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| **Protocol #13v1.** | **Immunoprecipitation (IP) and co-IP** |
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| **Aim** | To isolate protein or protein complexes |
| **Risk assessment** | * 9431: UQDI general laboratory work in a PC2 facility * 9474: UQDI RNA/DNA/Protein extraction from cells or tissue * 8315: Hill – Proteomic Techniques * 11079: UQDI Electrophoresis and blotting (DNA, RNA, Protein) * 9704: UQDI use of heating block |
| **Training requirement** | If unsure of any techniques ask another Hill group member or a floor manager. |
| **Materials to be prepared beforehand** | SDS PAGE gel (appropriate for protein size) – see SOP#8  Key Considerations:  **IP** = only targeting a single protein of interest, ie. antigen for the antibody you are using.  **Co-IP** = isolate binding proteins to the target protein.  Practically, the difference is in the solubilization of your protein sample.  To IP single protein only, use RIPA buffer for solubilization of cell/fraction, and also for IP (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, and 0.2% SDS with protease and phosphatase inhibitors).    For co-IP, the goal is to maintain protein complexes whilst removing insoluble material which can lead to undesired background binding. Suggested final co-IP buffer compositions with lower range for more transient complexes:    50 mM (10-50 mM)Tris-HCl, pH 7.5 (buffer)  150 mM (50-150 mM) NaCl (ionic strength)  0.5% (0.1% - 0.5%) detergent (generally use NP-40 or Triton X-100)  Protease and phosphatase inhibitors  Stock Solutions:   * 3M Sodium Acetate (2.46g Sodium acetate in 10mL water) * 10% Tween-20 (5mL Tween-20 with 45mL water) * 10% Triton X-100 (5mL TritonX-100 with 45mL water) * 2M NaCl (5.84g in 50mL water) * 1M Tris pH7.5 (6.06g Tris in 50mL water – pH adjusted)   Antibody Binding Buffer   |  |  |  | | --- | --- | --- | | Reagent | Final Concentration | Volume to add (15mL total) | | 3M Sodium Acetate | 0.1M | 500uL | | 10% Tween-20 | 0.05% | 75uL | | Water |  | 14.5mL |   IP Buffer   |  |  |  |  |  | | --- | --- | --- | --- | --- | | Reagent | Final Concentration | Volume (1mL) | Volume (7mL) 1xIP | Volume (21mL) 3xIP | | Water | NA | 771.5uL | 5.4mL | 16.2mL | | 10% Triton X | 1% for IP  (0.1-0.5% for co-IP) | 100uL | 700uL | 2.1mL | | 2M NaCl | 50mM | 25uL | 175uL | 525uL | | 1M Tris pH7.5 | 20mM | 20uL | 140uL | 420uL | | 50mM Na3VO4 (Van) | 0.5mM | 10uL | 70uL | 210uL | | 1mM Sodium Pyrophosphate |  | 7.5uL | 52.5uL | 157.5uL | | 200mM AEBSF | 200mM | 2.5uL | 17.5uL | 52.5uL | | 1000x Protease Inhibitor | 1x | 1uL | 7uL | 21uL | |
| **Procedures** | Couple antibody to magnetic beads   1. Determine which type of beads you need (protein A or G) – This will depend on the antibody type and species. 2. Mix the magnetic beads in the bottle 3. Use 20-30uL of beads for each IP. Resuspend in 200uL of antibody binding buffer. Place tubes on the magnetic rack to remove the binding buffer without removing the beads. Repeat three times. 4. Resuspend in 200uL of binding buffer and add your antibody. This is typically 1ug. Ensure that your control for non specific binding matches in concentration. E.g. phospho PKC Rabbit conc = 0.1mg/mL add 10uL while Normal Rabbit IgG conc = 1mg/mL add 1uL. 5. Incubate the antibody with the beads for 40 minutes at room temperature on a rotating wheel. 6. Wash the beads twice with 1mL binding buffer. Wash beads twice with 1mL CoIP buffer. Leave in solution to avoid beads drying out. 7. You only need one control per IP (eg normal rabbit IgG) for non specific binding (not per sample type)   Prepare the Sample   1. Prepare the protein sample in IP buffer, and then spin down insoluble material at top speed of mini-centrifuge in cold room for 5 minutes.   For co-IP from membranes, solubilize first using 5x conc of salt and detergent (e.g. 250 mM NaCl, 0.5% NP-40 or Triton X-100), pellet insoluble. Then dilute to final co-IP concentration.   1. If quantifying, resuspend in a small amount of buffer and perform a Bradford protein estimation. If not quantifying sample, you can add 1mL of CoIP buffer directly to the sample. NOTE: best to keep protein concentration <1 mg/ml to reduce non-specific binding. 2. Keep a portion of the sample as a control (starting material) for the western blot to ensure your protein of interest is actually present.   ImmunoPrecipitation   1. Ensure your sample is in 1mL of CoIP buffer before adding to beads. 2. Incubate at 4c for 1 hour (range 30min – 2 hours) with mixing (e.g. use spinning wheel or roller in cold room). \*30 minutes is sufficient for specific binding to antibody. Long incubation increases non-specific binding with only minor increase of specific binding. 3. Transfer the sample to a new tube. This prevents any proteins non- specifically bound to the tube from being eluted. 4. Using the magnet remove the supernatant and wash twice with 1mL of CoIP buffer 5. For the last wash, centrifuge the sample so that the beads pellet and remove the remaining CoIP buffer. 6. Resuspend the beads in 2x sample buffer and boil at 95C for 5 minutes. 7. Run your samples on a gel the same day, or store at -20C until use.   Loading the Gel  Samples should be loaded as follows:   1. Ladder 2. Start Material (known percentage of final IP) – Should be positive for probed ab 3. Control Rabbit/mouse IgG – Should be negative for probed ab 4. IP – Possibly positive for probed ab   You will also have to blot for the antibody used for the IP to show that it was successfully precipitated. |
| **Notes** | * Elution with SDS-PAGE sample buffer will elute all the IgGs. If your target protein is near 25 kDa or 55 kDa, it will be masked. * If the rotating wheel is in the cold room, take this out the day before so that any condensation inside has time to dry * Antibody coupled beads can be prepared in the same week * <https://tools.thermofisher.com/content/sfs/brochures/TR0064-Immunoprecipitation-guide.pdf> |