Chatham Lab General IP Protocol (author: Boglarka Laczy)

I. Preparation of tissue lysate (optimal cc.: 1-5 mg/mL)

- Make lysis buffer, pipette ~ **1,5 mL** into centrifuge tubes, add ~ **100 mg** tissue powder; keep the tubes on ice;
- Homogenize the samples: 20-40 sec (clean with dd. H_2O and methanol between each sample);
- Keep the homogenized samples on ice, wortex them /10-15 min;
- Centrifugation (after **45 min lysis**): **15000 x g for 15 min at 4ºC**;
- Take and aliquot (350 µL) the supernatant into Eppendorf tubes;
- Store at - 80ºC;

II. Protein A Agarose preparation

(Protein A agarose; 10 mL packed beads containing 6 mg/mL recombinant Protein A suspended as a 50% slurry in dd H_2O containing 0.01 % thiomersal; # 16-156; Upstate)

- Prior to use, **wash 2x the agarose beads** with an appropriate buffer to remove ethanol;
- Cut the tip of the pipette!
- Add PBS to Protein A slurry (1: 9); centrifuge 3 000 x g for 2 min, remove PBS;
- Add PBS to Protein A slurry (1: 10); centrifuge 3 000 x g for 2 min, remove PBS;
- Resuspend the beads in PBS and **restore to a 50 % slurry** (1:1);

III. Preclearing

- Preclear the lysate by adding **100 µL of Protein A agarose bead slurry (50%)** per **1 mL of lysate (30-40 µL/sample)**;
- Incubate **at 4 ºC for 60 min** with gentle agitation;
- Remove the beads by centrifugation **14 000 x g for 10 min at 4 ºC;**
- **Transfer and keep the supernatant** for IP (cave beads!); beads can be washed 1-2x in PBS and collected together;

IV. Protein measurement

- **Dilute the lysate** before beginning IP to roughly 1 µg/ µL total protein in a centrifuge tube with PBS;

V. Immunopreciptation

- **Add** 4 µg (= 16 µL) of IP antibody to **500**-1000 **µg** (500-1000 µL) the pre-cleared tissue sample lysate;

  CTR of Ab/agarose [BS]: mock lysate (only lysis buffer without sample) plus IP Ab;
  CTR of lysate [BA]: lysate including sample without IP Ab;
  Negative CTR [BL]: lysate without sample and without Ab;

- Gently rock the reaction mixture **at 4ºC** for **overnight**;

- Capture the immunocomplex by adding **100 µL** of washed Protein A agarose bead slurry;

- Gently rock the lysate-beads mixture **at 4ºC for 2hs**;

- Collect the agarose beads by pulse centrifugation: **10 000 x g for 2 min at 4ºC**;

- **Drain off the supernatant**; carefully aspirate the supernatant leaving 10 µL fluid above the pellet;

- **Wash the beads 3 x** with either ice-cold PBS (800 µL); Centrifuge **10 000 x g for 2 min at 4ºC** and **discard the supernatant**;

- Resuspend the agarose beads in **60 µL Laemmli sample buffer (2X)**; Do not wortex!

- Boil the beads for **5 min at 100 ºC**;

- Collect the beads: by pulse centrifugation: **10 000 x g for 2 min**;

- **Take the supernatant (= IP sample)** and apply SDS-PAGE, or IP sample can be stored at -20ºC (and should be reboiled prior to loading);