-25mM Tris-HCl, 0.15M NaCl buffered saline, pH7.2 containing 0.05% Tween-20

4. **Elution Buffer:**
   IgG Elution Buffer, pH 2.0 (Thermo Product No. 21028 or equivalent) or 0.1M glycine, pH 2.0

5. **Alternative Elution Buffer:**
   SDS-PAGE reducing sample buffer

6. **20X Coupling Buffer:**
   25ml, when diluted results in 0.01M sodium phosphate, 0.15M NaCl; pH 7.2

7. **100X Conditioning Buffer:** 5ml, neutral pH buffer

8. **SDS-PAGE reducing sample buffer**

9. **Antibody for immunoprecipitation**

10. **Neutralization Buffer:**
    High-ionic strength alkaline buffer such as a 1M phosphate or 1M Tris, pH 7.5-9

11. **Pierce Spin Columns:**
    Screw Cap, 50 columns, includes accessories

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II. Experimental Procedure

1. Preparation of lysates:

- Place the cell culture dish on ice and wash the cells two times with ice cold PBS.
- After aspirating PBS completely, add ice cold Cell Lysis Buffer into cells and mix well: 1ml per 107 cells/100mm2 dish/150cm2 flask; 0.5ml per 5x106 cells/60mm2 dish or 75cm2 flask.
- Incubate cells with lysis buffer on ice for 5 minutes with periodic mixing.
- Collect lysate into 1.5mL microcentrifuge tubes and centrifuge at 4°C using a microcentrifuge. * The speed and time of centrifugation depending on the cell type. A guideline is 10 minutes at 13,000 rpm but this must be determined by user.
- Remove the tubes from the centrifuge gently and place on ice.
- Transfer the supernatant to a new tube and keep on ice for protein concentration determination and further analysis. Discard the pellet.

2. Pre-clearing the lysates

- Add either 50µl of irrelevant antibody of the same species or same isotype as the IP antibody to 1ml of lysate. Incubate for one hour.
- Add 100µl of bead slurry into the lysate.
- Incubate sample at 4°C with gentle agitation for 10 to 30 minutes.
- Centrifuge sample at 14,000 x g at 4°C for 10 minutes.
- Discard bead pellet and keep supernatant for immunoprecipitation. To increase the yield, the beads can be washed 1 or 2 more times using lysis buffer, followed by collection of the supernatants together for immunoprecipitation.

3. Binding of Antibody to Protein A/G Plus Agarose

- Swirl the bottle of Pierce Protein A/G Plus Agarose gently to obtain an even suspension. Using a wide-bore or cut pipette tip, add 20µl of Protein A/G resin slurry into a Pierce Spin Column. Place column into a microcentrifuge tube and centrifuge at 1,000 × g for 1 minute. Discard the flow-through.
- Wash the resin with 200µl of 1X Coupling Buffer, centrifuge and discard the flow-through. Repeat this step one time.
- Tap the bottom of the column on a paper towel gently to remove any excess liquid. Insert the bottom plug.
- Prepare 3-10µg of antibody for coupling (see individual datasheet for exact amount). Adjust the volume to 10 µl with sufficient ultrapure water and 20X Coupling Buffer to produce 1X Coupling Buffer. For example, for a 1µg/µl of antibody solution, add 5µl of 20X Coupling Buffer and 85µl of water and 10µl of antibody. Add the ultrapure water, 20X Coupling Buffer and affinity-purified antibody to the resin in the column directly.
- Attach the screw cap to the column and incubate on a rotator or mixer at room temperature for 30-60 minutes, ensuring that the slurry remains suspended during incubation.
- Remove and retain the bottom plug and remove the cap. Place the column into a collection tube and centrifuge. Save the flow-through to verify antibody coupling.
- Wash the resin with 100µl of 1X Coupling Buffer, centrifuge and discard the flow-through.
- Wash the resin with 300µl of 1X Coupling Buffer, centrifuge and discard the flow-through. Repeat this step one time. Note: The following protocol is optimized for coupling 10µg of antibody but can be used for 2-50µg. For antibody amounts > 50µg, proportionally scale the resin, crosslinker and buffer volumes.

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4. Crosslinking the Bound Antibody:

- Puncture the foil covering of a single tube of DSS with a pipette tip and add 217µl of DMSO or DMF to prepare a 10X DSS solution (25mM). Use the pipette to mix the solution thoroughly (i.e., draw up and expel the solution) until the DSS is dissolved.
- Dilute the DSS solution 1:10 in DMSO or DMF (100µl of 10X DSS with 900µl solvent) to make 2.5 mM DSS.
- Tap the bottom of the column on a paper towel to remove excess liquid and insert the bottom plug.
- Add 2.5µl of 20X Coupling Buffer, 9µl of 2.5 mM DSS and 38.5µl of ultrapure water to the column. The total solution volume will be 50µl. The DSS is added at 10X molar excess to Protein A/G on the resin with a working concentration of 450µM.
- Incubate the crosslinking reaction for 30-60 minutes at room temperature on a rotator or mixer.
- Remove and retain the bottom plug and open the cap. Place the column into a collection tube and centrifuge.
- Add 50µl of Elution Buffer to the column and centrifuge. Save the flow-through to verify antibody crosslinking.
- Wash twice with 100 µl of Elution Buffer to remove non-crosslinked antibody and quench the crosslinking reaction.
- Wash twice with 200µl of cold IP Lysis/Wash Buffer and centrifuge after each wash. Note: Conventional IP can be performed by omitting crosslinking; however, if crosslinking is omitted, the antibody will co-elute with the antigen during the elution steps. Note: The DSS crosslinker is moisture-sensitive. Keep DSS in foil pouch after use. Dissolve DSS in DMSO or DMF immediately before use. DSS is not compatible with amine-containing buffers (e.g.,Tris, glycine).

5. Antigen Immunoprecipitation Protocol:

- If the antibody-crosslinked resin was stored in PBS, wash twice with IP Lysis/Wash Buffer. Discard the flow-through after each wash.
- Tap bottom of the column on a paper towel to remove excess liquid. Replace bottom plug.
- Dilute the cell extract in IP Lysis/Wash Buffer. The recommended sample volume in the spin column is 300-600µl. The suggested amount of total protein per IP reaction is 500-1,000µg as determined by the Pierce BCA Protein Assay.
- Add the pre-cleaned lysate sample to the antibody-crosslinked resin in the column. Attach the screw cap and incubate column with gentle end-over-end mixing or shaking for 1-2 hours or overnight at 4°C.
- Remove bottom plug, loosen the screw cap and place the column in a collection tube. Centrifuge column and save the flow-through. Do not discard flow-through until confirming that the IP was successful.
- Remove the screw cap, place the column into a new tube, add 200µl of Wash Buffer and centrifuge.
- Wash sample twice with 200µl Wash Buffer and centrifuge after each wash.
- Wash sample once with 100µl of 1X Conditioning Buffer.

6. Antigen Elution:

- Place the spin column into a new collection tube, add 10µl of Elution Buffer and centrifuge at 1,000 × g for 1 minute.
- Keep the column in the tube and add 5µl of Elution Buffer. Incubate for 5 minutes at room temperature. The column does not need to be closed or mixed. Note: For a more concentrated eluate, less Elution Buffer may be used; however, overall yield might be reduced.
- Centrifuge the tube and collect the flow-through. Analyze the eluate for presence of antigen. Perform additional elutions (i.e., Steps 4.1-4.2) as needed. Analyze each eluate separately to ensure that the antigen has completely eluted.

7. Sample Preparation for SDS-PAGE Analysis:

- Equilibrate the 5X Lane Marker Sample Buffer to room temperature. Gently mix the sample buffer by inverting 5-10 times. For a reducing gel, add 1M DTT to a final concentration of 100mM in the 5X Sample Buffer.
- Add 5X Sample Buffer to sample to make a 1X final solution (i.e., add 5µl of 5X Sample Buffer to 20µl of sample).
- Heat the sample at 95-100°C for ~5 minutes. Allow the sample to cool to room temperature before applying to the gel.

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8. Additional Notes:

- Perform all steps at 4°C unless otherwise indicated.
- Perform all resin centrifugation steps for 30-60 seconds at low speed (i.e., 1,000-3,000 × g). Centrifuging at speeds greater than 5,000 × g may cause the resin to clump and make resuspension difficult.
- When centrifuging spin columns, the flow-through volume should not exceed 600µl when using a 2ml collection tube and 300µl when using a 1. ml collection tube. Exceeding these volumes may result in back pressure in the column and incomplete washing or elution.
- Before performing the immunoprecipitation, pre-clear lysates using the Control Agarose Resin to reduce nonspecific protein binding.